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Regulation of the Synaptic Vesicle Cycle and Mitochondrial Morphology by Alpha-Synuclein

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Regulation of the Synaptic Vesicle Cycle and Mitochondrial Morphology by Alpha-Synuclein

by

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DISSERTATION

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Contribution of Others to the Presented Work – Thesis Advisor's Statement

This work was performed almost entirely by Venu Nemani. In Chapter 2, Wei Lu in Roger Nicoll's lab performed the electrophysiologic analysis shown in Figure 8. In Chapter 3, rotation student Jon Levy performed the experiments shown in Figures 12-15, and postdoctoral fellow Ken Nakamura the data shown in Figure 16.

The work described in this thesis meets the standards required for the completion of the Ph.D. program in Neuroscience at UCSF.

Robert H. Edwards, MD
Thesis Advisor

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ABSTRACT

Neurodegenerative diseases are characterized by the accumulation and aggregation of particular proteins. In most cases, very little is known about the physiological function of these proteins; further, the manner in which their normal functions may contribute to the initiation of disease has not been well studied.

The protein alpha-synuclein has been implicated in both sporadic and familial forms of Parkinson's disease. Three point mutations in alpha-synuclein cause dominantly inherited forms of the disease, implying a causative role in the disorder. Although it localizes to nerve terminals, its role in neurotransmitter release remains poorly understood. Recently, it has been shown that modest increases in alpha-synuclein protein expression are sufficient to cause Parkinson's disease, suggesting that an increase in its normal function may initiate degeneration.

To understand the role of increased alpha-synuclein expression on nerve terminal physiology, we overexpressed alpha-synuclein in hippocampal neurons along with a fluorescent reporter of neurotransmitter release, VGLUT1-pHluorin. We find that increased expression of alpha-synuclein inhibits neurotransmitter release, and does so by reducing the size of the synaptic vesicle recycling pool. A mutation in alpha-synuclein linked to Parkinson's disease (A30P) that inhibits membrane binding abolishes this

effect; however, the C-terminus is not required. Further, alpha-synuclein overexpression inhibits neurotransmitter release in hippocampal slices from transgenic mice. Importantly, we also show that increased alpha-synuclein expression inhibits neurotransmitter release in midbrain dopamine neurons. Biochemical experiments suggest that the decrease in recycling pool size may be due to a selective decrease in complexin and synapsin proteins.

We also present preliminary evidence indicating a role for alpha-synuclein in the control of mitochondrial morphology. Overexpression of alpha-synuclein causes mitochondrial fragmentation, while the A30P mutant has a diminished ability to fragment mitochondria.

These experiments clarify the function of alpha-synuclein at the nerve terminal, and suggest a role for increased expression of the protein and altered synaptic activity in the initiation of Parkinson's disease pathogenesis. Further, we provide evidence for a previously unappreciated link between alpha-synuclein and mitochondria, an organelle whose dysfunction is central to the degeneration of neurons in Parkinson's disease.

Table of Contents

List of Figures

Chapter 2 – Increased Alpha-Synuclein Expression Impairs Neurotransmitter Release by Reducing the Size of the Synaptic Vesicle Recycling Pool

Chapter 3 – Alpha-Synuclein Induces Mitochondrial Fragmentation

CHAPTER 1: INTRODUCTION

PARKINSON'S DISEASE

History

Parkinson's disease is the second most common neurodegenerative disorder in the United States. The incidence of the disease increases with age, with approximately 1% of the population over 65 affected, and 4-5% of the population over 85 affected (Rao et al., 2006). Parkinson's disease (PD) was originally described in a monograph written by James Parkinson in 1817 titled "An Essay on the Shaking Palsy." He described the characteristic symptoms of the disease as follows:

> Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured (Parkinson, 1817).

Although he was not able to conduct a pathological examination, Parkinson conjectured that the constellation of symptoms he was seeing in his patients stemmed from an injury to the superior aspect of the medulla. However, he did acknowledge that without examination of the brain tissue from his patients, he could not be certain as to the root cause of the disease. Today, we know that the disease which bears Parkinson's name results from the loss of neuromelanin containing dopaminergic neurons in the substantia nigra pars compacta, resulting in a marked loss of dopamine in the dorsal striatum. Although his prediction as to the lesion underlying the disease was incorrect, his clinical description was fairly accurate. The only symptom which he did not describe was rigidity, which was later described by Charcot (Charcot, 1888). Together, these

physicians captured the hallmark symptoms that describe prototypical PD today: rigidity, bradykinesia, resting tremor, and postural instability.

Clinical diagnosis

Despite our increased understanding of the pathological hallmarks of PD, the diagnosis remains a clinical one. Generally, patients have many, if not all, of the following symptoms: a 3-6 Hz resting tremor, bradykinesia, rigidity, and asymmetric onset (Rao et al., 2006). Additionally, patients must respond to a dose of levadopa or a dopamine agonist. There are several other conditions, however, that must be included in the differential diagnosis of PD. These include drug-induced parkinsonism, vascular parkinsonism, essential tremor, progressive supranuclear palsy, multiple system atrophy, corticobasal degeneration, and dementia with Lewy Bodies (Rao et al., 2006).

Treatment

Pharmacological treatment for PD is currently the mainstay of therapy. Depending on the symptoms, patients are usually started on a dopamine agonist and/or levodopa. These drugs, respectively, act directly on the postsynaptic dopamine receptors in the striatum, or lead to increased synthesis of dopamine in the surviving nerve terminals. Other medications which are used in the management of PD are anticholinergics, catechol-O-methyltransferase inhibitors, monoamine oxidase B inhibitors, or NMDA receptor inhibitors. Unfortunately, none of these drugs slow or stop the progression of the disease, but are used only to provide symptomatic relief.

In patients who become refractory to medical treatment, deep brain stimulation (DBS) has proven to be a very effective means of controlling the motor symptoms of PD. In these procedures, electrodes are implanted into the subthalamic nucleus or the globus pallidus internus to shut down activity of these neurons.

Future therapeutic strategies include gene therapy to increase production of proteins that ameliorate symptoms or alter disease progression. In the United States, there are currently three Phase I clinical trials underway investigating whether delivery of aromatic acid decarboxylase (AADC), glutamic acid decarboxylase (GAD), or Neurturin (NTN) via a viral vector can be performed safely, in anticipation of studies to determine if these treatments can help patients with PD (Fiandaca et al., 2008). Additionally, the generation of dopamine neurons from neural stem cells may one day be used to replace neurons lost to the degenerative process (Daniela et al., 2007).

Pathology

Pathologically, PD is characterized by the relatively specific loss of dopamine neurons in the substantia nigra. However, the specificity of PD for this neuronal subtype has become unclear in recent years as careful pathological studies have shown loss of other neuronal subtypes in PD, with some populations being affected even earlier than midbrain dopamine neurons (Braak et al., 2003; Braak et al., 2004). Unfortunately, the motor symptoms of PD typically do not manifest until relatively late in the disease progression, after 70% or more of substantia nigra dopamine neurons have already been lost. In addition to neuronal loss, PD is characterized by the accumulation of intracytoplasmic proteinaceous deposits called Lewy bodies. Indeed, Lewy bodies are

the pathological hallmark of PD, however they can also be found in several other diseases. Lewy bodies are characterized by a dense core surrounded by a filamentous halo. It is still unclear whether Lewy bodies play a causative, protective, or neutral role in the degenerative process.

Lewy bodies and Aggregation

The protein α-synuclein (αsyn) was demonstrated to be a major component of Lewy bodies in sporadic PD (Spillantini et al., 1998; Spillantini et al., 1997), indicating an important role for the protein in the pathogenesis of PD. In fact, Lewy bodies containing αsyn are present in several other neurodegenerative diseases such as Lewy body dementia, the Lewy body variant of Alzheimer's disease, neurodegeneration with brain iron accumulation type 1, and multiple system atrophy (Baba et al., 1998; Gai et al., 2003; Yamazaki et al., 2000). These diseases have been termed "synucleinopathies." It is unclear, however, how Lewy body formation is initiated, and whether αsyn plays a central role in this process or simply accumulates in Lewy bodies after their formation. It is generally thought that during the formation of a Lewy body, αsyn monomers coalesce into oligomers or protofibrils. These soluble protofibrils then come together to form fibrils, which aggregate into Lewy body inclusions (Maries et al., 2003). Mature Lewy bodies consist of a dense, eosinophilic core that has a high content of lipids and protein (Gai et al., 2000). Peripherally, mature Lewy bodies exhibit filamentous structures 7-12 nm in diameter which radiate out from the central core (Meredith et al., 2004). High concentrations of αsyn are associated with the peripheral structures, whereas ubiquitin is

present in large amounts in the core of the Lewy body (Gai et al., 2000; Meredith et al., 2004).

It remains unclear whether Lewy bodies play a causal, neutral, or protective role in the disease process of PD. Some investigators believe that soluble oligomers or protofibrils may in fact be the toxic species, and that Lewy bodies are an attempt by the cell to sequester these damaging structures into a more benign form (Conway et al., 2000a; Conway et al., 2000b; Lashuel et al., 2002; Mouradian, 2002; Volles and Lansbury, 2002; Volles et al., 2001). Supporting this hypothesis, it has been shown that two of the PD-linked mutations in αsyn, A53T and A30P, accelerate protofibril formation while the A30P mutation slows insoluble fibril formation (Conway et al., 2000b). However, more recent evidence has shown that the third PD-linked mutation, E46K, slows protofibril formation while accelerating fibril formation (Fredenburg et al., 2007). Because of the conflicting evidence, the debate concerning the toxic species of αsyn has not yet been resolved. Interestingly, it has been shown in a cell culture model of Huntington's disease that insoluble protein aggregates play a protective role, and that formation of an inclusion reduces the risk of death for individual neurons (Arrasate et al., 2004). A recent study showed that pharmacologically increasing inclusion formation in cells transfected with αsyn leads to a reduction in toxicity (Bodner et al., 2006). More work is needed to identify definitively the pathological and protective αsyn species in cells.

Role of Mitochondria

There is a growing body of data that implicates mitochondrial dysfunction in the pathogenesis of PD. The first evidence came from the accidental discovery of MPTP in a group of illicit drug users who were trying to synthesize an analog of meperidine, MPPP (Langston et al., 1983). The active metabolite of MPTP, MPP^+, is a substrate for the dopamine transporter (DAT) and is an inhibitor of complex I in the respiratory chain. It presumably causes selective toxicity by entering dopamine neurons through DAT and inhibiting mitochondrial respiration. Other inhibitors of complex I also cause dopamine neuron degeneration in rodent models (Betarbet et al., 2000; Thiruchelvam et al., 2000). Additionally, post-mortem studies have shown complex I deficiency in the substantia nigra of patients with PD (Schapira et al., 1989). It is not well understood why complex I inhibition preferentially causes the death of dopamine neurons. Interestingly, αsyn knockout mice are resistant to toxicity due to several mitochondrial toxins, including MPTP, malonate, and 3-nitropropionic acid (Dauer et al., 2002; Klivenyi et al., 2006; Robertson et al., 2004). The mechanism mediating their resistance is not known, but would greatly increase our understanding of the role of mitochondria in PD.

FAMILIAL FORMS OF PD

The vast majority of PD cases are sporadic, meaning that there has been no identified genetic cause. The contribution of environmental and genetic factors to the overall incidence of PD remains unclear. However, to date 12 different chromosomal loci have been linked to familial forms of PD (Mizuno et al., 2008). From these chromosomal loci, 8 genes have been identified that are linked to familial forms of PD: α-synuclein (αsyn), parkin, DJ-1, UCH-L1, PINK1, LRRK2, ATP13A2, and Omi/HtrA2

(Mizuno et al., 2008). Although these genetic causes likely represent no more than 5% of all cases of PD, the discovery of these genes allows molecular insight into some of the cellular process that may be disregulated in both sporadic and genetic forms of PD. The genes that have been identified to date function in the ubiquitin-protesome pathway (parkin, UCH-L1), mitochondrial function (PINK1, Omi/HtrA2), the oxidative stress response (DJ-1), lysosomal function (ATP13A2), or have an unclear function (αsyn, LRRK2). Of these proteins, the most well studied are parkin, PINK1, DJ-1, LRRK2, αsyn which will be reviewed below.

Parkin

Mutations in parkin cause an early-onset, recessive form of PD. Parkin encodes an E3-ubiquitin ligase (Imai et al., 2000; Shimura et al., 2000; Staropoli et al., 2003; Zhang et al., 2000), which facilitates the addition of a polyubiquitin chain to lysine residues of proteins destined for degradation by the proteasome. The discovery of the function of parkin was quite exciting, because it made sense that a malfunctioning ubiquitin-proteasome system (UPS) would lead to the accumulation of misfolded protein, such as those present in Lewy bodies. Also, it is thought that derangement of the UPS is a common insult in several different neurodegenerative diseases (Ciechanover and Brundin, 2003; Giasson and Lee, 2003; Moore et al., 2003). Strikingly though, patients with PD due to mutations in parkin have no Lewy bodies (Mori et al., 1998; Takahashi et al., 1994). Because disease causing mutations in parkin affect its E3-ubiquitin ligase activity, it suggests that this function is essential for the formation of Lewy bodies. Also, because Lewy bodies are absent in familial PD due to parkin mutations, it suggests that

Lewy bodies themselves are not essential for the toxicity observed in midbrain dopamine neurons and may simply represent a downstream event. Although many substrates for parkin have been discovered, the interactions which are physiologically relevant remain unclear. Interestingly, *Drosophila* null for parkin show muscle degeneration due to mitochondrial pathology, demonstrating the importance of parkin function in the maintenance of mitochondrial function (Greene et al., 2003).

PINK1

Mutations in PINK1 cause an early-onset autosomal recessive form of PD (Valente et al., 2004). The protein has 581 amino acids, with a N-terminal mitochondrial targeting sequence and a kinase domain similar to serine/threonine kinases of the Ca^{2+} calmodulin family (Thomas and Beal, 2007). Although its exact function is still unknown, its targeting to mitochondria and the fact that most of the PD-linked mutations are in the kinase domain suggests that it plays an important role in mitochondrial function. The precise substrates of PINK1 are still unknown. Deletion of PINK1 in *Drosophila* leads to muscle and dopamine neuron degeneration due to mitochondrial dysfunction. Intriguingly, this degeneration is rescued by crossing these mutants with flies overexpressing parkin, identifying a genetic interaction between the two proteins (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). These experiments further show that PINK1 functions upstream of parkin, however their precise functions and the other molecules involved in this pathway remain to be elucidated. These experiments provide strong evidence that mitochondrial dysfunction likely plays a central role in the pathogenesis of PD.

DJ-1

Mutations in DJ-1 cause a rare autosomal recessive form of PD (Bonifati et al., 2003; Hague et al., 2003; Hering et al., 2004). There is now a considerable body of evidence supporting a role for DJ-1 as an antioxidant protein. Overexpression of DJ-1 protects cells against oxidative stress, whereas inactivation of DJ-1 sensitizes cells to oxidative stress (Abeliovich and Flint Beal, 2006). Structurally, DJ-1 contains a cysteine residue at position 106 that becomes modified under oxidizing conditions. Interestingly, modification of this residue is essential for the protective action of DJ-1 against oxidative stress (Canet-Aviles et al., 2004; Kinumi et al., 2004; Meulener et al., 2006). Although mitochondrial localization of DJ-1 has been reported (Zhang et al., 2005), the function of DJ-1 in mitochondria is unclear.

LRRK2

Mutations in LRRK2, also known as dardarin, cause an autosomal dominant form of PD. LRRK2 is an extremely large protein, consisting of 2527 amino acids that includes a Rho/Ras-like GTPase domain, a MAPKKK family domain, a WD40-repeat domain, and a leucine-rich repeat domain (Paisan-Ruiz et al., 2004). The discovery of this protein as the cause for *Park8*-linked PD was particularly exciting because of its relatively high prevalence – a single mutation (G2019S) was found to represent approximately 5% of all familial forms of PD (Di Fonzo et al., 2005; Nichols et al., 2005) and the same mutation was found in 1.6% of screened patients with idiopathic PD (Gilks

et al., 2005). Thus, mutations in LRRK2 occur at a relatively high frequency even in sporadic PD.

The function of LRRK2 is still unclear, however its multi-domain structure suggests that it likely has several roles. Recent experiments have shown that LRRK2 associates with lipid rafts (Hatano et al., 2007), regulates neurite morphology (MacLeod et al., 2006), and affects synaptic vesicle endocytosis (Shin et al., 2008). Most interestingly, it has been shown recently that mutations in the kinase domain of LRRK2 that reduce its kinase activity reduce neuronal toxicity, and PD-linked mutations augment its kinase activity, suggesting that the kinase activity of LRRK2 is important for mediating cell toxicity (Gloeckner et al., 2006; Greggio et al., 2006; Smith et al., 2006; West et al., 2005). Ongoing work to identify the substrates phosphorylated by LRRK2 will undoubtedly shed light on the molecular mechanisms of PD due to LRRK2 mutations, but will also hopefully translate into increased understanding of toxicity in sporadic PD.

α-synuclein

Mutations in αsyn cause a rare, autosomal dominant form of PD. Three different point mutations, and genomic duplication or triplication of the gene locus containing αsyn all cause familial forms of PD (Chartier-Harlin et al., 2004; Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003; Zarranz et al., 2004). Interestingly, genomic triplication causes a widespread, and very early onset, form of the disease compared to the duplication or sporadic forms of the disease, suggesting a dose dependent phenomenon. Because the normal function of αsyn is still not well

understood, it is unclear whether PD due to increased expression of the wild-type protein reflects an abnormal gain-of-function, or simply an increase in the normal function. The work in this dissertation examines the effects of increased expression of αsyn on neuronal function and its potential relationship to PD pathogenesis.

α-SYNUCLEIN

The first, and most extensively studied, protein with a link to PD is αsyn. Despite 20 years of work on this protein, its precise function still remains unclear.

Cloning and initial characterization

Alpha-synuclein was initially cloned as a synaptic vesicle associated protein from *Torpedo californica* (Maroteaux et al., 1988). There are 2 other proteins in the family, β-synuclein (βsyn) and γ-synuclein (γsyn). The three family members all share primary structure similarity, in that they consist of a variable number of N-terminal repeats (KTKEGV) and an acidic C-terminal tail. Although αsyn was initially described as having both synaptic and nuclear localization (Maroteaux et al., 1988), it has become clear from subsequent studies that αsyn primarily localizes to nerve terminals, where it colocalizes with other presynaptic markers (Fortin et al., 2004). Interestingly, αsyn does not contain a lipid anchor, transmembrane domain, or other targeting motif that would suggest its exclusive localization to the nerve terminal. It preferentially binds to membranes containing high curvature and acidic phospholipids (Davidson et al., 1998), and associates with lipid membranes at phase transitions (Kubo et al., 2005). The mechanism underlying the precise targeting of αsyn to synapses remains to be fully

elucidated, although its association with lipid rafts plays an important role (Fortin et al., 2004).

Physiological function – knockout animals

Several lines of evidence point to a role for αsyn in the regulation of neurotransmitter release. First, αsyn is specifically localized to the nerve terminal, a structure highly specialized to mediate the rapid exo- and endocytosis of synaptic vesicles in response to membrane depolarization. Because this structure must be highly efficient in this process, it is unlikely that there would be proteins resident in the nerve terminal that do not aid or somehow regulate this process.

Second, three knockouts of αsyn have been generated, two of which examined and showed alterations in neurotransmitter release (Abeliovich et al., 2000; Cabin et al., 2002; Schluter et al., 2003). Unfortunately, the deficits observed in these two studies are not in agreement. In the first study, the authors found no defect in synaptic protein content or synapse morphology, although quantitation of this data was not shown (Abeliovich et al., 2000). They showed, however, that paired stimulus depression was reduced in knockout mice compared to wildtype in striatal slices containing midbrain dopamine terminals. Additionally, they found an 18% reduction in striatal dopamine content, and a reduced locomotor response to amphetamine in the knockout animals (Abeliovich et al., 2000). Taken together, this data suggested that αsyn was acting as a negative regulator of neurotransmitter release, but did not offer any insight into the mechanism of its action.

