

Parkinson's Disease Associated Protein α -Synuclein-E46K Demonstrates Strain and Ploidy-Specific Toxicity in Budding Yeast

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Summary

Parkinson's disease (PD) is a fatal neurodegenerative disorder that affects 1 in 100 individuals over the age of 60. PD results from dopaminergic neuronal atrophy localized within the substantia nigra of the midbrain; the pathology consists of fibrillar inclusions, Lewy Bodies, within substantia nigral neurons. The principal component of Lewy Bodies is the protein α -synuclein. Though 90-95% of PD cases are sporadic, familial forms result from the missense mutations A30P, A53T, or E46K in the α -synuclein gene. Little is known about the properties of the recently discovered E46K mutation. We hypothesized that the E46K mutation alters the conformation of α -synuclein in a potentially toxic manner that results in increased α -synuclein misfolding and alters its plasma membrane localization. Therefore, the purpose of this thesis was to characterize E46K in budding yeast (*Saccharomyces cerevisiae*). To further compare all familial mutants, double and triple E46K isoforms were synthesized with A30P and A53T mutations and tagged with green fluorescent protein (GFP). All familial mutants, and control GFP or parent vector pYES2 were expressed in several naturally occurring and commonly studied budding yeast haploid strains and a diploid strain. Growth curves and viability assays of one haploid strain revealed E46K to be toxic. Consequently, we wanted to determine if E46K toxicity was strain specific. Identical growth and viability assays were performed on several other haploid strains. Interestingly, no E46K toxicity was observed in any of the strains except the opposite-mating type of the toxic strain. Therefore, to test if E46K toxicity was ploidy-specific, these analyses were performed on the diploid strain formed by the mating of the two toxic strains. Unexpectedly, E46K and E46K variations were not toxic in the nearly genetically identical diploid. Thus, strain and ploidy differences between strains must account for this selective E46K toxicity. GFP fluorescence live-cell microscopy revealed that α -synuclein membrane localization was correlated with toxicity. Further study of the underlying mechanisms for E46K toxicity in yeast may provide insight into its role in neuronal toxicity and familial PD.

Introduction

The brain is the central entity that defines each individual and when damaged, their personality may be

altered beyond recognition. The localization of brain damage determines which functions and characteristics are lost. Though external brain trauma and cancers are readily identifiable causes of damage, neurological degeneration resulting from the misfolding of proteins is not. Neurodegenerative diseases manifest themselves within the brain or nervous system differently, but all result in the chronic depletion of nervous tissue (Purves et al., 2004). As these neurons that provide fundamental characteristics such as cognition, memory, and movement die, bodily functions cease to occur and patients slowly waste away to death.

Parkinson's disease (PD) is the second most common fatal neurodegenerative disorder of the central nervous system. It afflicts 1 in 100 individuals over the age of 60, of which 5 to 10% are diagnosed before the age of 40 (PD Foundation, 2006). Though 90 to 95% of all cases are sporadic, early onset familial forms of PD occur in 5 to 10% of patients (PD Foundation, 2006). The disease has no cure, and patients are destined to die from motor initiation failure. The principal symptoms of PD are resting tremor, postural deficits, rigidity, and bradykinesia (Purves et al., 2004). In addition, psychological studies suggest that PD patients exhibit motor learning deficits (Heindel et al., 1989; Helmuth et al., 2000). As the disease progresses, the intensity of each symptom increases until the patient is at a complete loss to initiate voluntary movements.

Chronic increases in motor initiation deficits are the direct result of neuronal atrophy localized within the substantia nigra in the midbrain (Purves et al., 2004; Figure 1). This vital structure consists of a thin black strip (1-2 mm wide) of dopaminergic neurons pigmented with neuromelanin (Purves et al., 2004). They function as an integral part of the basal ganglia which, is a complex circuit responsible for initiating voluntary movements. Through numerous inhibitory and excitatory synapses, the substantia nigra functions as an initiator of voluntary movements (Figure 2).