The second study looking at the effect of deleting αsyn showed a striking reduction in undocked vesicles by electron microscopy (Cabin et al., 2002). Surprisingly, however, they did not find any reduction in synaptic proteins using quantitative immunoblotting. Next, they used electrophysiology to measure the response to high frequency stimulation in CA1 of the hippocampus. In line with their observations using electron microscopy, they found a deficit in the ability of knockout slices to maintain release during a high frequency stimulus train (Cabin et al., 2002). Further, these slices recovered basal release properties more slowly after the train. These experiments suggested that rather than acting as a negative regulator of release, αsyn maintains a population of vesicles important for supporting release during high frequency stimulation (Cabin et al., 2002). It is difficult to reconcile the results of these two groups. Whereas Abeliovich and colleagues examined dopamine release in the striatum directly using cyclic voltammetry (Abeliovich et al., 2000), Cabin and colleagues used postsynaptic recording in CA1 of the hippocampus (Cabin et al., 2002). The strains of mice used in these two studies were also different, but it is unlikely that strain differences could account for such a large difference in physiological phenotype. It is also possible that αsyn functions in opposing roles in these two neuronal populations, but parsimony suggests that this is not the case. Of note, the third knockout generated was not examined for synaptic function, but quantitative immunoblotting showed no change in synaptic vesicle proteins (Schluter et al., 2003). Since it has been previously shown that synaptic vesicle protein amounts scale in relation to the number of synaptic vesicles (Rosahl et al., 1995), it can be inferred that this knockout also had no change in the number of synaptic vesicles. More recently, a double knockout for αsyn and βsyn was generated. This

mouse also had no overt functional synaptic phenotype, but interestingly, showed an increase in the amount of complexins, proteins that are involved in the regulation of neurotransmitter release (Tang et al., 2006).

Physiological function – overexpression studies

Several labs have used overexpression experiments to try to understand the physiological function of αsyn. Overexpression studies, unfortunately, are sometimes more difficult to interpret than the corresponding knockout experiment. However, because αsyn is likely playing a regulatory role at the synapse, and because it normally associates very loosely with synaptic vesicles (Fortin et al., 2005), overexpression of the protein may provide more insight into its normal function than examining its loss of function. Additionally, because overexpression of αsyn causes PD in humans, these studies have direct relevance for understanding how the normal function of αsyn is related to the pathogenesis of PD.

Some labs have harnessed the simplicity and genetic power of *Saccharomyces cerevisiae* to study the function of αsyn. Expression of αsyn in yeast recapitulates some of the properties of αsyn described in neurons and described biochemically, such as membrane localization and inhibition of phospholipase D2 (Outeiro and Lindquist, 2003) (Jenco et al., 1998). However, because yeast are such simple cells which lack the extreme complexity and polarity of neurons, and also lack endogenous expression of αsyn, it is difficult to assess the relevance for the function of αsyn in its endogenous cell type. However, several findings in yeast have been reproduced in neurons. Cooper and colleagues found that overexpression of αsyn blocks endoplasmic reticulum (ER) to

Golgi traffic, and that this defect is rescued by overexpression of the Rab GTPase Ypt1P (Cooper et al., 2006). They similarly overexpressed the Ypt1P mammalian homolog Rab1 in *Drosophila*, cultured midbrain neurons, and *C. elegans* and showed rescue of dopamine cell death caused by αsyn overexpression in these model systems (Cooper et al., 2006). Notably, however, the authors did not demonstrate the ER to Golgi block in these systems. The relevance of these findings to αsyn biology in neurons is unclear, because the ER and Golgi apparatus are located in the neuronal soma, while αsyn is located in the nerve terminal which is usually quite far removed from the soma.

Another study examined the effect of overexpression of αsyn on catecholamine release in chromaffin cells and PC12 cells (Larsen et al., 2006). They found that overexpression of either wildtype αsyn or the A30P mutant led to an inhibition of release, and concluded that αsyn inhibited a priming step after vesicle docking to the plasma membrane.

In addition to the work in yeast and cell lines, a recent study has examined the effect of overexpression of αsyn on the neural degeneration caused by the loss of a synaptic chaperone protein. Chandra and colleagues performed a genetic experiment where they crossed a mouse overexpressing αsyn with a mouse containing a targeted deletion of cysteine string protein alpha (CSP) (Chandra et al., 2005). Mice with a deletion of CSP are normal at birth, but undergo progressive neurodegeneration that results in synaptic defects and death at 1-4 months (Fernandez-Chacon et al., 2004). Remarkably, overexpression of αsyn rescues the CSP knockout phenotype. Interestingly, overexpression of the A30P mutant, which is defective in membrane binding (Fortin et al., 2004; Jo et al., 2002), does not rescue the CSP knockout phenotype. Although the

mechanism mediating this rescue was not elucidated, the authors speculated that αsyn acts downstream of CSP as a co-chaperone to protect synapses from degeneration in the setting of loss of function of CSP (Chandra et al., 2005).

Main findings of the dissertation

Current therapies treat the symptoms of PD, but do not address the underlying neurodegenerative process because the initial steps underlying PD pathogenesis are not understood. The most effective medications used to treat PD function either by activating directly postsynaptic dopamine receptors, or by increasing synthesis and regulated release from the surviving dopamine neurons. From a therapeutic standpoint, the ultimate goal is to develop pharmacological approaches to slow or stop the loss of neurons before symptoms become debilitating. Most current research is focused on the aggregation and fibrillization properties of αsyn, however these are undoubtedly late events in the disease progression. Because increased expression of αsyn causes familial forms of PD (Chartier-Harlin et al., 2004; Singleton et al., 2003), and is also implicated in sporadic PD (Chiba-Falek et al., 2006), we chose to investigate the effect of increased expression of αsyn on neuronal physiology. In Chapter 2 of the dissertation, we show that modest overexpression of αsyn causes a substantial reduction in synaptic vesicle exocytosis in both cultured hippocampal neurons and cultured midbrain dopamine neurons. Further, we show that increased expression of αsyn does not affect the kinetics of vesicle fusion, but rather causes a reduction in the size of the recycling pool of vesicles. We also show that αsyn inhibits neurotransmitter release *in vivo*, by using electrophysiology to record basal synaptic transmission in hippocampal slices from αsyn transgenic mice. Further,

we show that αsyn overexpression causes a selective reduction in synapsins and complexins, proteins important in controlling recycling pool size and SNARE fusion, respectively, with no effect on various other synaptic vesicle proteins.

In chapter 3 of this dissertation, we present preliminary evidence that αsyn overexpression regulates mitochondrial morphology. Because of the link between mitochondrial function and PD, these results provide intriguing evidence linking both a protein and an organelle that play central roles in PD pathogenesis.

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CHAPTER 2:

Increased α-Synuclein Expression Impairs Neurotransmitter Release by Reducing the Size of the Synaptic Vesicle Recycling Pool

ABSTRACT

Mutations in the presynaptic protein α-synuclein cause autosomal dominant Parkinson's disease. More intriguingly, duplication or triplication of the α-synuclein gene locus resulting in overexpression of the wildtype protein causes widespread, early-onset Parkinson's disease. Despite its role in both the genetic and sporadic disorders, its normal function is not well understood. In this study, we use several different model systems to show that increased expression of α-synuclein inhibits neurotransmitter release both *in vitro* and *in vivo*. We show that this is a general property of the protein, functioning in both hippocampal and midbrain dopamine neurons. This reduction is dependent on membrane binding by α-synuclein, and causes a selective reduction in the size of the recycling pool of synaptic vesicles, and is accompanied by specific reductions in the levels of synapsins and complexins, nerve terminal proteins important for neurotransmitter release. This study provides mechanistic detail into the normal function of α-synuclein, which will be important to understand the pathophysiology of Parkinson's disease.

INTRODUCTION

The accumulation of characteristic proteins is a defining feature of neurodegenerative disease. AB peptide accumulates in senile plaques of Alzheimer's disease, and the microtubule associated protein tau in the neurofibrillary tangles of many degenerative disorders. Human genetics has demonstrated that mutations affecting these proteins can be causative. However, it remains unclear whether the aggregates detected morphologically are themselves toxic. In the case of HD, considerable evidence suggests a protective role for huntingtin aggregates, raising the possibility that a gain in function of the normal protein causes disease.

The discovery of rare, genetic forms of Parkinson's disease (PD) has elucidated pathways that likely play an important role in the initial progression of PD. Mutations in the proteins parkin, DJ-1, and PINK1 cause autosomal recessive forms of PD, presumably through the loss of function in the ubiquitin-proteasome system, antioxidant defense, and mitochondrial function, respectively. Alternatively, mutations in the proteins LRRK2 and α-synuclein (αsyn) cause autosomal dominant forms of PD. The normal function of these proteins are not entirely understood, so it is unclear whether PD-linked mutations in these proteins act through a gain-of-function mechanism, or simply amplify the normal function of the protein to cause disease.

Point mutations in αsyn cause autosomal dominant forms of PD, indicating that the protein plays a causative role in the disease. Importantly, αsyn also accumulates in the Lewy bodies and dystrophic neurites characteristic of idiopathic PD, suggesting an important role in the absence of inherited mutations. Further, a simple duplication or

triplication of the wildtype αsyn gene suffices to cause PD, suggesting that an increase in the normal function of the protein could be pathogenic.

The normal function of αsyn is not well understood, although recent work suggests a role for the protein in vesicular trafficking and neurotransmitter release. Mice null for αsyn show no gross phenotype, but have subtle, although conflicting, changes in neurotransmitter release properties (Abeliovich et al., 2000; Cabin et al., 2002; Yavich et al., 2004). Recent work in yeast has intriguingly shown that overexpression of αsyn blocks vesicular trafficking, particularly of vesicles moving from the endoplasmic reticulum to the Golgi apparatus (Cooper et al., 2006; Gitler et al., 2008). Similarly, αsyn overexpression inhibits catecholamine release in PC12 and chromaffin cells (Larsen et al., 2006). Whether αsyn similarly affects vesicle trafficking in neurons, however, is unknown.

Since an increase in expression due to increased gene dosage causes PD, we have examined the effect of αsyn overexpression on the function of the nerve terminal where αsyn normally resides. We show that a modest increase in αsyn expression inhibits neurotransmitter release in both hippocampal neurons and dopamine neurons. Further, this inhibition occurs not through an effect on the fusion event itself, but rather through a reduction in the size of the recycling pool of synaptic vesicles. These results suggest a potential link between the normal function of αsyn in the modulation of synaptic transmission and a pathogenic role of increased expression of αsyn in PD.

RESULTS

Alpha-synuclein coexpresses with VGLUT1-pHluorin at hippocampal nerve terminals

Previous studies attempting to understand the role of alpha-synuclein in neurotransmitter release have examined dopamine release in mice null for αsyn (Abeliovich et al., 2000; Yavich et al., 2004). However, the observed effects were quite small making it difficult to use those experimental systems to more fully understand the defect in synaptic transmission. In this study, we use an optical reporter of synaptic transmission, VGLUT1-pHluorin, expressed in cultured neurons to study how overexpression of αsyn affects the function of individually resolved nerve terminals. This reporter allows the real-time visualization of both exocytosis and endocytosis of synaptic vesicles (SVs), and thus provides an extremely sensitive assay of nerve terminal function (Balaji and Ryan, 2007; Voglmaier et al., 2006). Coexpression of human αsyn with VGLUT1-pHluorin in rat hippocampal neurons results in the colocalization of αsyn with VGLUT1-pHluorin at nerve terminals (Figure 1A). To ascertain the degree of overexpression achieved in our system, we used quantitative immunofluorescence for αsyn using an antibody, syn-1, that detects both the endogenous rat protein as well as the expressed human protein (Figure 1B). Quantitation of αsyn fluorescence revealed that αsyn was expressed at 2-3 times the endogenous level at nerve terminals colocalized with VGLUT1-pHluorin compared to untransfected terminals (Figure 1C). This level of overexpression is quite similar to that found in patients with early-onset PD due to triplication of the gene locus containing αsyn (Miller et al., 2004), underscoring the relevance of our model for understanding the function of αsyn.

Overexpression of αsyn inhibits synaptic vesicle release at hippocampal synapses

To examine how increased expression of αsyn affects the function of nerve terminals, we subjected neurons expressing either VGLUT1-pHluorin alone (vector) or VGLUT1-pHluorin with αsyn (αsyn) to a 10Hz field stimulus for 60 seconds (Figure 2A). We found that compared to the vector condition, neurons overexpressing αsyn exposed less VGLUT1-pHluorin on the surface during the stimulus, resulting in a lower peak $\Delta F/F_0$ (Figure 2B). It has been suggested that αsyn overexpression blocks ER to Golgi trafficking in a variety of cell types, including neurons (Cooper et al., 2006). To rule out an effect on trafficking of VGLUT1-pHluorin to the nerve terminal, or simply a decrease in expression of the reporter, we alkalinized the pool of synaptic vesicles using NH_4Cl, revealing the total amount of VGLUT1-pHluorin at the nerve terminal (Figure 2A, 2C). This shows that the amount of the reporter was the same in the two groups, and can not account for the difference observed during electrical stimulation. To rule out any bias in the selection of nerve terminals used for quantitation, we selected every nerve terminal that could be visualized over a series of imaging fields and plotted the response of those terminals as a frequency histogram. As shown in Figure 2D, overexpression of αsyn results in a uniform left shift of the histogram towards a smaller peak $\Delta F/F_0$. This analysis shows that there is not a discrete population of synapses which shut down release, but rather that all synapses have a decreased ability to externalize the VGLUT1-pHluorin reporter.

The decrease in the peak $\Delta F/F_0$ could result from either a decrease in exocytosis or an increase in endocytosis. To examine the efficacy of endocytosis, we calculated the

time constant for endocytosis after the stimulus and found that there was no change between the two groups (Figure 2E), suggesting a decrease in exocytosis rather than an acceleration of endocytosis underlies the decrease in release seen with overexpression of αsyn.

In these experiments, the neurons in the control condition still expression endogenous rat αsyn. To rule out any effects due to an interaction of the endogenous rat protein with the transfected human protein, we overexpressed human αsyn in hippocampal cultures from mice with a targeted deletion of αsyn (Abeliovich et al., 2000). In response to a 10 Hz stimulus for 60 seconds, these nerve terminals showed a similar reduction in VGLUT1-pHluorin exocytosis compared to the experiments performed in rat hippocampal neurons containing endogenous αsyn (Figure 2F).

Previous studies have examined neurotransmitter release in αsyn knockout animals but the changes seen have been small, and the data somewhat conflicting (Abeliovich et al., 2000; Cabin et al., 2002; Yavich et al., 2004). We examined VGLUT1-pHluorin release in hippocampal cultures from wild-type or αsyn knockout littermates, and found no effect of loss of αsyn in this paradigm (Figure 2G).

Overexpression of αsyn decreases the size of the recycling pool of synaptic vesicles

To characterize the nature of the defect in exocytosis, we examined uptake of the styryl dye FM 4-64 in neurons coexpressing αsyn and GFP, or GFP alone. Neurons were subjected to a 10Hz stimulus for 60 seconds in the presence of 15 μM FM 4-64. The neurons were exposed to the dye for another 60 seconds after the stimulus for endocytosis to be completed. After extensive washing, the neurons were stimulated again at 10Hz for

120 seconds to completely unload dye specifically loaded into nerve terminals during the first stimulus (Figure 3A). The amount of dye unloaded during the second stimulus thus provides a measure of the amount of exocytosis and endocytosis that occurred during the first stimulus. In nerve terminals coexpressing αsyn and GFP, there is an approximately 50% reduction in the amount of FM dye loaded during the first stimulus compared to terminals expressing GFP alone (Figure 3B, 3C). This result provides independent experimental verification that αsyn overexpression inhibits synaptic vesicle release. Interestingly, the rate of dye efflux during the second stimulus from loaded nerve terminals is unaffected, but rather only the extent of dye loading is affected (Figure 3D). This suggests that the exocytic fusion of synaptic vesicles with the synaptic plasma membrane is not affected, but rather that the size of the recycling pool of vesicles is reduced.

Synaptic vesicles fall into several distinct pools, categorized functionally by their propensity for release (Rizzoli and Betz, 2005). To further test the idea that αsyn overexpression reduces the size of the recycling pool of synaptic vesicles, we stimulated neurons expressing VGLUT1-pHluorin in the presence of bafilomycin, an inhibitor of the vacuolar ATPase. This manipulation allows the visualization of exocytic events in isolation from endocytosis, because reacidification of synaptic vesicles is blocked (Sankaranarayanan and Ryan, 2001). If the rate of exocytosis is slowed but the size of the recycling pool of vesicles is unchanged, then an extremely long stimulus should eventually lead to the release of all release-competent vesicles. Alternatively, if the size of the recycling vesicle pool is smaller and there are less release-competent vesicles, then even a very long stimulus will not lead to the exocytosis of those vesicles. Figures 3E

and 3F show that even after stimulation for 150 seconds at 10 Hz, nerve terminals overexpressing αsyn undergo less synaptic vesicle exocytosis than control terminals. Alternatively, lowering the external Ca^{2+} concentration to 1mM from 2mM, which decreases the rate of exocytosis but should not effect recycling pool size, does not lead to a decrease in the amount of exocytosis after 150 seconds of stimulation at 10 Hz (Figure 3E, 3F). Thus, αsyn overexpression decreases the size of the recycling pool, and because the size of the total vesicle pool is unchanged (Figure 2C), concomitantly increases the size of the reserve pool of synaptic vesicles.

Overexpression of αsyn inhibits release from the readily releasable pool of synaptic vesicles

The readily releasable pool of synaptic vesicles mediates responses to small numbers of action potentials at nerve terminals, and likely mediates the majority of ongoing synaptic transmission *in vivo*. To determine if neurotransmitter release mediated by vesicles from the readily releasable pool is inhibited by αsyn overexpression, we delivered a 30 Hz stimulus for 3 seconds, which has been used previously to selectively activate this vesicle pool (Pyle et al., 2000). Similar to the longer stimuli, release from the readily releasable pool was also inhibited by αsyn overexpression (Figure 4A, 4B). In addition to brief intense electrical stimulation, release from this vesicle pool can also be elicited using hypertonic sucrose (Rosenmund and Stevens, 1996). We find that synaptic vesicle release elicited by hypertonic sucrose is also inhibited by αsyn overexpression (Figure 4C). Because the readily releasable pool of vesicles are a subset of the recycling pool of vesicles, the inhibition of release from this pool is likely due to the overall

reduction in recycling pool size. Further, because hypertonic sucrose stimulated exocytosis is calcium independent (Rosenmund and Stevens, 1996), αsyn overexpression does not reduce recycling pool size through a calcium-sensitive mechanism.

PD-associated mutations modulate the effect of αsyn overexpression on synaptic vesicle release

Three point mutations in the N-terminus of αsyn cause autosomal dominant PD (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). One of the mutations, A30P, abolishes the synaptic localization of αsyn (Fortin et al., 2004) whereas the other mutations, A53T and E46K, do not affect its localization to the nerve terminal (Fortin et al., 2004). We sought to determine how the mutations that cause PD affect the ability of αsyn overexpression to mediate a reduction in the size of the recycling vesicle pool. We find that the A30P mutation abolishes the inhibitory effect of αsyn overexpression on neurotransmitter release (Figure 5A, 5B). Quantitative immunofluorescence using a human αsyn specific antibody (15G7) showed no difference in the expression level of the wildtype versus A30P mutant protein (data not shown), ruling out reduced expression of the mutant protein as a cause for the loss of inhibition. Because this mutation disrupts the association of αsyn with membranes (Fortin et al., 2004; Jo et al., 2002), this experiment shows that the localization of αsyn to the nerve terminal and its interaction with synaptic vesicle membranes are critical to its inhibitory effect on neurotransmission. Conversely, the A53T and E46K mutations do not affect the inhibitory action of αsyn on synaptic vesicle release (Figure 5C, 5D).

The N-terminal domain of αsyn is sufficient for its inhibitory effect on synaptic vesicle release

Alpha-synuclein is a small natively unstructured protein in solution. However, upon binding to membranes, the N-terminus adopts an amphipathic alpha-helical structure while the C-terminus remains unstructured. This N-terminal domain is necessary and sufficient for the synaptic localization of αsyn (Specht et al., 2005). To determine if the N-terminal membrane binding domain of αsyn is sufficient for its ability to mediate a reduction in recycling pool size, we coexpressed a truncated form of αsyn containing only the first 110 amino acids (αsyn 1-110) with VGLUT1-pHluorin in hippocampal neurons. We found that overexpression of the N-terminal domain of αsyn was sufficient to mediate the reduction in recycling pool size (Figure 6A, 6B).

Increased alpha-synuclein expression inhibits synaptic transmission *in vivo*

To test whether αsyn inhibits neurotransmitter release *in vivo*, we generated transgenic mice expressing human αsyn under control of the Syrian hamster prion promoter to drive expression of αsyn in all neurons (Figure 7). These mice express αsyn approximately 3 fold over endogenous levels, which mirrors the level of overexpression used in our dissociated cultures and also is similar to the level of overexpression in humans with triplication of the gene locus containing αsyn (Singleton et al., 2003). We measured basal synaptic transmission by recording field excitatory postsynaptic potentials (fEPSPs) from synapses made by CA3 Schaffer collateral terminals onto CA1 stratum radiatum dendrites in the hippocampus. Remarkably, we found that increased expression of αsyn inhibited basal synaptic transmission *in vivo* (Figure 8A, 8B, 8C). To

confirm that the reduction observed was due to a presynaptic deficit in neurotransmission, we measured the paired pulse ratio (PPR) with a 40 ms interstimulus interval. This synapse is characteristically displays paired pulse facilitation with short interstimulus intervals, presumably due to residual Ca^{2+} remaining in the terminal. Manipulations that cause a presynaptic reduction in the probability of release are usually manifested by an increase in the PPR. Accordingly, we observe a significant increase in the PPR, confirming our imaging experiments that αsyn inhibits neurotransmitter release presynaptically (Figure 8D).

Alpha-synuclein inhibits neurotransmitter release in midbrain dopamine neurons

Although it is now clear that many cell types other than dopamine neurons are affected by PD (Braak et al., 2003; Braak et al., 2004), sporadic PD is classically characterized by the loss of dopamine neurons from the substantia nigra. Therefore, it is of great interest how the physiology of dopamine neurons is altered by increased expression of αsyn. To test how overexpression of αsyn affects synaptic vesicle recycling in dopamine neurons, we cotransfected human αsyn with VGLUT1-pHluorin into postnatal cultures from the ventral midbrain. These cultures typically contain greater than 90% dopaminergic neurons, as detected by staining for tyrosine hydroxylase (TH) (data not shown). After live imaging, coverslips were fixed and immunostained for TH to ensure that only TH+ transfected boutons were used for analysis (Figure 9A). We found that, similar to hippocampal neurons, dopamine neurons that overexpress αsyn have less synaptic vesicle exocytosis when compared to control transfected dopamine neurons (Figure 9B, 9C). This data shows that the physiological mechanisms that

mediate the reduction in recycling pool size with increased expression of αsyn are similar between hippocampal neurons and midbrain dopamine neurons.

Alpha-synuclein overexpression selectively reduces levels of synapsins and complexins

Because increased expression of αsyn reduces the size of the recycling pool of synaptic vesicles, we hypothesized that the molecular composition of nerve terminals would be altered in the αsyn transgenic mice. To test this idea, we performed quantitative Western analysis for many synaptic proteins, including peripheral proteins of SVs, integral membrane proteins of SVs, synaptic SNARE proteins, and active zone proteins. We find that the overall levels of integral membrane proteins of SVs and SNARE proteins are unchanged, making it unlikely that the decrease in neurotransmitter release observed in αsyn transgenic mice is due to an overall reduction in the number of synaptic vesicles (Rosahl et al., 1995). However, we do find relatively large reductions in synapsin IIb and complexin 2, with smaller reductions of synapsins Ia, Ib, IIa, and complexin 1 (Figure 10).

DISCUSSION

In this study we find that modest overexpression of αsyn inhibits neurotransmitter release by selectively reducing the size of the recycling pool of synaptic vesicles. Further, a mutation linked to autosomal dominant PD, A30P, completely abolishes this inhibition, presumably through its inability to bind to synaptic vesicles and localize to the nerve terminal. We also find that increased αsyn expression in transgenic mice causes a reduction in neurotransmitter release in *ex vivo* hippocampal slices. Increased expression of αsyn affects neurotransmitter release in both hippocampal and midbrain dopamine neurons. Since αsyn is normally expressed widely in many neuronal subtypes, these results suggest that the regulation of recycling pool size represents a physiological function of αsyn rather than an abnormal function only in neurons susceptible to degeneration in PD.

To study the effect of αsyn overexpression on nerve terminal function, we elected first to use rat hippocampal neurons for several reasons. First, the properties of exocytosis and endocytosis in the nerve terminal have been extensively characterized in synapses from these neurons (Fernandez-Alfonso and Ryan, 2006; Lisman et al., 2007). Second, αsyn is most highly expressed in cortex and hippocampus (Iwai et al., 1995), suggesting that these neurons, in particular, would serve as a good model system to understand the normal function of αsyn. Finally, humans with early onset Parkinsonism due to triplication of the gene locus containing αsyn have significant Lewy body formation and neuronal loss in the hippocampus and cortex, in addition to the substantia nigra (Singleton et al., 2003). This clinical data emphasizes the importance of understanding the function of αsyn in a variety of neuronal subtypes.

The data presented in this paper, along with a growing body of evidence from other labs, clearly establishes a role for αsyn as an inhibitory factor in neurotransmitter release. We used overexpression of human αsyn to study the normal function of this protein for several reasons. First, the data obtained from knockout animals have shown small effects that are somewhat conflicting. The first knockout of αsyn showed a subtle acceleration of neurotransmitter release as evidenced by decreased paired pulse depression at nigrostriatal synapses. (Abeliovich et al., 2000). Because the different synuclein isoforms are most likely functionally redundant, it is possible that compensation by either βsyn or γsyn underlie the subtle phenotype in the αsyn knockout. In line with this reasoning, a recent study looking at dopamine release in the striatum found increased release in response to single stimuli or burst stimuli in αsyn and γsyn double knockout mice, but not in the single knockouts (Senior et al., 2008). However, no impairment of neurotransmission was detected in a double knockout of both αsyn and βsyn (Chandra et al., 2004). Another experiment performed using *in vivo* voltammetry from the striatum in mice carrying a spontaneous deletion of αsyn showed increased filling of the readily releasable pool of vesicles (Yavich et al., 2004). Another knockout of αsyn showed a severe reduction in distal pool synaptic vesicles, and deficits in the response to prolonged stimulation (Cabin et al., 2002). However, the amounts of synaptic vesicle proteins are unchanged making the reduction in synaptic vesicle number by electron microscopy and the corresponding physiology data difficult to interpret. Another reason we believed that overexpression of αsyn would yield insight into its normal function is that the protein normally associates very loosely with synaptic vesicles (Fortin et al., 2005). Therefore, deletion of the protein may not reveal an experimentally

detectable defect. Indeed, we were not able to resolve a significant increase in recycling pool size with deletion of αsyn alone (Figure 2).

Conversely, overexpression of αsyn has yielded several important insights that converge on a role for αsyn in vesicle trafficking and presynaptic function. Cooper and colleagues showed that αsyn overexpression in yeast blocked ER to Golgi trafficking, and that overexpression of the Rab GTPase Ypt1p rescued this phenotype (Cooper et al., 2006). They also showed that overexpressing the Ypt1p mammalian homolog Rab1 rescued toxicity due to expression of the αsyn mutant A53T in several model systems, although a block in ER to Golgi trafficking in these systems was not shown. Our biochemical data shows that the expression level of many SV proteins is unaffected, and our imaging data shows that the targeting of VGLUT1-pHluorin to nerve terminals is unperturbed, suggesting that impaired ER to Golgi traffic does not explain our results. However, a general role for αsyn in the inhibition of vesicular trafficking is consistent with our data showing that αsyn inhibits SV exocytosis.

Another recent study showed that αsyn acts as a protective factor preventing neurodegeneration in mice lacking cysteine string protein α (CSPα) (Chandra et al., 2005). In this study, overexpression of αsyn rescued neuron loss, the behavior motor phenotype, and the decrease in SNARE complex formation caused by deletion of CSPα. CSPα is a nerve terminal chaperone protein that prevents the accumulation of misfolded molecules that accumulate during synaptic activity. Interestingly, αsyn A30P did not rescue the CSPα KO, suggesting that membrane binding is necessary for the function of αsyn in this respect. An intriguing explanation for this rescue would be that overexpression of wildtype αsyn, but not A30P, reduced neurotransmitter release in the

CSPα KO mouse, thus reducing the need for a synaptic chaperone. Further experiments would be necessary to test this hypothesis.

Finally, in PC12 cells and chromaffin cells, αsyn overexpression substantially inhibited catecholamine release measured by directly recording dopamine release (Larsen et al., 2006); however, αsyn is not normally expressed at high levels in these cell types. Our observation that αsyn inhibits SV release in both hippocampal neurons and dopamine neurons agrees nicely with this previous data. However, we show that the A30P mutation, which greatly reduces membrane binding and abolishes nerve terminal localization, also prevents inhibition of SV release by αsyn. It is possible that the degree of overexpression used by Larsen and colleagues was enough to overcome the reduced membrane binding capacity of αsyn A30P. Alternatively, this discrepancy might represent a fundamental difference in the cell types used (cell lines versus primary neurons). In this case, it emphasizes the importance of using neurons to confirm and extend observations on both the physiological and pathological functions of αsyn.

The advantage of the approach we use in our experiments is the ability to visualize the entire SV cycle. We have identified a selective defect in SV exocytosis, and further show that increased expression of αsyn reduces the size of the SV recycling pool, and effectively increases the size of the SV reserve pool. The molecular determinants of recycling pool and reserve pool size are poorly understood. Morphologically, in both mammalian and *Drosophila* synapses, recycling pool vesicles are present closer to the active zone whereas reserve pool vesicles are generally further from the active zone (Kuromi and Kidokoro, 1998; Schikorski and Stevens, 2001). Similarly, αsyn and the synapsins also preferentially localize to vesicles further from the active zone (Clayton

and George, 1999; Pieribone et al., 1995; Tao-Cheng, 2006). It is possible that αsyn acts by reducing synapsin levels to control recycling pool size. Indeed, synapsin knockouts show reduced recycling pool size and increased depression in response to repetitive sitmulation (Gitler et al., 2004; Rosahl et al., 1995; Ryan et al., 1996). It is also possible that αsyn interacts with the complexins to regulate synaptic vesicle pools. Interestingly, mice null for both αsyn and βsyn have increased complexin levels (Chandra et al., 2004), confirming that the decrease in complexin levels we observe likely represents a functional relationship between the two proteins. Although much is known about the proteins that constitute generic synaptic vesicles (Takamori et al., 2006), further understanding of the molecular makeup of vesicles from individual functional pools will be needed to fully understand the interactions of αsyn at the nerve terminal.

Finally, these experiments clarify our understanding of the normal function of αsyn, but it remains unknown whether autosomal dominant forms of PD are caused by a pathological gain of function or an increase in normal function. However, PD-linked mutations of LRRK2 have been shown to increase its kinase function (Gloeckner et al., 2006; West et al., 2005). It is possible that impairment of neurotransmitter release sensitizes midbrain dopamine neurons to an endogenous or exogenous toxin that leads to their degeneration. However, it remains unclear how the A30P mutant could cause PD through this mechanism because it has a greatly diminished capacity to inhibit neurotransmitter release. However, rat αsyn A53T does not cause toxicity when expressed in mice whereas human αsyn A53T does . Therefore, it is possible that species differences could underlie the lack of inhibition by αsyn A30P in our experiments.

EXPERIMENTAL PROCEDURES

Reagents

The rat monoclonal antibody to human αsyn (15G7) was obtained from Alexis Biochemicals (San Diego, CA); the rabbit polycloncal and mouse monoclonal antibodies to GFP were obtained from Invitrogen (San Diego, CA); the mouse monoclonal antibody to recognizing both rat and human αsyn (syn-1) was obtained from BD Transduction labs (San Jose, CA); the mouse monoclonal antibody to actin was obtained from Millipore (Billerica, MA); the rabbit tyrosine hydroxylase antibody was obtained from Pelfreez (Rogers, AR); the VAMP2, rab3 and munc13 mouse monoclonal antibodies were obtained from Synaptic Systems; the SNAP25, synapsin I & II, synaptotagmin, complexin I & II, and proton pump rabbit polyclonal antibodies were obtained from Synaptic Systems (Goettingen, Germany); the synaptophysin rabbit polyclonal antibody was obtained from Zymed; the syntaxin mouse monoclonal antibody was obtained from Sigma; the SV2 antibody was a generous gift of Regis Kelly (University of California, San Francisco). Secondary antibodies conjugated to Cy3 or Cy5 were purchased from Jackson ImmunoResearch (West Grove, PA). Secondary antibodies conjugated to Alexa 488 or Alexa 594 were purchased from Invitrogen (San Diego, CA). Secondary antibodies conjugated to IR800 were purchased from Rockland (Philadelphia, PA).

Molecular Biology

pCAGGS-VGLUT1-pHluorin was generated as described (Voglmaier et al., 2006). Human αsyn was subcloned into the chicken actin vector pCAGGS (gift of J. L. R. Rubenstein, University of California, San Francisco) with a preceding 5' Kozak

consensus sequence to optimize translation in mammalian cells. Point mutations were introduced using Quikchange (Stratagene). For C-terminal deletions, a stop codon was introduced at the appropriate residue with the rest of the construct unchanged. For FM experiments, human αsyn was inserted upstream of the internal ribosomal entry site (IRES) sequence in pCAGGS-IRES2-GFP. All constructs were verified by sequence analysis.

Neuronal Culture & Transfections

Primary hippocampal neurons were prepared as previously described (Li et al., 2005) with modifications. Briefly, hippocampi from embryonic day 19 (E19) rats were dissociated in Hanks' Balanced Salt Solution (HBSS) containing trypsin EDTA (Gibco BRL), treated with trypsin inhibitor, washed several times in DMEM with GlutaMAX (Gibco BRL) containing 10% FBS, triturated, and electroporated with 3μg total DNA per 3 x 10^6 cells (Amaxa). In cotransfection experiments, 1.5μg VGLUT1-pHluorin DNA and 1.5μg pCAGGS or pCAGGS-αsyn DNA was used. After transfection, cells were recovered for 10 minutes in RPMI and plated in DMEM with GlutaMAX containing 10% fetal bovine serum (FBS) at a density of 477 cells/mm^2 onto coverslips coated with poly-L-lysine (Sigma). After 3-5 hours, the media was changed to Neurobasal containing 2% B27, 1% GlutaMAX, 1% FBS, 0.5% insulin-transferrin-selenium supplement (Gibco BRL), and 0.2% Primocin (Amaxa). Uridine and 5-fluorodeoxyuridine (5-FU) at 10μM were added at 5 days *in vitro* (DIV) to inhibit glial growth. Neurons were fed with FUDR and uridine containing media every 4-6 days. For FM experiments, neurons were

grown as above except that neurons were plated at 177-283 cells/mm^2 and grown inverted on a monolayer of glial cells (reference – Banker).

For hippocampal cultures from mice, hippocampi from P0 – P1 αsyn knockout (KO) (Abeliovich et al., 2000) or wildtype littermate pups were dissociated in 0.25% trypsin, washed several times in HBSS containing 10 mM HEPES and 20mM glucose, triturated, and electroporated with 0.6μg total DNA per 500,000 cells (Amaxa). In cotransfection experiments, 0.3μg VGLUT1-pHluorin DNA and 0.3μg pCAGGS or pCAGGS-αsyn DNA was used. After transfection, cells were recovered for 10 minutes and plated in MEM containing 21 mM glucose, 5% FBS, 2% B27, 1% Glutamax, and Mito+ serum extender (BD Biosciences) at a density of 1768 cells/mm^2 onto coverslips coated with poly-L-lysine (Sigma). Uridine and 5-FU at 10μM were added at 3-5 days *in vitro* (DIV) to inhibit glial growth. Neurons were fed with FUDR and uridine containing media every 4-6 days.

Postnatal rat midbrain cultures were prepared from the ventral midbrain of P0-P1 rats as described with modifications (Mena et al., 1997). Briefly, the ventral midbrain was dissected, the tissue digested in 20 units/mL papain for 15 minutes, mechanically dissociated, and electroporated with 0.6 μg total DNA per 500,000 cells (Amaxa). Neurons were plated onto astrocyte feeder layers at a density of 1415 – 2830 cells/mm^2. After 2 hours, 10 μg/mL GDNF, 6.7 μg/mL 5-FU, and 16.5 μg/mL uridine were added to the media to promote dopamine neuron survival and inhibit mitotic cell proliferation.

Immunofluorescence

After 14-28 DIV, neurons were fixed in 4% paraformaldehyde, then permeabilized and blocked in IF buffer (0.1% Triton X-100 or 0.05% saponin and 5% FBS in phosphate buffered saline (PBS)). Neurons were incubated with primary antibody overnight at 4°C, washed 3 times in IF buffer, incubated with appropriate secondary antibodies for 1.5 hours at room temperature in the dark, washed again 3 times in IF buffer, rinsed in PBS, then mounted onto slides using Prolong Gold Antifade reagent (Invitrogen).

For staining tissue sections, animals were transcardially perfused with 4% paraformaldehyde in PBS, then the brains were removed and postfixed in the same solution overnight at 4°C. The brains were cryoprotected in 30% sucrose for 2-3 days, frozen in 2-methylbutane, and sectioned using a cryostat. Floating sections were permeabilized in 0.1% Triton X-100 for 15 minutes, blocked in IF buffer, incubated with primary antibody overnight at 4°C, washed 3 times in IF buffer, incubated with appropriate secondary antibodies for 1.5 hours at room temperature in the dark, washed again 3 times in IF buffer, rinsed in PBS, then mounted onto slides using Fluoromount-G (Southern Biotech).

Generation of αsyn transgenic mice

A human αsyn cDNA was subcloned into the cosSHa.Tet cosmid vector (a gift from S. B. Prusiner, University of California San Francisco) downstream of the Syrian hamster prion protein promoter. The transgene construct was linearized and injected into fertilized ova from FVB mice. Transgene expression in brain was confirmed by Western and immunofluorescence analyses.

Biochemistry

To quantitate the level of αsyn and other synaptic proteins in transgenic animals, cortices from animals sacrificed for electrophysiology were homogenized using a Dounce homogenizer in buffer containing 320 mM sucrose, 4 mM HEPES-NaOH pH 7.5, 1% Triton X-100, Complete protease inhibitor cocktail (Roche), 1mM MgEGTA, 200μM PMSF, and 1 μg/mL Pepstatin A. The homogenate was centrifuged at 1300 x g to remove nuclei and cell debris. Equal amounts of protein from the supernatant were separated on a SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and processed for Western analysis using appropriate antibodies against synaptic vesicle proteins, an actin antibody as a loading control, and secondary antibodies conjugated to IR800 (Rockland) or Cy5. The fluorescence protein bands were detected using the Licor imaging system (Licor). Protein expression level was quantitated by normalizing each band intensity to the actin band intensity to control for protein loading and transfer efficiency. Samples from each animal were run in triplicate, and the levels from 3 different animals for each genotype averaged.