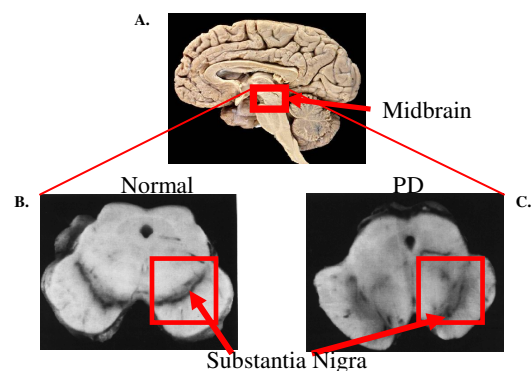


Figure 1: Parkinson's Disease Pathology. Image A is a sagittal section along the longitudinal fissure that shows the location of the substantia nigra in the human brain, which is enclosed by a red box. Image B is of the substantia nigra from a normal human brain depicted from a horizontal section. Image C shows the substantia nigra in a PD brain. In

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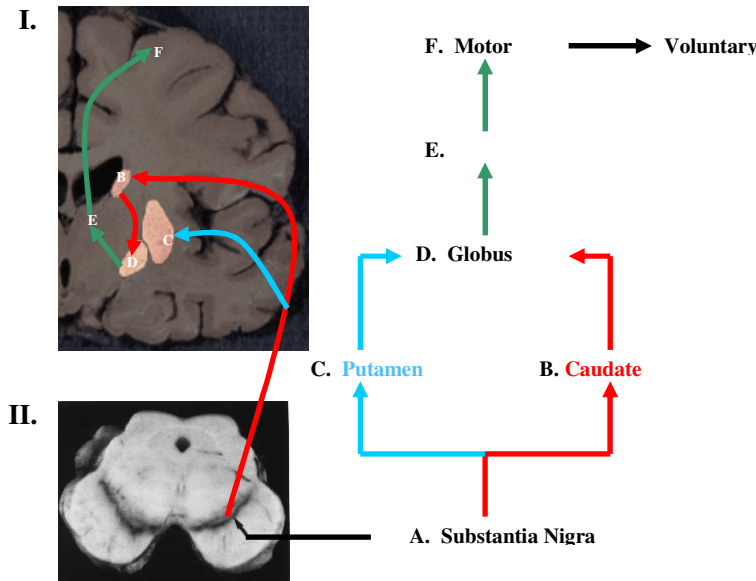


Figure 2: The Basal Ganglia and Voluntary Movement. This depiction of the basal ganglia details the flow of stimuli through it resulting in motor initiation. The colored words and lines, and their assigned letters correspond to the exact location of their structures and synaptic targets in image I. The substantia nigra, image II, synapses on both the caudate nucleus, B, and putamen, C. This is indicated on image I by the red and blue lines. The caudate nucleus, B, and putamen, C, synapse on the globus pallidus, D. On image I, this is represented by the convergence of the blue and red lines on a single structure. The globus pallidus, D, synapses on the thalamus, E, which is indicated by the green arrow on image I. The thalamus, E, synapses on the motor cortex, F, which is located at the end of the green arrows in image I. Excitatory stimuli traveling up from the substantia nigra and through the basal ganglia result in activation of the globus pallidus, relieving the thalamus of tonic inhibition. Consequently, an excitatory stimulus is sent to the motor cortex and movement is initiated.

the PD brain, there is a significant decrease in the number of substantia nigral neurons compared to the normal brain. This can be seen by the decreased black pigmentation of the substantia nigra in C. When cells in the *pars compacta* region of the substantia nigra die, the cascade of inhibitory and excitatory synapses through the caudate nucleus, putamen, and globus pallidus fail to relieve the thalamus of tonic inhibition (Purves et al., 2004). Consequently, the pre-motor and motor cortexes are not stimulated and movement is not initiated (Purves et al., 2004; Figure 2). Interestingly, symptoms of PD are not always expressed until 60 to 80% of substantia nigral neurons are dead (Lansbury et al., 2003; Purves et al., 2004).