Live Cell Imaging

Transfected neurons were imaged between 14-21 DIV. Coverslips were mounted in a laminar-flow perfusion chamber on an inverted Nikon TE300 fluorescence microscope and imaged in Tyrode's buffer (containing 126.5 mM NaCl, 10 mM HEPES-NaOH pH 7.4, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2.5 mM KCl, 30 mM Glucose, 10 μM 6-cyano-7 nitroquinoxaline-2,3-dione (CNQX) and 10 μM 3-(2-carboxypiperazin-4-yl)propyl-1-

phosphonic acid (CPP)). Images were obtained under epifluorescence illumination using a 63x 1.2NA water objective and an Orca ER CCD cameria (Hamamatsu), using 2x2 on-chip pixel binning. The fluorescence of individual nerve terminals was quantitated by manually placing 4x4 regions of interest (ROIs) over the center of fluorescence puncta. The fluorescence in these regions was averaged, subtracted by the average of 3 ROIs placed in regions of the field without cell bodies or processes, and then the fractional change in fluorescence over time normalized to the initial fluorescence ($\Delta F/F_0$) was calculated and plotted. Traces represent a single experiment in which 20 boutons were selected per coverslip, and the data from 3 coverslips were averaged. For quantitation of peak $\Delta F/F_0$, 20 boutons were selected per coverslip, and 9 coverslips from 3 independent transfections were averaged. All imaging and quantitation was performed blind to the transfected constructs.

To measure recycling pool size using FM 4-64, neurons between 14 – 21 DIV and transfected with either pCAGGS-IRES2-GFP or pCAGGS-αsyn-IRES2-GFP were incubated in Tyrode's buffer containing 15 μM FM 4-64 and stimulated at 10 Hz for 60 seconds. Dye was left on the neurons for 1 additional minute after the end of the stimulus to allow for complete endocytosis to occur. After extensive washing in Tyrode's buffer for 10 – 15 minutes, FM 4-64 was unloaded by stimulating at 10 Hz for 120 seconds. The pool size was determined by quantitating the amount of FM fluorescence released by the second stimulus at boutons expressing GFP.

Electrophysiology

Transverse hippocampal slices for fEPSP recording (400 μm thick) were prepared from 24- to 36-day-old animals hemizygous for the human αsyn transgene and their wild type littermates in ice-cold high sucrose cutting solution, containing (in mM): NaCl 50, KCl 2.5, $CaCl_2$ 0.5, $MgCl_2$ 7, NaH_2PO_4 1.0, $NaHCO_3$ 25, glucose 10 and sucrose 150. Freshly cut slices were placed in an incubating chamber containing prewarmed (37°C) artificial cerebrospinal fluid (ACSF), containing NaCl 119, KCl 2.5, $CaCl_2$ 2.5, $MgSO_4$ 1.3, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, and glucose 11, and recovered at 37 °C for 1 h. Slices were then maintained in ACSF at room temperature (23 °C–26 °C) prior to recording.

fEPSPs were evoked in CA1 stratum radiatum by stimulation of Schaffer collaterals with a bipolar tungsten stimulating electrode, and were recorded with ACSF filled 3–6 MΩ glass pipettes using an MultiClamp 700A amplifier (Axon Instruments). The perfusion rate was ~2ml/min. Test stimuli consisted of 100 μs pulses of constant voltage delivered by stimulus isolation units (Digitimer Ltd). The stimulation rate was 0.1 Hz for all fEPSP recording experiments. Paired pulse facilitation was performed by delivery of two stimuli at an interval of 40 ms. The slices from transgenic mice and wild type littermates were interleaved, and the experimenter was blind to genotype. fEPSPs were filtered at 2 kHz, digitized at 10 kHz, and stored on computers using IgorPro (Wavemetrics Inc.).

Statistical Analysis

All data are presented as mean ± SEM unless otherwise indicated in the figure legends. Comparisons were made using unpaired, two-tailed t-tests or one-way ANOVA as appropriate.

FIGURES

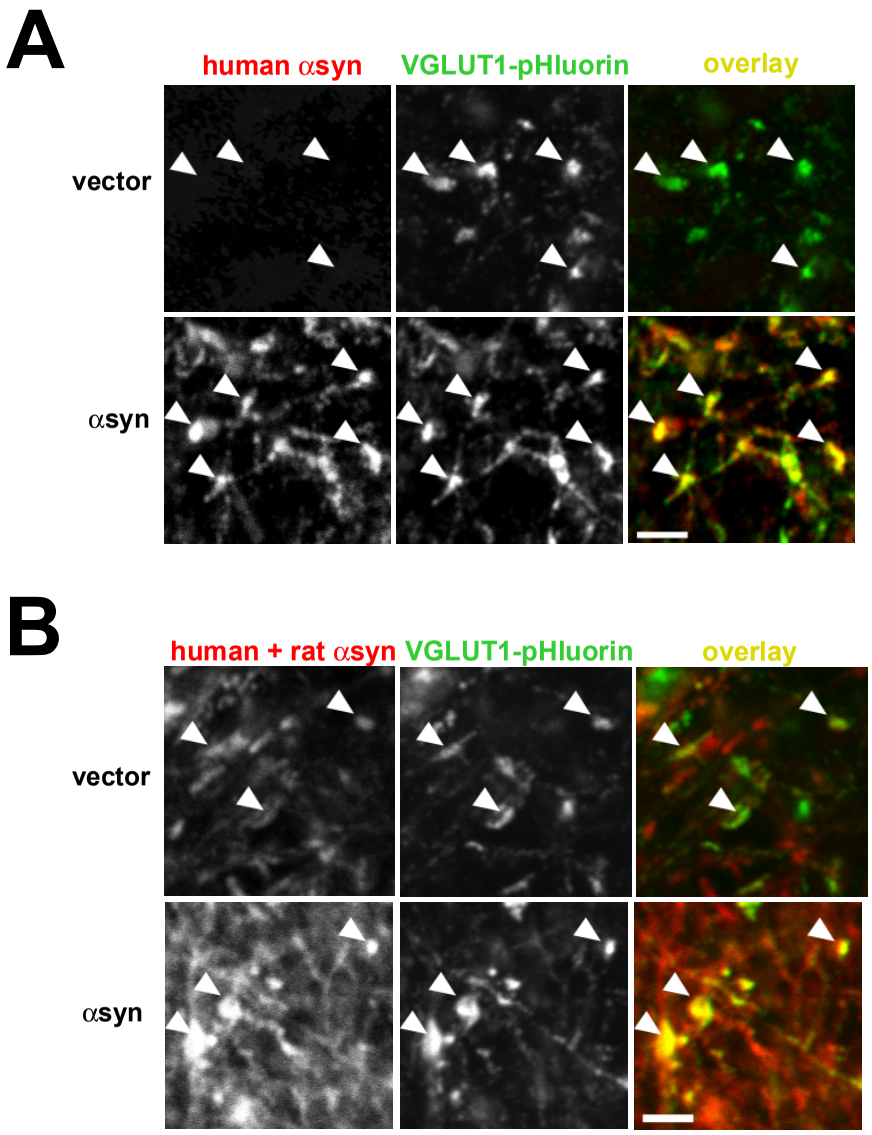
Figure 1 – Overexpression of αsyn at nerve terminals coexpressing VGLUT1-pHluorin

Hippocampal neurons were transfected with human αsyn and VGLUT1-pHluorin, then immunostained after 2-3 weeks in culture.

(A) Human αsyn is detected using the human specific αsyn antibody, 15G7, and VGLUT1-pHluorin is detected using a polyclonal antibody against GFP. Arrowheads highlight colocalization of αsyn and VGLUT1-pHluorin at nerve terminals. Scale bar = 2.5 μm.

(B) αsyn is detected using an antibody that recognizes both the rat and human proteins, and VGLUT1-pHluorin is detected using a polyclonal antibody against GFP. Arrowheads highlight VGLUT1-pHluorin positive nerve terminals showing increased expression of αsyn. Scale bar = 2.5 μm.

(C) αsyn expression at VGLUT1-pHluorin positive nerve terminals was measured using quantitative immunofluorescence using the syn-1 antibody to detect both the human and rat proteins. n = 12 coverslips (300 nerve terminals) per group from 2 independent transfections. The values represent mean ± SEM.


A
human αsyn
VGLUT1-pHluorin
overlay
vector
αsyn
B
human + rat αsyn
VGLUT1-pHluorin
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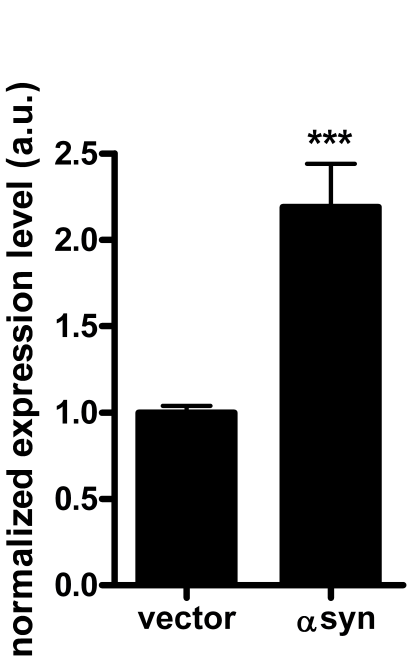

C
normalized expression level (a.u.)
2.5
2.0
1.5
1.0
0.5
0.0
***
vector
αsyn

Figure 1

Figure 2 – Overexpression of αsyn inhibits synaptic vesicle release at hippocampal synapses

(A) Timecourse of VGLUT1-pHluorin fluorescence during a 10 Hz stimulus for 60 seconds in neurons coexpressing either vector control or human αsyn. n = 3 coverslips, 60 nerve terminals for each condition from a single transfection. The error bars represent SEM.

(B) Peak $\Delta F/F_0$ values at the end of the stimulus. Values are normalized to the response in the vector control condition. n = 9 coverslips, 180 nerve terminals for each condition from 3 independent transfections. The values represent mean ± SEM. Two-tailed, unpaired t-test, $p < 0.05$.

(C) Fluorescence at nerve terminals after addition of Tyrode's buffer containing 50mM NH_4Cl. Values are normalized to the fluorescence obtained in the vector control condition. n = 9 coverslips, 180 nerve terminals for each condition from 3 independent transfections. The values represent mean ± SEM.

(D) Frequency histogram showing the peak $\Delta F/F_0$ VGLUT1-pHluorin response over a large number of synapses in response to a 10 Hz stimulus for 60 seconds. n = 9 coverslips, 1809 nerve terminals for the vector condition, 1428 nerve terminals for the αsyn condition, from 3 independent transfections.

(E) The rate of endocytosis after the end of a 10 Hz stimulus for 60 seconds. Fluorescence decay was fit by a single exponential. n = 9 coverslips, 180 nerve terminals for each condition from 3 independent transfections. Values represent mean ± SEM.

(F) Timecourse of VGLUT1-pHluorin fluorescence during a 10 Hz stimulus for 60 seconds in hippocampal neurons cultured from αsyn knockout mice coexpressing either

vector control or human αsyn. n = 6 coverslips, 120 nerve terminals for each condition from 2 independent transfections. The error bars represent SEM.

(G) Peak $\Delta F/F_0$ values after a 10 Hz stimulus for 60 seconds in either wildtype or αsyn knockout neurons expressing VGLUT1-pHluorin alone. n = 6 coverslips, 120 nerve terminals for each condition from 2 independent transfections. Values represent mean ± SEM.

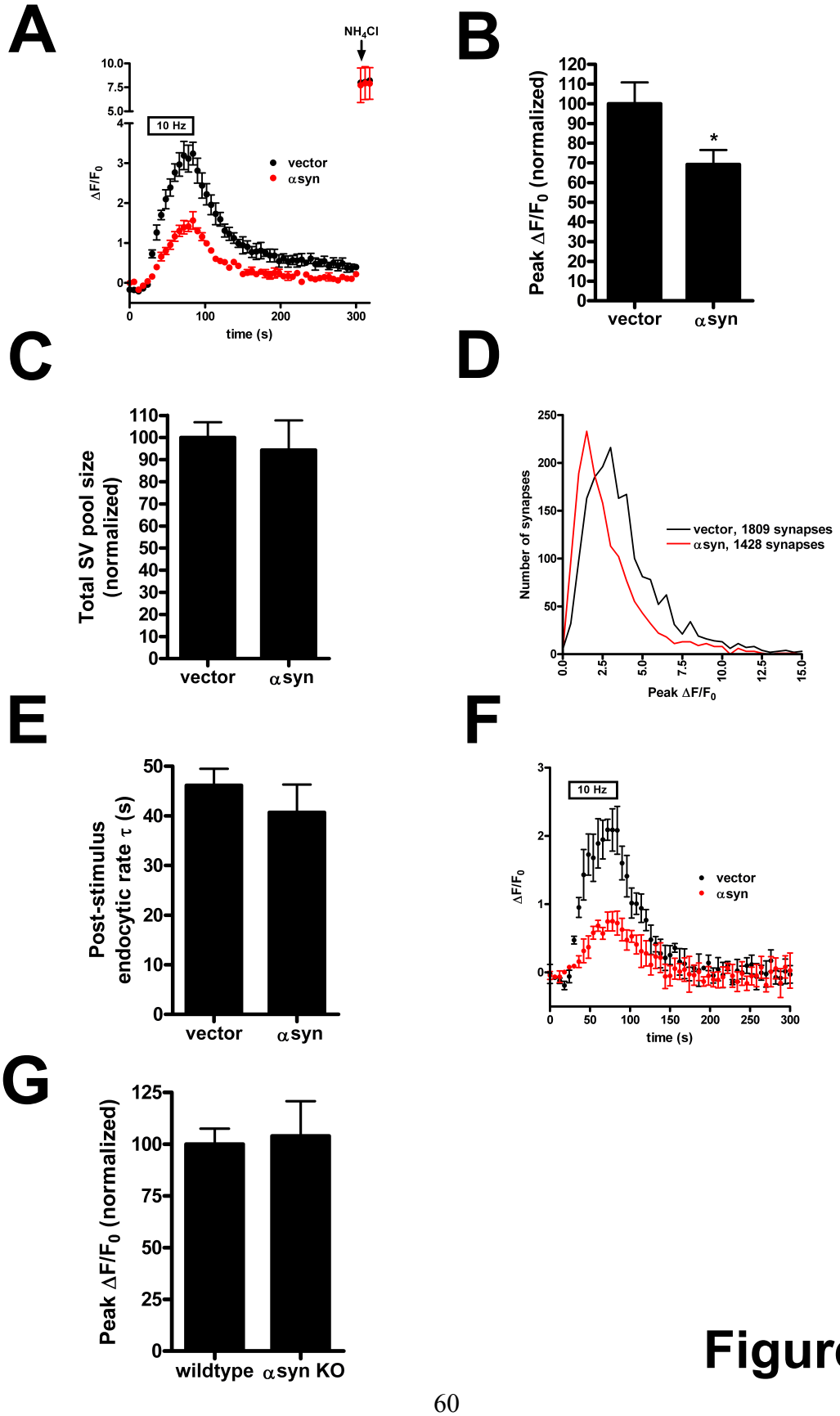

A
NH4Cl
10.0
7.5
5.0
4
3
2
1
0
10 Hz
vector
αsyn
ΔF/F0
0
100
200
300
time (s)
B
Peak ΔF/F0 (normalized)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
vector
αsyn
C
Total SV pool size (normalized)
110
100
90
80
70
60
50
40
30
20
10
0
vector
αsyn
D
Number of synapses
250
200
150
100
50
0
vector, 1809 synapses
αsyn, 1428 synapses
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Peak ΔF/F0
E
Post-stimulus endocytic rate τ (s)
50
40
30
20
10
0
vector
αsyn
F
3
2
1
0
10 Hz
ΔF/F0
vector
αsyn
0
50
100
150
200
250
300
time (s)
G
Peak ΔF/F0 (normalized)
125
100
75
50
25
0
wildtype
αsyn KO

Figure 2

Figure 3 – Overexpression of αsyn decreases the size of the recycling pool of synaptic vesicles

(A) Schematic showing details of FM experiment.

(B) Hippocampal neurons transfected with either IRES2-GFP or αsyn-IRES2-GFP were loaded with 15 μM FM 4-64 during a 10Hz stimulus for 60 seconds. After 60 additional seconds of dye exposure to allow for complete endocytosis, extracellular dye was washed off by extensive washing. The trace shows the subsequent destaining of GFP positive FM 4-64 labeled terminals. n = 3 coverslips, 73 nerve terminals for the vector condition, 71 nerve terminals for the αsyn condition, from a single transfection. Values represent mean ± SEM.

(C) Extent of specific FM 4-64 dye loading measured as releasable dye during a 10 Hz stimulus for 120 seconds. n = 6 coverslips, 208 nerve terminals for the vector condition, 153 nerve terminals for the αsyn condition, from 2 independent transfections. Values represent mean ± SEM.

(D) Kinetics of FM 4-64 dye release during the initial 60 seconds of stimulation at 10 Hz. n = 3 coverslips, 73 nerve terminals for the vector condition, 71 nerve terminals for the αsyn condition, from a single transfection. Error bars represent SEM.

(E) Timecourse of VGLUT1-pHluorin fluorescence during a 10 Hz stimulus for 150 seconds in the presence of 1 μM bafilomycin to inhibit synaptic vesicle reacidification after endocytosis. Vector transfected neurons were stimulated in Tyrode's buffer containing either 2 mM or 1 mM Ca^{2+} whereas human αsyn transfected neurons were stimulated in Tyrode's buffer containing 2 mM Ca^{2+}. n = 3 coverslips, 60 boutons per condition from a single transfection.

(F) Peak $\Delta F/F_0$ values after a 10 Hz stimulus for 150 seconds in the noted conditions. n = 6 coverslips, 120 boutons per condition from 2 independent transfections. Data represents mean ± SEM. One-way ANOVA, $p < 0.01$. Tukey's post hoc tests: vector 2 mM Ca^{2+} versus vector 1 mM Ca^{2+}, $p > 0.05$; vector 2 mM Ca^{2+} versus αsyn 2 mM Ca^{2+}, $p < 0.01$; vector 1 mM Ca^{2+} versus αsyn 2 mM Ca^{2+}, $p < 0.05$.

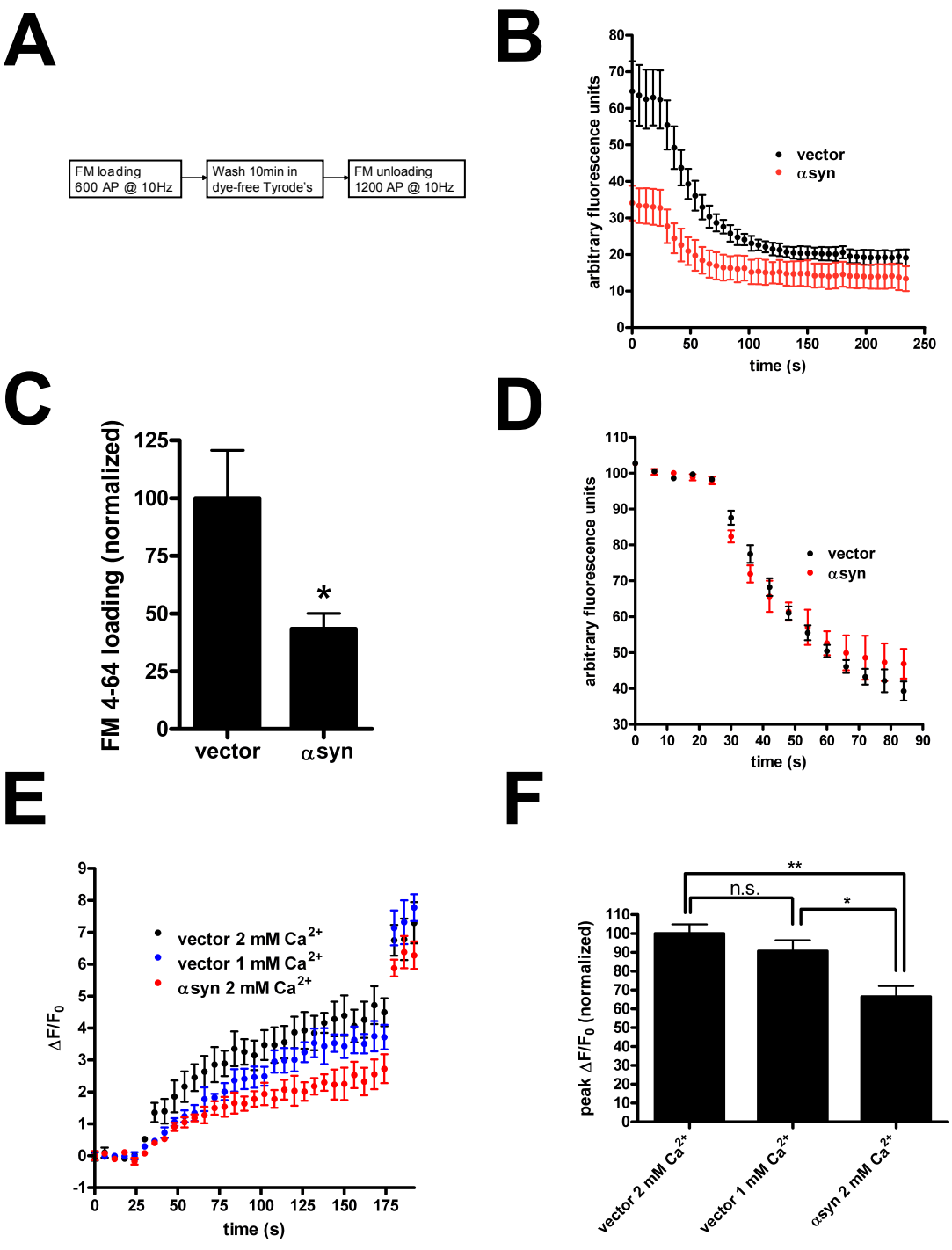

A
FM loading
600 AP @ 10Hz
Wash 10min in
dye-free Tyrode's
FM unloading
1200 AP @ 10Hz
B
arbitrary fluorescence units
time (s)
vector
αsyn
C
FM 4-64 loading (normalized)
vector
αsyn
*
D
arbitrary fluorescence units
time (s)
vector
αsyn
E
ΔF/F0
time (s)
vector 2 mM Ca2+
vector 1 mM Ca2+
αsyn 2 mM Ca2+
F
peak ΔF/F0 (normalized)
n.s.
**
*
vector 2 mM Ca2+
vector 1 mM Ca2+
αsyn 2 mM Ca2+

Figure 3

Figure 4 – Overexpression of αsyn inhibits release from the readily releasable pool of synaptic vesicles

(A) Time course of VGLUT1-pHluorin fluorescence during a 30 Hz stimulus for 3 seconds to selectively activate the readily releasable pool in neurons coexpressing either vector control or human αsyn. n = 3 coverslips, 60 boutons per condition from a single transfection. The error bars represent SEM.

(B) Peak $\Delta F/F_0$ values at the end of the stimulus. Values are normalized to the response in the vector control condition. n = 6 coverslips, 120 boutons per condition from 2 independent transfections. The values represent mean ± SEM. Two-tailed, unpaired t-test, $p < 0.0001$.

(C) VGLUT1-pHluorin expressing neurons were stimulated with 500mM hypertonic sucrose in Tyrode's solution in the presence of 1μM bafilomycin to prevent reacidification of endocytosed vesicles. The change in $\Delta F/F_0$ fluorescence signal after the stimulus compared to before the stimulus is plotted. n = 9 coverslips, 180 boutons per condition from 3 independent transfections. The error bars represent SEM. Two-tailed, unpaired t-test, $p < 0.05$.

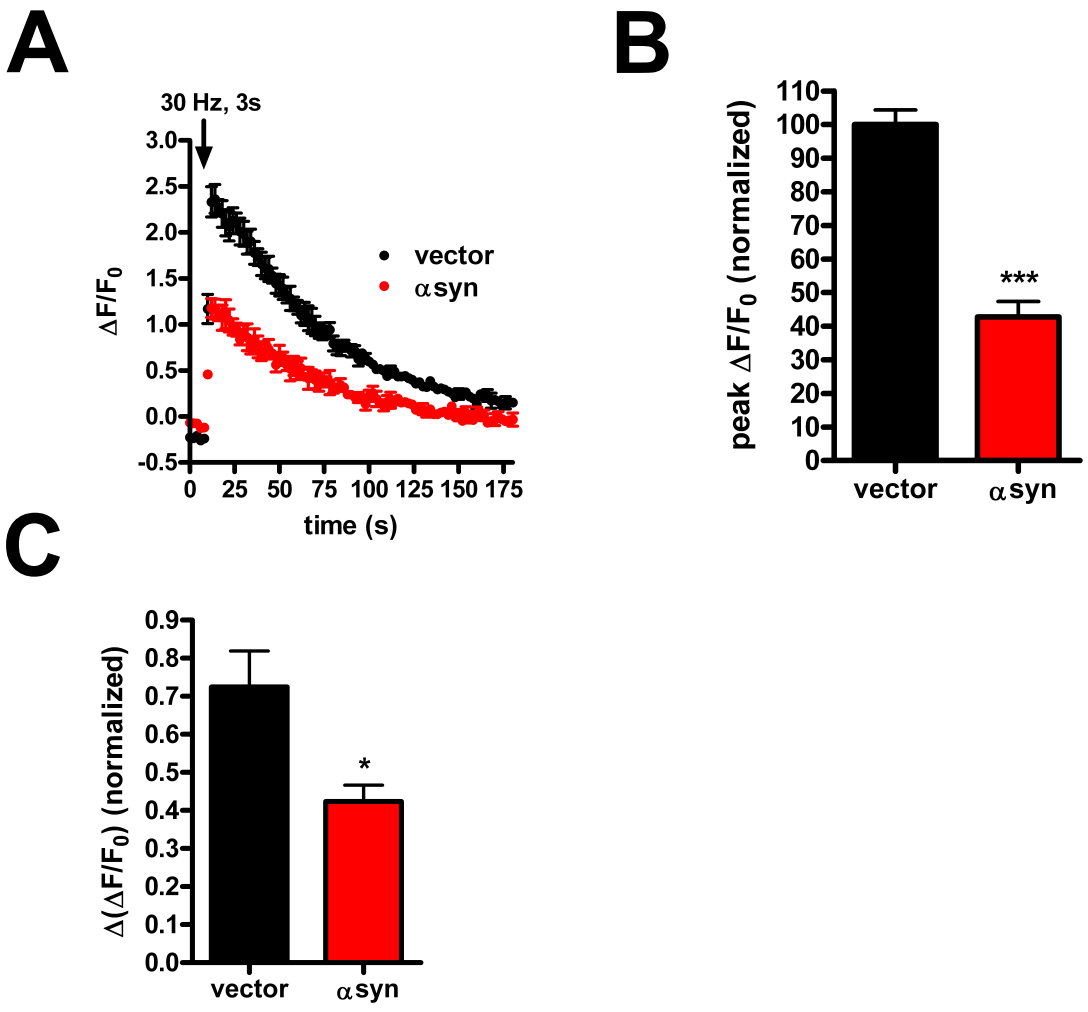

A
30 Hz, 3s
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
ΔF/F0
0 25 50 75 100 125 150 175
time (s)
vector
αsyn
B
peak ΔF/F0 (normalized)
110
100
90
80
70
60
50
40
30
20
10
0
***
vector
αsyn
C
Δ(ΔF/F0) (normalized)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
vector
αsyn

Figure 4

Figure 5 – Parkinson's Disease associated mutations have differential effects on the inhibitory effect of αsyn overexpression on synaptic vesicle release

(A) Time course of VGLUT1-pHluorin fluorescence during a 10 Hz stimulus for 60 seconds in neurons coexpressing either vector control, human αsyn, or the PD-associated mutant αsyn A30P. n = 3 coverslips, 60 boutons per condition from a single transfection.

(B) Peak $\Delta F/F_0$ values at the end of the stimulus. Values are normalized to the response in the vector control condition. n = 9 coverslips, 180 boutons per condition from 3 independent transfections. One-way ANOVA, $p < 0.0001$. Tukey's multiple comparison test: vector vs. αsyn, $p < 0.001$; vector vs. A30P, $p > 0.05$; αsyn vs. A30P, $p < 0.001$.

(C) Time course of VGLUT1-pHluorin fluorescence during a 10 Hz stimulus for 60 seconds in neurons coexpressing either vector, αsyn, or the PD-associated mutants αsyn A53T and E46K. n = 3 coverslips, 60 boutons per condition from a single transfection.

(D) Peak $\Delta F/F_0$ values at the end of the stimulus. Values are normalized to the response in the vector control condition. n = 9 coverslips, 180 boutons per condition from 3 independent transfections. One-way ANOVA, $p < 0.0001$. Tukey's multiple comparison test: vector vs. αsyn, $p < 0.001$; vector vs. A53T, $p < 0.001$; vector vs. E46K, $p < 0.001$.

All data shown are mean ± SEM.

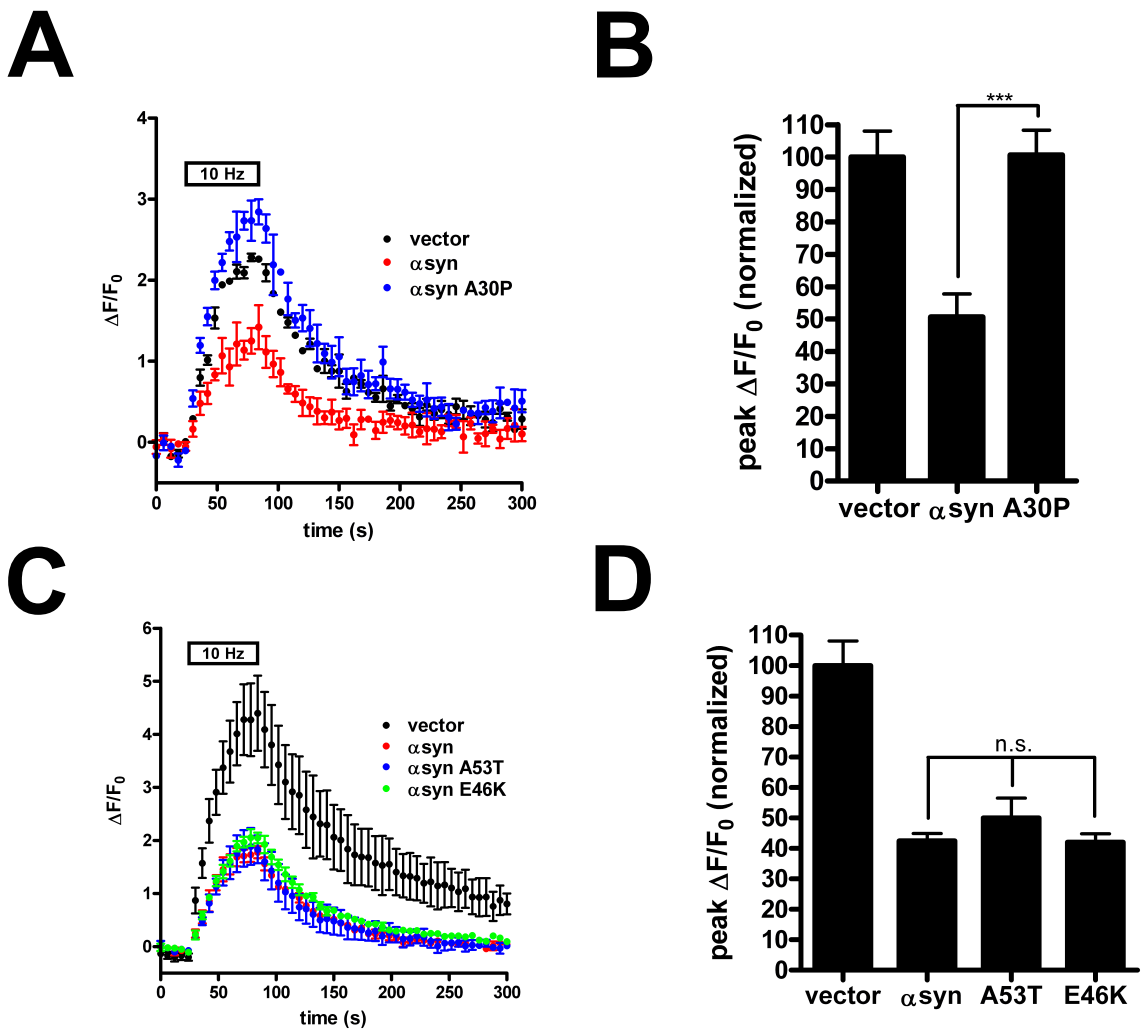

A
10 Hz
vector
αsyn
αsyn A30P
ΔF/F0
time (s)
B
peak ΔF/F0 (normalized)
***
vector
αsyn
A30P
C
10 Hz
vector
αsyn
αsyn A53T
αsyn E46K
ΔF/F0
time (s)
D
peak ΔF/F0 (normalized)
n.s.
vector
αsyn
A53T
E46K

Figure 5

Figure 6 – The C-terminus of αsyn is not required for its inhibitory effect on synaptic vesicle release

(A) Time course of VGLUT1-pHluorin fluorescence during a 10 Hz stimulus for 60 seconds in neurons coexpressing either vector control, human αsyn, or the C-terminally truncated mutant αsyn 1-110. n = 3 coverslips, 60 boutons per condition from a single transfection.

(B) Peak $\Delta F/F_0$ values at the end of the stimulus. Values are normalized to the response in the vector control condition. n = 6 coverslips, 120 boutons per condition from 2 independent transfections. One-way ANOVA, $p < 0.01$. Tukey's multiple comparison test: vector vs. αsyn, $p < 0.05$; vector vs. 110, $p < 0.05$; αsyn vs. 1-110, $p > 0.05$.
All data shown are mean ± SEM.

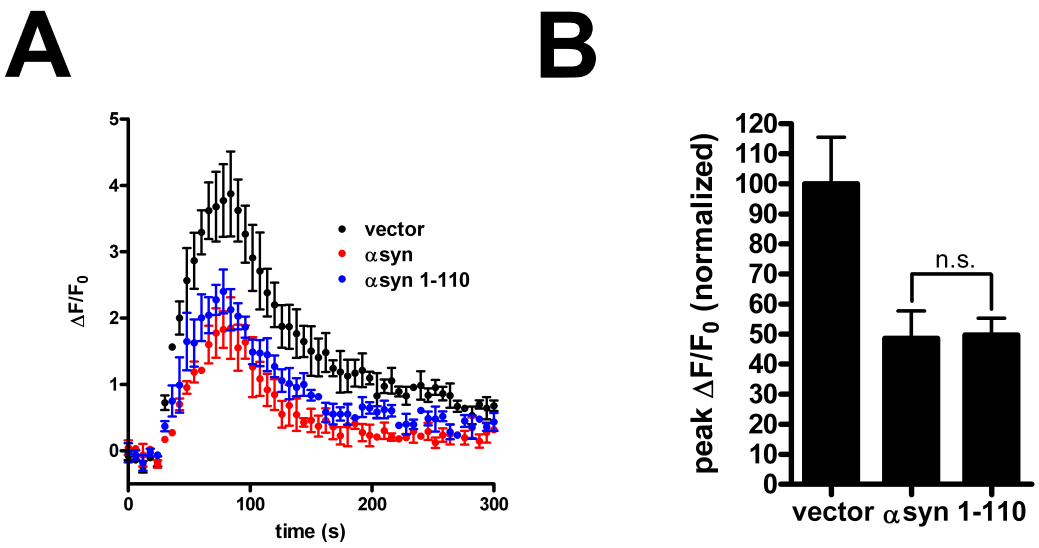

A
B
ΔF/F0
time (s)
vector
αsyn
αsyn 1-110
peak ΔF/F0 (normalized)
n.s.
vector
αsyn 1-110

Figure 6

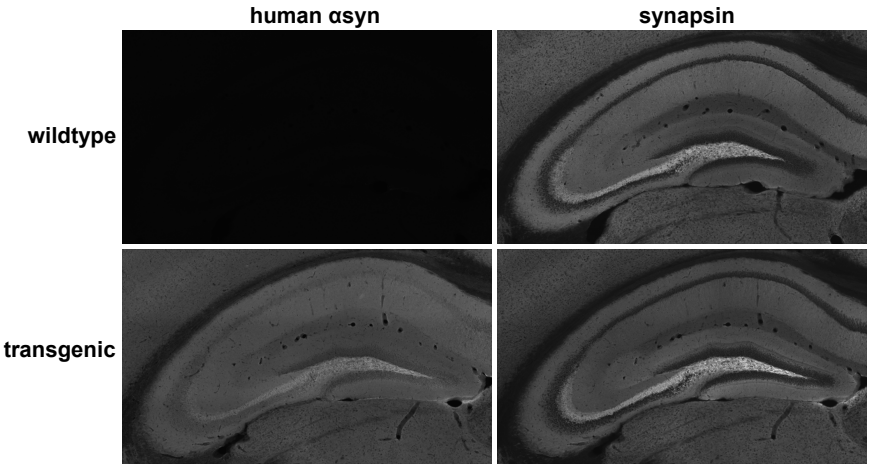
Figure 7 – Generation of transgenic mice with increased αsyn expression

(A) Brain sections (35 μm thick) were immunostained for human αsyn using the 15G7 antibody, and for synapsins using an antibody that recognizes both synapsins I and II.

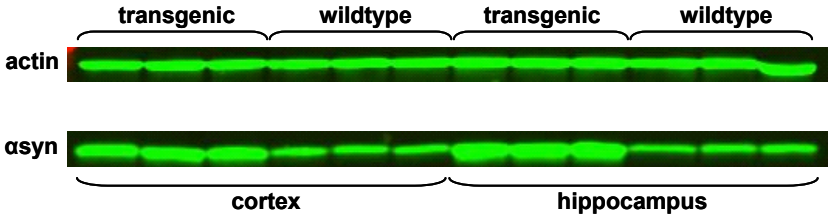
(B) Equal amounts of protein from cortex or hippocampus from αsyn transgenic mice and their wildtype littermates were separated on a SDS polyacrylamide gel and processed for Western analysis using an antibody against actin and the syn-1 antibody against αsyn, which recognizes both the mouse and human protein**.**

(C) Expression of αsyn was quantitated in the cortex of both wildtype and human αsyn transgenic mice using the syn-1 antibody. Samples from each animal were loaded in triplicate, and syn-1 antibody signals were normalized to the amount of actin detected in the same lane. n = 3 animals.

A


human αsyn
synapsin
wildtype
transgenic

B


transgenic
wildtype
transgenic
wildtype
actin
αsyn
cortex
hippocampus

C

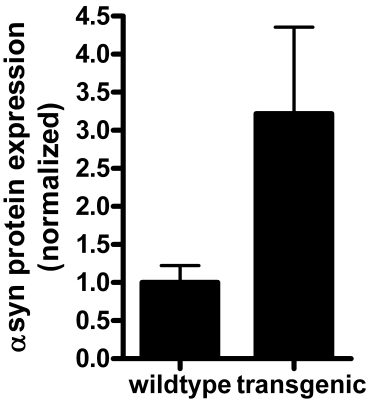

αsyn protein expression (normalized)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
wildtype
transgenic

Figure 7

Figure 8 – Increased αsyn expression inhibits synaptic transmission in αsyn transgenic mice

(A) Representative field EPSP traces from CA3 stratum radiatum in response to increasing stimulation of Schaffer collateral fibers in CA1 of hippocampal slices from wildtype mice.

(B) Representative field EPSP traces from CA3 stratum radiatum in response to increasing stimulation of Schaffer collateral fibers in CA1 of hippocampal slices from transgenic mice overexpressing αsyn.

(C) Input-output curve plotting CA3 statum radiatum field EPSP slope versus fiber volley amplitude in response to single action potentials elicited in CA1 Schaffer collaterals in hippocampal slices from wildtype versus αsyn transgenic mice. Error bars represent SEM.

(D) Paired pulse ratio recorded in CA1 from two successive stimuli delivered to Schaffer collaterals with a 40 ms interstimulus interval. The right side shows representative fEPSPs recorded from slices taken from both wildtype and transgenic animals. Two-tailed, unpaired t-test, $p < 0.05$.