Upon autopsy, PD pathology consists of neurofibrillary inclusions, Lewy Bodies, within substantia nigral neurons. The principal component of Lewy Bodies is the protein α -synuclein (Spillantini et al., 1998). This protein is expressed throughout the brain and exists in a natively unfolded state localized to the plasma membranes of pre-synaptic terminals (Purves et al., 2004). α -synuclein aggregation is specific to the substantia nigra of PD patients, suggesting that other unique features of these neurons are involved (Purves et al., 2004). Although α -synuclein is intimately related to the pathogenesis of PD, its function remains unknown.

Cellular Basis of PD

Though the exact cellular basis of PD has yet to be discovered, and the vast majority of cases are sporadic, genetic studies have linked autosomal dominant familial forms of PD to α -synuclein mutations A30P (Kruger et al., 1998), A53T (Polymeropoulos et al., 1997), and recently E46K (Zarranz et al., 2004). In addition, mutations in *parkin* (Kitada et al., 1998) and DJ-1 (Leroy et al., 1998) are associated with adolescent

forms of PD (Dawson et al., 2003). DJ-1 and *Parkin*, an E3 ligase, function within the ubiquitin-proteasomal pathway. (Dawson et al., 2003). The dysfunction of *parkin* or DJ-1 is thought to exacerbate the potentially toxic properties of mutant α -synuclein in addition to being capable of causing PD on their own (Dawson et al., 2003; Purves et al., 2004). Proteins to be degraded in the proteasome are tagged with the molecule ubiquitin (Purves et al., 2004). UCH-L1, which is involved in both addition (ligase) and removal (hydrolase) of ubiquitin to/from α -synuclein, is also implicated in autosomal dominant familial PD because the S18Y mutation reduces its hydrolase activity (Liu et al., 2002). The loss of hydrolase function could account for fibrillar α -synuclein being poly-ubiquitinated (Stefanis et al., 2001). Mutations within PINK1 (Valente et al., 2004) and LRRK2 (Funayama et al., 2002) are also capable of causing familial forms of PD. Though several proteins can cause PD, α -synuclein remains the principal target of investigations because it is always misfolded in PD, unlike the other proteins, and forms the hallmark feature of the disease, Lewy Bodies.

Several questions on PD remain unanswered; 1) Why is neuronal atrophy specific to the Substantia Nigra? 2) Are Lewy Bodies the toxic or neuroprotective form of α -synuclein? 3) What are the characteristics of α -synuclein-E46K, and how do they compare to A30P and A53T? *This thesis will focus on the latter question by comparative evaluation of E46K characteristics and previously discovered familial mutants A30P and A53T within budding yeast (Saccharomyces cerevisiae).*

Characteristics of α -Synuclein

α -synuclein is a small, 140 amino acid, 14 kDa, natively unfolded protein localized within the pre-synaptic terminal (Uversky et al., 2002). It acquires an α -helical

conformation, *in vitro*, when exposed to fluorinated alcohols, detergents, or vesicles/membranes (Weinreb et al., 1996; Davidson et al., 1998). The amino acid sequence is divided into three regions based on significantly different secondary-structure characteristics. The N-terminus is amphipathic and contains residues 1-60 along with seven imperfect repeats of XKTKEGVXXXX that are thought to resist aggregation (Kessler et al., 2003; Uversky et al., 2002). All familial PD mutants occur in this region. Amino acids 61-95 form the hydrophobic core, and are involved in facilitating aggregation (Uversky et al., 2002). Specifically, a stretch of 12-uncharged amino acids within that hydrophobic domain are required for fibril formation (Giasson et al., 2001). This result is supported by the finding that the introduction of various charged amino acids into the central region decreases the rate of fibril formation (Giasson et al., 2001). β -synuclein, an α -synuclein homologue, lacks an uncharged sequence at this locus and consequently, does not aggregate (Lee et al., 2001). The C-terminus, residues 96-140, is hydrophilic and unfolded due to many proline residues (Uversky et al., 2002).