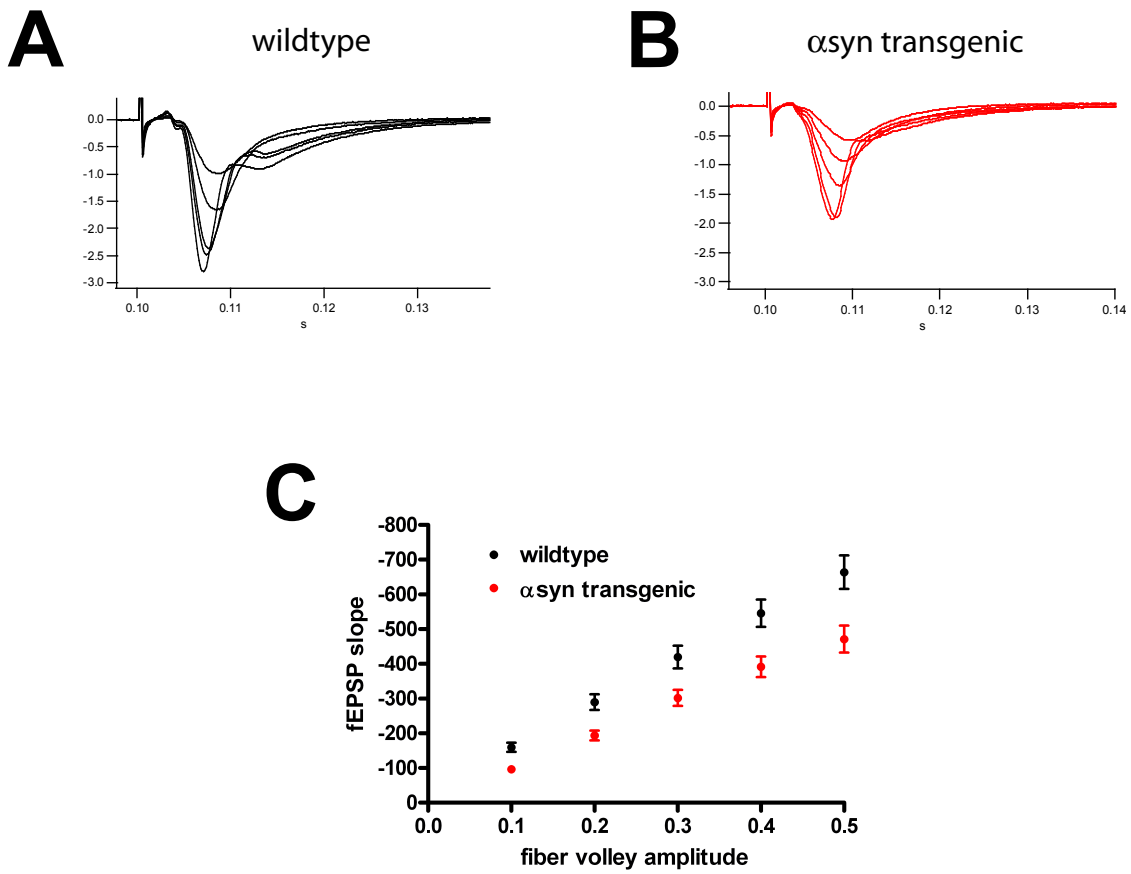

A
wildtype
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
0.10
0.11
0.12
0.13
s
B
αsyn transgenic
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
0.10
0.11
0.12
0.13
0.14
s
C
-800
-700
-600
-500
-400
-300
-200
-100
0
fEPSP slope
wildtype
αsyn transgenic
0.0
0.1
0.2
0.3
0.4
0.5
fiber volley amplitude
D
Paired-pulse ratio
1.5
1.0
0.5
0.0
*
wildtype
transgenic
transgenic
wildtype

Figure 8

Figure 9 – αsyn inhibits neurotransmitter release in midbrain dopamine neurons

Postnatal midbrain neurons were transfected with human αsyn and VGLUT1-pHluorin, then immunostained after 2-3 weeks in culture.

(A) Human αsyn is detected using the human specific αsyn antibody, 15G7; VGLUT1-pHluorin is detected using a monoclonal antibody against GFP; and tyrosine hydroxylase is detected using a polyclonal antibody. Arrowheads highlight areas of colocalization. Scale bar = 5 μm.

(B) Time course of VGLUT1-pHluorin fluorescence during a 10 Hz stimulus for 60 seconds in tyrosine hydroxylase positive nerve terminals coexpressing either vector control or human αsyn. n = 3 coverslips, 40 nerve terminals for vector and n = 4 coverslips, 31 nerve terminals for αsyn from a single transfection. Error bars represent SEM.

(C) Peak $\Delta F/F_0$ values at the end of the stimulus. Values are normalized to the response in the vector control condition. n = 3 coverslips, 40 nerve terminals for vector and n = 4 coverslips, 31 nerve terminals for αsyn from a single transfection. Data represents mean ± SEM.

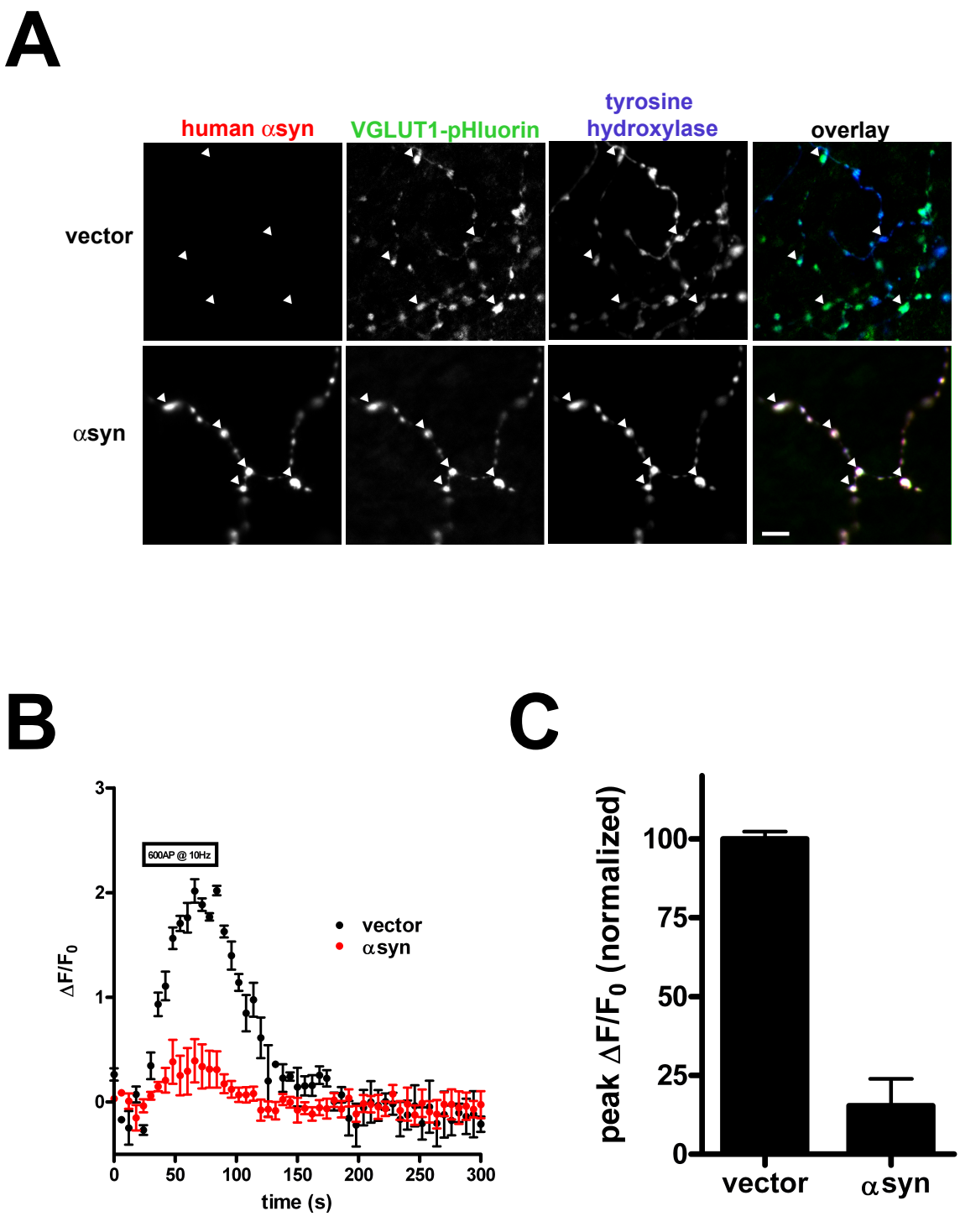

A
human αsyn
VGLUT1-pHluorin
tyrosine hydroxylase
overlay
vector
αsyn
B
600AP @ 10Hz
vector
αsyn
ΔF/F0
time (s)
C
peak ΔF/F0 (normalized)
vector
αsyn

Figure 9

Figure 10 – αsyn overexpression causes a selective reduction in synapsins and complexins

(A), (B) Equal amounts of protein from cortical lysates from αsyn transgenic mice and their wildtype littermates were separated on SDS polyacrylamide gels and processed for Western analysis using the indicated primary antibodies and fluorescent secondary antibodies.

(C) Cortical lysates were processed for Western analysis and probed with the indicated antibodies. Secondary antibodies conjugated to fluorescent molecules were used to obtain a quantitative measure of protein expression. Values obtained were normalized to an actin loading control in the same lane, and the change in protein expression in αsyn transgenic mice versus littermate wildtype mice was quantified. Data represent mean ± SEM, from n = 3 animals for each genotype.

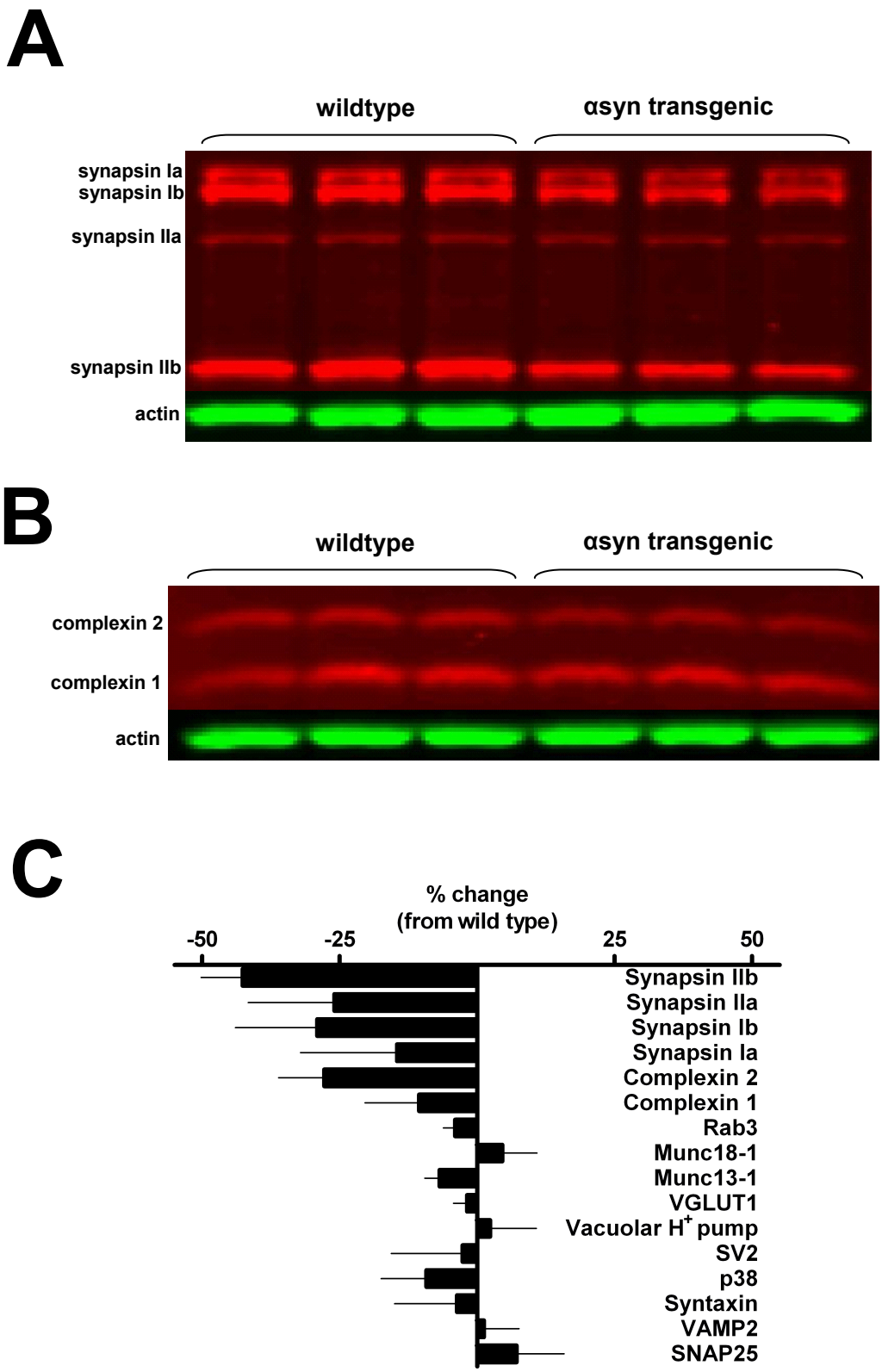

A
wildtype
αsyn transgenic
synapsin Ia
synapsin Ib
synapsin IIa
synapsin IIb
actin
B
wildtype
αsyn transgenic
complexin 2
complexin 1
actin
C
% change
(from wild type)
-50
-25
25
50
Synapsin IIb
Synapsin IIa
Synapsin Ib
Synapsin Ia
Complexin 2
Complexin 1
Rab3
Munc18-1
Munc13-1
VGLUT1
Vacuolar H+ pump
SV2
p38
Syntaxin
VAMP2
SNAP25

Figure 10

ACKNOWLEDGMENTS

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CHAPTER 3:

Alpha-Synuclein Induces Mitochondrial Fragmentation

ABSTRACT

Mitochondrial dysfunction plays a central role in the pathogenesis of Parkinson's disease. However, the initial insults that lead to mitochondrial pathology are unknown. Mutations in proteins important for mitochondrial fusion and fission have recently been described for several neurodegenerative diseases. Additionally, 2 proteins implicated in familial Parkinson's disease have been shown recently to regulate mitochondrial morphology. We have recently found that α-synulcein, a protein linked to both familial and sporadic Parkinson's disease, interacts specifically with mitochondria. Our preliminary evidence now shows that α-synulcein expression in cells dramatically changes mitochondrial morphology from tubular to fragmented. These effects are specific to mitochondria, and are partially blocked by a mutation in α-synulcein that reduces its affinity for membrane binding. These results may indicate a potential mechanism through which α-synulcein could regulate synaptic vesicle pool size, but rather may represent an initial pathogenic effect on mitochondria in the progression of Parkinson's disease.

INTRODUCTION

Mitochondria play an important role in the pathogenesis of Parkinson's disease (PD), but their precise contribution to the disease process is not well understood. It is known that various mitochondrial toxins can cause Parkinsonian symptoms in both animal models (Schober, 2004) and humans (Langston et al., 1983). Further, mutations in proteins implicated in mitochondrial function cause familial forms of PD (Clark et al., 2006; Greene et al., 2003; Park et al., 2006; Yang et al., 2006). It is still not known, however, what aspects of normal mitochondrial physiology are important for maintaining the survival of the dopamine neurons that degenerate in PD.

Although mitochondria are classically described as small, cigar-shaped structures, they form extensive tubular networks in most cell types, and their morphologies are highly dynamic (Detmer and Chan, 2007). Mitochondria constantly undergo repeated fusion and fission with neighboring mitochondria, and are transported rapidly throughout the cell. In recent years, many of the molecules that mediate these reactions have been described. In mammalian cells, three GTPases mediate mitochondrial fusion - Mfn1, Mfn2, and OPA1 – while the GTPase Drp1 and the mitochondrial outer membrane protein Fis1 mediate mitochondrial fission (Detmer and Chan, 2007). In normal conditions, fusion and fission reactions are balanced so that the mitochondrial network retains a stable structure. However, manipulations that affect mitochondrial energy production or mutations in proteins involved in fusion and fission destabilize the tubular network of mitochondria and result in their fragmentation when fission dominates, or aggregation when fusion dominates. The precise manner in which cells balance mitochondrial fusion and fission is not well understood.

Recent evidence from our laboratory indicates that the protein αsyn binds tightly and specifically to mitochondria (Ken Nakamura, unpublished data). Three point mutations in αsyn cause familial autosomal dominant PD, and overexpression of the wildtype protein also causes PD, suggesting that an increase in the normal function of the protein may play a role in disease pathogenesis. Indeed, αsyn also accumulates in Lewy bodies and dystrophic neurites in sporadic PD, demonstrating a role for the protein in PD in the absence of inherited mutations. We demonstrate in chapter 2 of this dissertation that increased expression of αsyn in neurons inhibits neurotransmitter release by reducing the size of the recycling pool. We also show that decreased levels of synapsins may account for this reduction. However, since synapsin knockouts show only partial defects in neurotransmitter release (Rosahl et al., 1995; Ryan et al., 1996), it is possible that this does not fully account for the defect in release seen with αsyn overexpression

Because mitochondria localize to nerve terminals and are important for synaptic function, we hypothesized that increased αsyn expression may alter normal mitochondrial physiology. To examine the effect of αsyn overexpression on mitochondria, we first used HeLa cells, a thin, flat cell line where mitochondria are easily visualized using GFP targeted to mitochondria. We chose to perform our initial experiments in this cell line because the complex geometry of primary neurons could mask subtle effects on mitochondrial morphology or distribution. Surprisingly, our preliminary evidence indicates that increased αsyn expression causes robust mitochondrial fragmentation. The A30P mutation, which markedly reduces the ability of αsyn to bind membranes and inhibit neurotransmitter release, decreases the capacity of αsyn to fragment mitochondria. These results suggest mitochondrial fragmentation as a potential mechanism for

inhibition of neurotransmitter release by αsyn, or alternatively a pathogenic event in the initiation and progression of PD.

RESULTS

Alpha-synuclein overexpression causes mitochondrial fragmentation

It has become evident in recent years that the regulation of mitochondrial morphology plays an important role in protecting neurons from degeneration (Detmer and Chan, 2007), both in mouse models (Chen et al., 2007) and in human disease (Alexander et al., 2000; Delettre et al., 2000; Waterham et al., 2007; Zuchner et al., 2004). Recent evidence from our laboratory indicates that αsyn binds to mitochondria with relatively high affinity, but the physiological relevance of this interaction is not known. To assess whether expression of αsyn has effects on mitochondrial morphology, we generated HeLa cells stably expressing MitoEGFP, a fluorescent protein targeted to the mitochondrial matrix, to label mitochondria. HeLa cells normally have an extensive tubular network of mitochondria (Figure 11A, left panel), and do not normally express αsyn. Expression of αsyn in this cell type causes a dramatic shift in mitochondrial morphology, with virtually all of the mitochondria in the cell becoming fragmented (Figure 11A, right panel). This phenomenon is quite robust, as a vast majority of cells expressing αsyn shift their mitochondrial network to a fragmented morphology (Figure 11B).

The effects of alpha-synuclein on organelle morphology are specific for mitochondria

It has been shown previously that overexpression of αsyn in yeast and other cell types disrupts vesicular trafficking from endoplasmic reticulum to Golgi (Cooper et al., 2006; Gitler et al., 2008). Therefore, it is possible that αsyn has more general effects on

organelle morphology that are not specific to mitochondria. Alternatively, it is possible that overexpression of αsyn causes non-specific toxicity to the cell, which could cause generalized disruption of cellular organelles. To test these possibilities, we performed immunostaining for markers of endoplasmic reticulum, Golgi, lysosomes, and tubulin while simultaneously visualizing mitochondrial morphology using MitoEGFP. We found that there was little to no disruption of these structures in cells that had extensively fragmented mitochondria (Figures 12, 13, 14, 15), suggesting that αsyn overexpression specifically affects mitochondrial morphology while sparing other organelles.

Alpha-synuclein overexpression causes mitochondrial fragmentation in midbrain neurons

While HeLa cells' flat morphology makes effects on mitochondria easy to visualize, the relevance to understanding the biology or pathobiology of αsyn is unclear. To test whether αsyn overexpression causes mitochondrial fragmentation in neurons, we cotransfected MitoEGFP and either a vector control or human αsyn into primary midbrain neurons. The mitochondria in these midbrain neurons, notably, have a fragmented morphology at baseline. However, we find that overexpression of human αsyn causes additional mitochondrial fragmentation in these cells (Figure 16), showing that this phenomenon acts even in cells that endogenously express αsyn.

PD-linked mutations in αsyn differentially regulate mitochondrial morphology

The data presented in Chapter 2 shows a striking difference in the behavior of the three PD-linked mutants on their effects on neurotransmitter release. While the A53T

and E46K mutants behave similarly to the wildtype protein, the A30P mutant has no effect on synaptic vesicle exocytosis. Presumably, this is due to the impaired membrane binding capability of this mutant. To determine if PD-linked mutations affect the ability of αsyn to alter mitochondrial morphology, we transfected these mutants into HeLa cells under the same conditions as used for wildtype αsyn. Interestingly, we find that the A30P mutation reduces the ability of αsyn to mediate mitochondrial fragmentation. The A53T and E46K mutants behave similar to the wildtype protein (Figure 17).

DISCUSSION

Our preliminary evidence shows that expression of αsyn in HeLa cells dramatically changes mitochondrial morphology from tubular to fragmented without affecting other organelles. Additionally, the PD-linked mutation, A30P, reduces the ability of αsyn to cause mitochondrial fragmentation. Overexpression of αsyn in ventral midbrain neuron culture also causes mitochondrial fragmentation. We are not certain yet whether αsyn induces fragmentation by inhibiting mitochondrial fusion, or accelerating mitochondrial fission. Nevertheless, these results suggest that mitochondrial fragmentation may play a role in the reduction of recycling pool size by αsyn overexpression, or alternatively, may represent a pathogenic mechanism for mitochondrial dysfunction in PD.

The role of mitochondria in synaptic function remains unclear. At *Drosophiila* neuromuscular junction (NMJ), depletion of mitochondria at synapses by introducing a mutation that prevents mitochondrial fission (Drp1 K38A) results in a specific presynaptic defect in the mobilization of reserve pool vesicles during prolonged stimulation (Verstreken et al., 2005). However, basal neurotransmission is unaffected. Additionally, there is substantial evidence in other model systems that mitochondria are Ca^{2+} sinks at nerve terminals during intense neuronal activity; however, at the *Drosophiila* neuromuscular junction (NMJ) loss of synaptic mitochondria has almost no effect on Ca^{2+} buffering (Guo et al., 2005; Verstreken et al., 2005). Postsynaptically, mitochondria are important for controlling both spine number and spine plasticity (Li et al., 2004).

Because it is well established that manipulations that affect mitochondrial fusion and fission also generally affect mitochondrial motility, it will be informative to examine mitochondrial distribution in neurons overexpressing αsyn. However, since we have shown in Chapter 2 that even basal synaptic transmission is reduced in both cultured neurons and transgenic mice overexpressing αsyn, it is unlikely that mitochondrial distribution away from synapses alone could account for the effects on neurotransmitter release.

The effects we observe on mitochondrial morphology could alternatively represent an early pathogenic event in the progression of disease. Defective mitochondrial function is clearly important to the degeneration observed in PD. Notably, both PINK1 and parkin have recently been shown to regulate mitochondrial morphology by promoting mitochondrial fission, and their loss-of-function mutations cause mitochondrial pathology (Poole et al., 2008). Mitochondrial fusion and fission have not previously been shown to play a direct role in the mitochondrial pathology observed in patients with PD. However, earlier studies have shown that defects in mitochondrial fission cause increased reactive oxygen species production and DNA damage, insults which are important in PD pathogenesis (Benard et al., 2007; Lee et al., 2007; Sherer et al., 2002). Therefore, it is possible that defects in mitochondrial fusion or fission result in increased production of reactive oxygen species, which then lead to dopamine neuron degeneration. Indeed, mutations in proteins involved in fusion and fission lead to several different neurodegenerative diseases (Alexander et al., 2000; Delettre et al., 2000; Waterham et al., 2007; Zuchner et al., 2004). Our evidence now links a third protein

involved in PD to the precise control of mitochondrial morphology. Further work will be necessary to establish a causative role in PD pathogenesis.

EXPERIMENTAL PROCEDURES

Reagents

The antibody to LAMP1 was obtained from BD Biosciences. The antibody to endoplasmic reticulum (anti-KDEL) was a generous gift from David Erle (University of California, San Francisco) and originally obtained from Stressgen. The GM130 antibody was a generous gift from S. Baekkeskov, and originally obtained from BD Biosciences. The tubulin antibody was obtained from Oncogene.

Molecular Biology and Cell Culture

Human αsyn was subcloned into the chicken actin vector pCAGGS (gift of J. L. R. Rubenstein, University of California, San Francisco) with a preceding 5' Kozak consensus sequence to optimize translation in mammalian cells. Point mutations were introduced using Quikchange (Stratagene). EGFP was subcloned into the pAcGFP1-Mito vector (Clontech) to replace AcGFP1 and generate MitoEGFP. MitoEGFP was then subcloned into pCAGGS. Azurite (gift of David Piston, Vanderbilt University) was subloned into pCAGGS. All constructs were verified by sequence analysis.

HeLa cells stably transfected with Mito-EGFP were grown in DMEM with 10% cosmic calf serum (HyClone, Logan, UT) at 5% CO_2 on either plastic or glass coverslips (for immunofluorescence) and transfected using electroporation at the time of plating (Amaxa, Germany).

For midbrain neuron culture, the ventral midbrain from E14 rat embryos were dissociated in trypsin, transfected by electroporation (Amaxa) at the time of plating, and plated onto glass coverslips.

Fluorescence Microscopy

HeLa cells or midbrain neurons growing on glass coverslips were fixed in 4% paraformaldehyde in PBS and mounted on slides using Prolong Gold Antifade (Invitrogen, Carlsbad, CA). Images were acquired on a Zeiss upright fluorescence microscope using a 63x 1.25 NA oil objective and GFP and DAPI filtersets to visualize MitoEGFP and Azurite, respectively. Cell counting and image analysis was performed by selecting transfected cells based on Azurite fluorescence alone, then classifying that transfected cell as having either "tubular" or "fragmented" mitochondrial morphology. Cells were classified as tubular based on the criteria that at least 3 mitochondria in the cell have a tubular appearance (long axis length approximately 3 times greater than the short axis length).

FIGURES

Figure 11 – Overexpression of human αsyn in HeLa cells causes mitochondrial fragmentation

(A) HeLa cells stably expressing Mito-EGFP were cotransfected with Azurite and either a vector control or αsyn at the time of plating, then fixed and processed for imaging 48 hours later. The left and right panels demonstrate mitochondrial morphology after transfection with a vector control or αsyn, respectively. Insets highlight tubular versus fragmented morphology in the area bordered by a red box. Asterisks indicate transfected cells identified by Azurite fluorescence. Scale bar = 20 μm.

(B) Quantitation of HeLa cells displaying tubular versus fragmented mitochondrial morphology. N = 6 coverslips per condition, with 30 cells counted per coverslip. Data represents mean ± SEM.

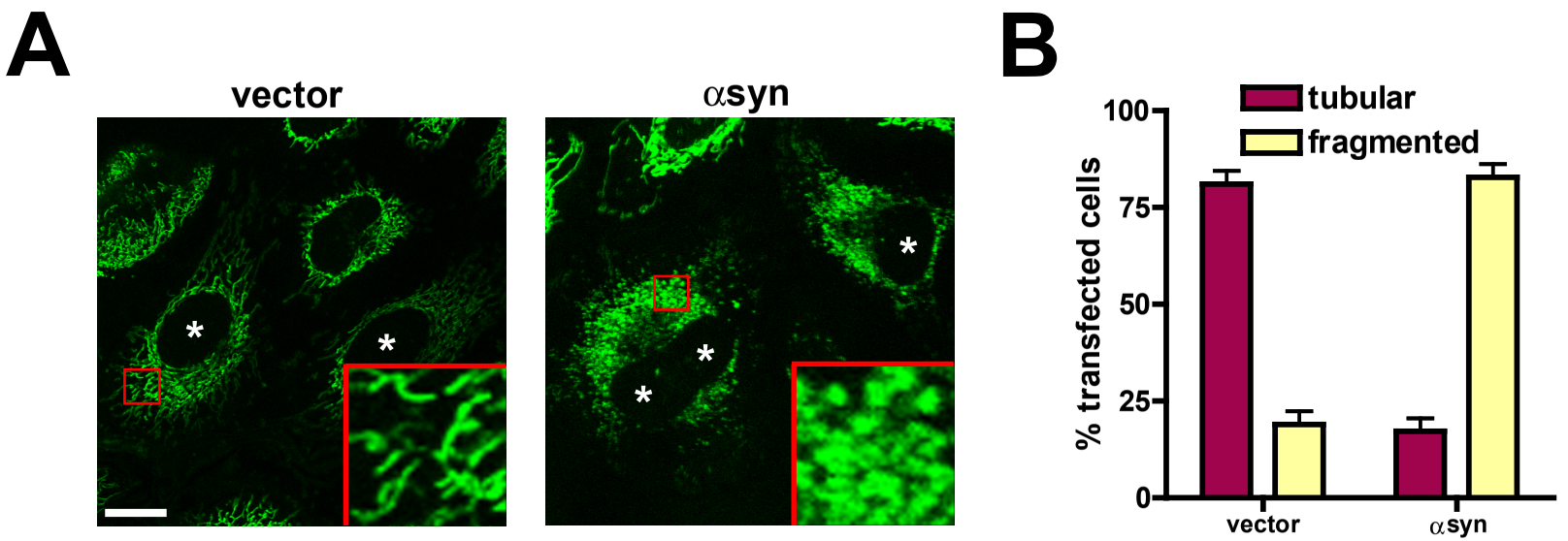
A
vector
αsyn
B
tubular
fragmented
% transfected cells
100
75
50
25
0
vector
αsyn

Figure 11

Figure 12 – Overexpression of human αsyn in HeLa cells does not affect endoplasmic reticulum morphology

HeLa cells stably expressing Mito-EGFP were cotransfected with Azurite and either a vector control or αsyn at the time of plating, fixed after 48 hours, and immunostained using an anti-KDEL antibody to label endoplasmic reticulum.

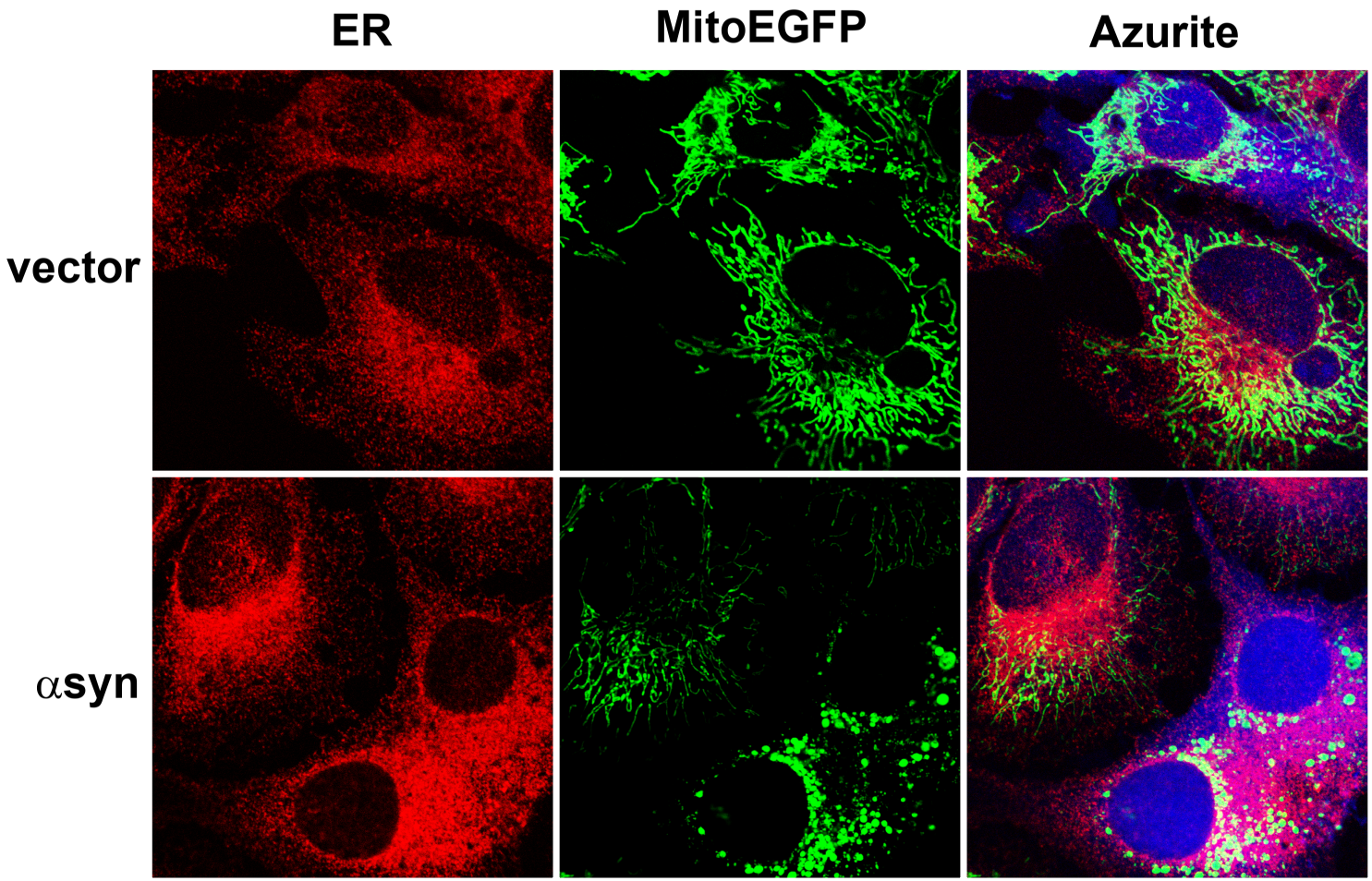

Figure 12

Figure 13 – Overexpression of human αsyn in HeLa cells does not affect Golgi morphology

HeLa cells stably expressing Mito-EGFP were cotransfected with Azurite and either a vector control or αsyn at the time of plating, fixed after 48 hours, and immunostained using an anti-GM 130 antibody to label Golgi.

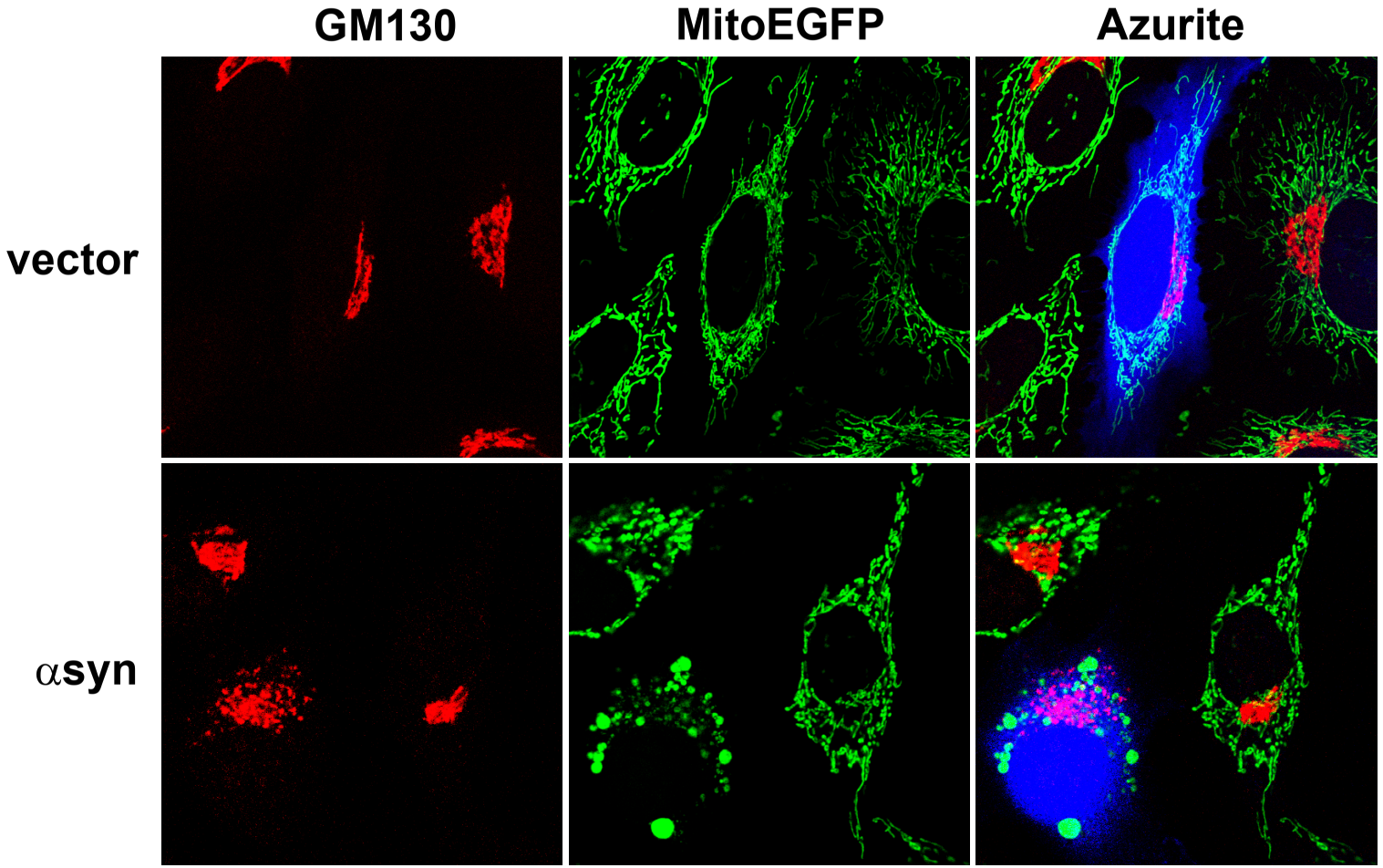

Figure 13

Figure 14 – Overexpression of human αsyn in HeLa cells does not affect lysosome morphology

HeLa cells stably expressing Mito-EGFP were cotransfected with Azurite and either a vector control or αsyn at the time of plating, fixed after 48 hours, and immunostained using an anti-LAMP1 antibody to label lysosomes.