Initial studies of PD were aimed at determining the function of α -synuclein. The several 11-amino acid repeats in α -synuclein resemble those of apolipoproteins thus, Davidson et al. hypothesized that α -synuclein bound to vesicular membranes (1998). Their *in vitro* results indicated that α -synuclein bound to 20 to 25 nm vesicles composed of acidic phospholipids. Furthermore, α -synuclein conformationally stabilized into an α -helix rich structure upon membrane binding (Davidson et al., 1998). When analyzing the α -synuclein familial PD mutants, A53T slightly decreases membrane binding affinity while A30P decreases it significantly compared to WT (Jensen et al., 1998; Perrin et al., 2000). Detailed analysis of α -synuclein membrane binding properties shows that the N-terminus binds and becomes α -helical while the C-terminus remains unfolded in the cytoplasm (Perrin et al., 2000; Eliezer et al., 2001). These secondary structural and binding characteristics, and the finding that the C-terminus is free to interact and conform to a variety of substrates, suggests that α -synuclein is involved in vesicular trafficking (Perrin et al., 2000; Eliezer et al., 2001).

Focusing on interactions between single α -synuclein molecules, Conway and colleagues were the first to detail the process of monomeric α -synuclein conversion into fibrils (1998). Specifically, they tested the hypothesis that familial PD results from acceleration of fibril formation by A30P and A53T. This was accomplished by comparing the rates of fibril formation between A30P, A53T, and WT, *in vitro* (Conway et al., 1998). Familial mutants A53T and A30P aggregated into fibrils faster than WT, with A53T as the fastest overall (Conway et al., 1998). This study provided the first evidence for A30P and A53T familial mutants causing PD by accelerating the rate of fibril formation.

Subsequent research by Conway et al. further characterized the monomer to fibril transition by determining the rate of monomeric α -synuclein consumption relative to the rate of fibril formation (2000). Interestingly, the increased rate of monomeric A30P and A53T consumption did not account for their respective rates of fibril formation (Conway et al., 2000). As a result, they proposed that a protofibrillar intermediate must exist between monomeric and fibrillar α -synuclein conformations. The existence of

protofibrils was confirmed when they were separated from monomeric and fibrillar α -synuclein using gel-filtration chromatography, and visualized with atomic force microscopy (AFM; Conway et al., 2000). The AFM images revealed that protofibrils exist in one of two states: chains or rings of spheres (Conway et al., 2000). Significantly, protofibrils were observed to bind and form pores in synthetic and mouse brain-derived membranes similar to the β -barrel pores formed by bacterial toxins (Volles et al., 2001; Ding et al., 2002). Thus, Lewy Bodies may actually be the neuroprotective form of α -synuclein if protofibrils are the toxic species because fibril formation consumes the oligomeric intermediates (Volles et al., 2001; Ding et al., 2002; Figure 3).

Neurodegeneration is Specific to the Substantia Nigra

α -synuclein is expressed throughout the brain and yet, aggregation and neuronal atrophy occurs only in the substantia nigra of the PD brain (Purves et al., 2004). The reason for this pathological specificity remains unknown. However, an oxidized form of dopamine (L-dopa) binds and stabilizes protofibrils *in vitro* (Conway et al., 2001). If protofibrils are the toxic agent in PD, then L-dopa would prolong the duration that α -synuclein is in this toxic conformation (Conway et al., 2001). Unlike the vast majority of neurons in the brain, the neurotransmitter of the substantia nigra is dopamine. Thus, oxidation of dopamine in the presence of protofibrils within substantia nigra neurons may rationalize PD specificity to this structure (Conway et al., 2001).

Model Organisms

In addition to primary neuronal cultures, several model organisms exist for the study of PD. A line of transgenic mice overexpressing human α -synuclein was established and found to exhibit the PD characteristics of substantia nigra atrophy, motor initiation deficits, and intraneuronal aggregates (Masliah et al., 2000). Interestingly, these aggregates are non-fibrillar and less organized in contrast to the highly organized fibrils in PD (Goldberg and Lansbury, 2000). *Drosophila* transgenic for human α -synuclein and familial mutants A30P and A53T are also an established PD model (Feany and Bender, 2000). *Drosophila* expressing human α -synuclein exhibit features characteristic of PD including dopaminergic neuronal atrophy, motor deficits such as irregular flying, and intraneuronal aggregates (Feany and Bender, 2000). Interestingly, a line of mice expressing both human α and β -synucleins have a decrease in Lewy Body concentration and do not manifest PD symptoms (Hashimoto et al., 2001). Together, these organisms serve as models that adequately represent the pathological and behavioral components of PD observed in humans (Lansbury et al., 2003).