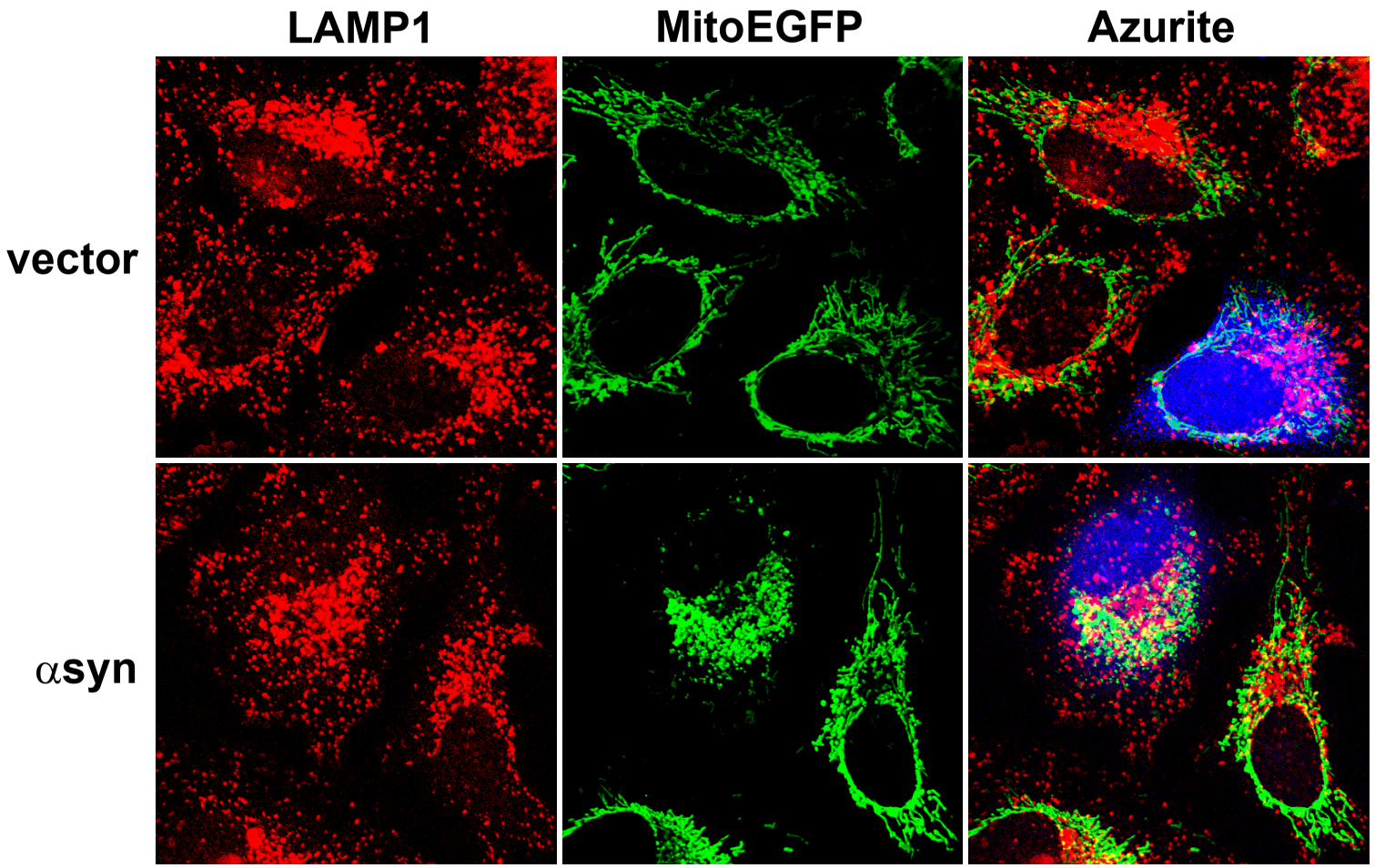

Figure 14

Figure 15 – Overexpression of human αsyn in HeLa cells does not affect tubulin cytoskeleton morphology

HeLa cells stably expressing Mito-EGFP were cotransfected with Azurite and either a vector control or αsyn at the time of plating, fixed after 48 hours, and immunostained using an anti-tubulin antibody to label the tubulin cytoskeleton.

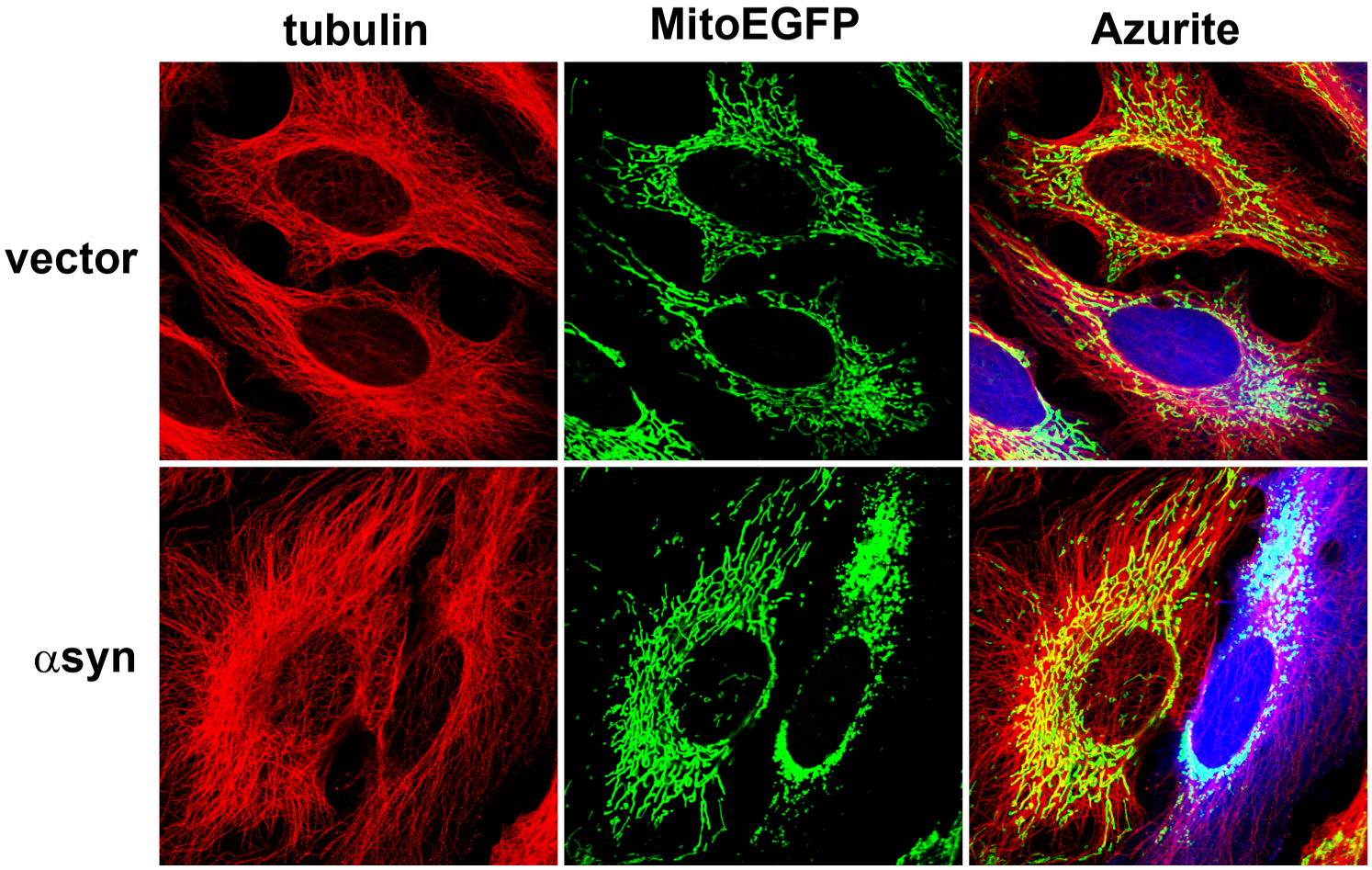

Figure 15

Figure 16 – Overexpression of human αsyn in midbrain neurons causes mitochondrial fragmentation

Ventral midbrain cultures from embryonic rats were transfected with MitoEGFP at the time of plating, and imaged live after 2 weeks in culture.

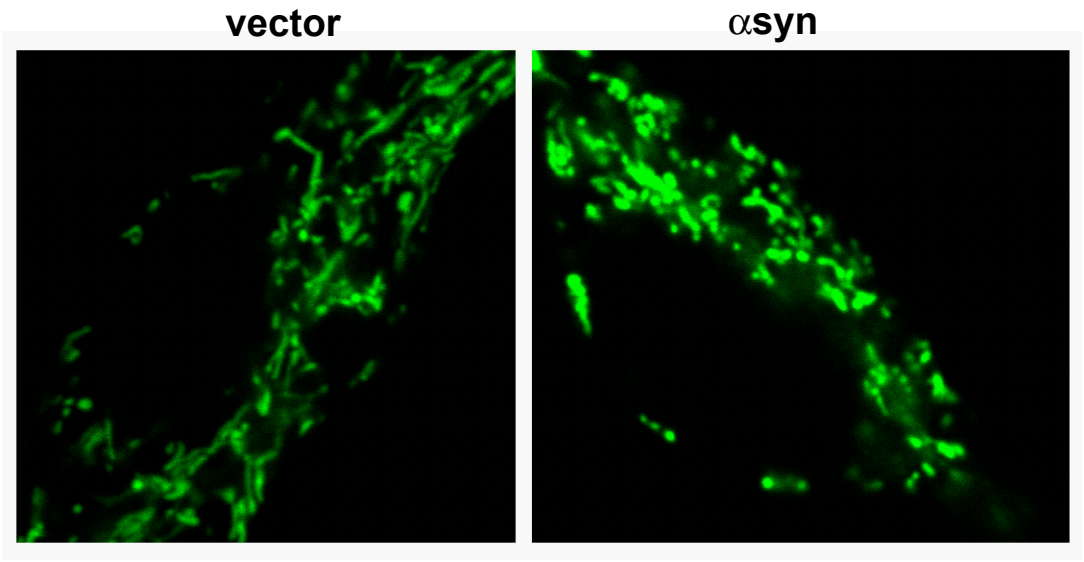

Figure 16

Figure 17 – Effect of PD-linked mutations in αsyn on mitochondrial morphology in HeLa cells

HeLa cells stably expressing Mito-EGFP were cotransfected with Azurite and either a vector control or the indicated αsyn constructs at the time of plating, then fixed and processed for imaging 48 hours later. The number of HeLa cells displaying tubular versus fragmented mitochondrial morphology was determined. N = 3 coverslips per condition, with 30 cells counted per coverslip. Data represents mean ± SEM.

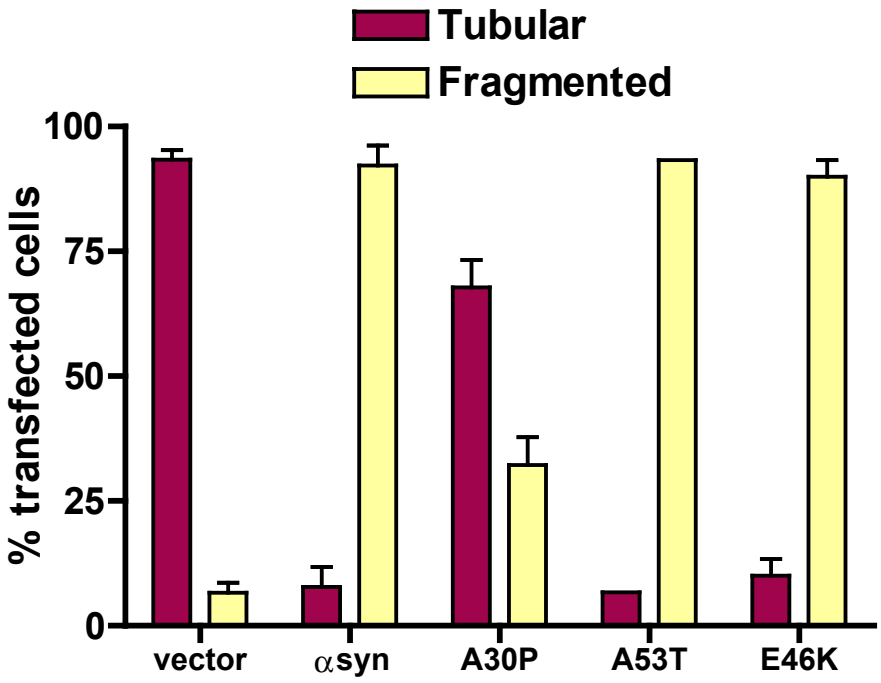
Tubular
Fragmented
% transfected cells
100
75
50
25
0
vector
αsyn
A30P
A53T
E46K

Figure 17

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CHAPTER 4: CONCLUDING REMARKS

SUMMARY OF FINDINGS

Alpha-synuclein is a small, synaptic protein that has been the subject of investigation for 20 years. Originally cloned as a synaptic vesicle associated protein (Maroteaux et al., 1988), αsyn has been intensely studied in the past decade due to its role in Parkinson's disease. It is highly abundant in Lewy bodies in the sporadic disorder (Spillantini et al., 1997), but more interestingly, point mutations and increased gene dosage cause dominantly inherited forms of PD, suggesting that the protein plays a causative role (Chartier-Harlin et al., 2004; Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003; Zarranz et al., 2004). Despite increasing understanding about the formation, contents, and structure of Lewy bodies, little is known about the earliest events that initiate the degenerative process in PD. Because increased expression of wildtype αsyn is sufficient to cause degeneration, we chose to investigate the physiological effect of overexpression of αsyn at the nerve terminal.

In Chapter 2 of this dissertation, I show that increased expression of αsyn inhibits synaptic vesicle exocytosis. Alpha-synuclein is normally expressed widely throughout the brain (Iwai et al., 1995), and accordingly αsyn inhibits neurotransmitter release in both primary hippocampal neurons (Figure 2) and primary midbrain dopamine neurons (Figure 9) cultured *in vitro*. Further I show that αsyn inhibits release not by altering the kinetics of synaptic vesicle fusion, but rather by reducing the size of the recycling pool of synaptic vesicles (Figure 3). Interestingly, a mutation in αsyn (A30P) that impairs its membrane binding and disrupts its synaptic localization abolishes its ability to inhibit neurotransmitter release (Figure 5). Two other point mutants, A53T and E46K, inhibit neurotransmitter release to the same degree as the wildtype protein. To test whether the

helical N-terminal domain or the unstructured C-terminal domain of αsyn were important for release, I generated a deletion mutant of αsyn that consisted of only the first 110 amino acids. Expression of this mutant in hippocampal neurons inhibited release to the same extent as the wildtype protein, showing that the membrane binding domain of αsyn is sufficient to inhibit SV release (Figure 6). Finally, to test whether αsyn inhibits neurotransmitter release *in vivo*, I generated transgenic mice overexpressing αsyn under control of the Syrian hamster Prion promoter (Figure 7). Electrophysiological recording of basal synaptic transmission at hippocampal Schaffer collateral synapses showed that αsyn inhibits neurotransmitter release *in vivo* (Figure 8). Quantitative western analysis from the brains of these transgenic mice showed selective decreases in synapsins and complexins (Figure 10), proteins known to be important for the control of synaptic vesicle pool size and neurotransmitter release. These results clearly establish a role for αsyn in the regulation of the synaptic vesicle cycle, and further show that increased expression of αsyn mediates a physiological reduction in synaptic function before the formation of morphologically detectable aggregates.

In Chapter 3 of this dissertation, I present preliminary data describing a role for αsyn in the control of mitochondrial morphology. Mitochondria clearly play a central role in the pathogenesis of PD, and there is considerable evidence implicating mitochondrial function in normal synaptic physiology. The tight regulation of mitochondrial fusion and fission is important for neuronal survival (Chen et al., 2007); further, proteins mutated in familial forms of PD have recently been implicated in the regulation of these processes (Poole et al., 2008; Yang et al., 2008). My preliminary evidence shows that overexpression of αsyn in a cell line that normally does not express

αsyn causes widespread mitochondrial fragmentation (Figure 11). This is a very robust phenomenon, affecting cells expressing even modest levels of αsyn. The effect on mitochondrial morphology is very specific, with no effect on endoplasmic reticulum, Golgi, lysosome, or tubulin cytoskeleton morphology (Figures 12 – 15). Further, the A30P mutant has a reduced ability to fragment mitochondria, most likely due to its decreased ability to bind membranes, while the A53T and E46K mutants behave similar to the wildtype protein (Figure 16). This evidence implicates αsyn in the regulation of mitochondrial fusion and fission, and suggests that mitochondrial function may play a role in regulation of synaptic vesicle recycling pool size. Alternatively, fragmentation of mitochondria by αsyn could represent an initial pathogenic insult that leads to cell death in certain neuronal populations.

FUTURE DIRECTIONS

In Chapter 2, I show that αsyn overexpression reduces the size of the synaptic vesicle recycling pool, but the precise molecular mechanism underlying this phenomenon remains unclear. We show using biochemical techniques that the levels of synapsins and complexins are selectively decreased in the brains of mice overexpressing αsyn (Figure 10). Interestingly, reductions in both of these proteins have been shown to inhibit neurotransmitter release (Gitler et al., 2004; Lonart and Simsek-Duran, 2006; Reim et al., 2001; Rosahl et al., 1995; Ryan et al., 1996). In particular, the synapsins are thought to regulate the reserve pool of synaptic vesicles (Hilfiker et al., 1999). Because the synapsins and αsyn both localize to synaptic vesicles distal to the active zone (Tao-Cheng, 2006), associate peripherally with synaptic vesicles (Fortin et al., 2005), and

behave similarly in response to neuronal activity (Fortin et al., 2005; Tao-Cheng, 2006), it would make sense that they might share a functional relationship. Whether these proteins interact directly or indirectly to regulate recycling pool size, and indeed, the mechanism through which this occurs, remains to be determined. It would be quite informative to overexpress αsyn in primary neurons derived from the synapsin triple knockout mice (Gitler et al., 2004). The prediction would be that knockout of synapsins would cause a reduction in recycling pool size measured using VGLUT1-pHluorin. If αsyn has no additional effect on recycling pool size in these neurons, then it could be concluded that αsyn acts through synapsin to mediate a reduction in the size of the SV recycling pool. If αsyn overexpression in these neurons led to a further reduction in recycling pool size, then it could be concluded that the change in synapsin levels I see do not mediate the effects of αsyn on neurotransmitter release but underlie some other undetected change in synaptic physiology.

The relationship of αsyn with complexins is more enigmatic. In mice with deletions of both αsyn and βsyn, the levels of complexin II increase by 30% (Chandra et al., 2004). With αsyn overexpression, we see a significant decrease in complexin II by 45%, and a smaller reduction in complexin I (Figure 10). Complexins are believed to bind to SNARE complexes, and to clamp then in a metastable state that allows for rapid synaptic vesicle exocytosis in response to calcium entry (Tang et al., 2006). The change in levels of the complexins with αsyn overexpression may represent a functional relationship between these proteins, but more experiments are needed to understand how αsyn might function in the regulation of SNARE complex formation. Intriguingly, however, complexin II levels have been shown to undergo progressive depletion in a

mouse model of Huntington's disease (Morton and Edwardson, 2001). It will be quite interesting to see if complexins and their role in synaptic transmission turn out to play a more general role in neurodegenerative disease, including PD.

Finally, the mechanisms through which recycling pool vesicles are specified remain unclear. In mature nerve terminals, synaptic vesicles segregate into three distinct functional pools: the readily releasable pool (RRP), the recycling pool, and the reserve pool (Rizzoli and Betz, 2005). The readily releasable pool consists of synaptic vesicles that are immediately available upon stimulation and are thought to be equivalent to the morphologically "docked" vesicles. The recycling pool contains vesicles that replenish the RRP during moderate stimulation. The reserve pool contains vesicles that are only recruited for release during intense stimulation, if they are recruited at all. Although it is not yet possible to examine the biochemical makeup of these individual pools of vesicles, it is likely that they will consist of different complements of proteins, or at the very least the same proteins in different stoichiometric ratios (Bonanomi et al., 2006; Voglmaier and Edwards, 2007). Further, the proteins present in these vesicles is undoubtedly specified at the time of endocytosis from the plasma membrane, or in some cases during budding from an endosomal structure (Santos et al., 2008). Using techniques available today, it is difficult to assess the many steps in the synaptic vesicle endocytic cycle after scission of the nascent vesicle from the plasma membrane. It remains possible that overexpression of αsyn affects a step in the maturation of synaptic vesicles between vesicle scission and the reavailability of that vesicle for another round of exocytosis, a process commonly referred to as "repriming" (Ryan et al., 1993). Experiments to examine this possibility are underway.

In Chapter 3, I present preliminary evidence examining a role for αsyn in the processes of mitochondrial fusion and fission. Experiments are currently underway to investigate the precise role of αsyn in these processes. We have already shown that this effect is specific to mitochondria, and also causes mitochondrial fragmentation in cultured midbrain neurons. After determining whether αsyn overexpression causes a decrease in fusion or an increase in fission, epistasis experiments will be performed to delineate which fusion or fission proteins αsyn might be acting through. Further, we will investigate whether αsyn overexpression in neurons affects mitochondrial localization to synapses, and whether this may play a role in the regulation of neurotransmitter release.

PERSPECTIVES

The data presented in this dissertation substantially adds to the body of knowledge concerning the function of αsyn at the nerve terminal. Ongoing experiments in our laboratory will be focused on determining how the decrease in neurotransmitter release observed with overexpression of αsyn could initiate the degenerative process observed in PD.

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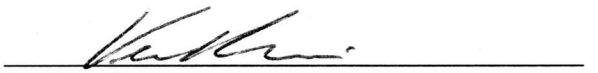

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