Our lab uses a *Saccharomyces cerevisiae* model that was recently established as a powerful means for the study of PD (Sharma et al., 2006). The power of the yeast model is seen through several characteristics of the organism. First, the yeast genome is completely sequenced, allowing for detailed mapping of yeast genes. Second, rapid turnover of generations allows for a genetically based disease study over several generations in a number of days. Third, gene deletion, introduction, or overexpression can be performed in a few days with relative ease.

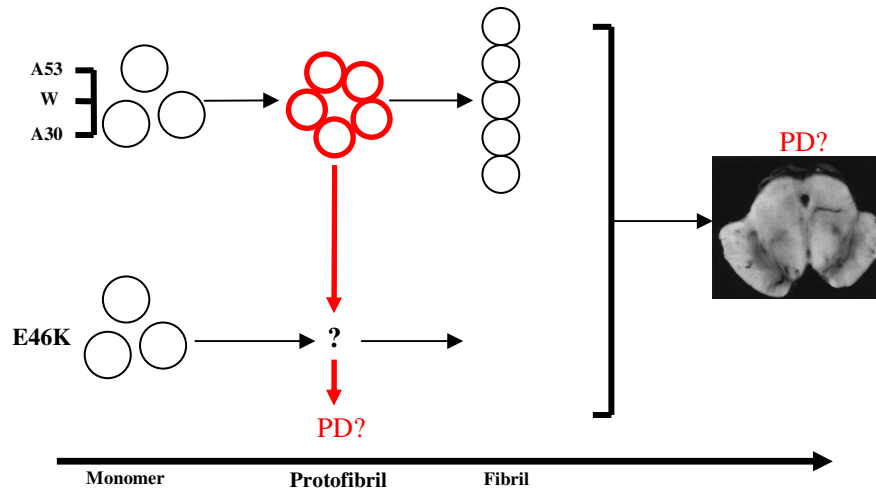


Figure 3: α -Synuclein Aggregation and the Toxic Protofibril Hypothesis.

The toxic Parkinson's disease (PD) causing conformation of α -synuclein is unknown but toxic protofibrillar intermediates are gaining support as the causative agent. WT- α -synuclein and familial mutants A30P, A53T, and E46K aggregate to form fibrils, Lewy Bodies, that are present in the substantia nigra of all PD brains. Within the pathway toward aggregation, WT, A30P, and A53T form a protofibrillar intermediate between the monomeric and fibrillar states. This pathway is not understood for E46K and the existence of an intermediate is not known.

Fourth, the Yeast Genome Project, as well as various corporations, have synthesized thousands of different genetic knock-out strains that are readily available to the scientific community (Outeiro et al., 2004).

Prior to my research, three yeast models existed for the study of PD or similar neurodegenerative disorders (Outeiro et al., 2003; Dixon et al., 2005; Zabrocki et al., 2005). In addition to these, our lab has established a budding yeast, *Saccharomyces cerevisiae*, model that expresses human α -synuclein-A30P, A53T, and A30P/A53T (Sharma et al., 2006). When WT, A30P, A53T, or A30P/A53T are expressed in the wild-type yeast strains BY4741, BY4743, and TSY623, no growth impairment is observed (Sharma et al., 2006).

Fluorescence microscopy and GFP tags used to study α -synuclein intracellular properties when expressed in BY4741, BY4743, and TSY623 revealed three general phenotypes of α -synuclein expression: 1) Cytoplasmically diffuse, 2) Plasma membrane or vacuolar localized, or 3) Aggregated (Sharma et al., 2006). Both A53T and WT forms of α -synuclein localize to the plasma membrane and form intracellular inclusions (Sharma et al., 2006). α -synuclein-A30P is observed to be cytoplasmically diffuse while the double mutant, A30P/A53T, exists as either cytoplasmically diffuse or localized to the plasma membrane (Sharma et al., 2006). In addition, these experiments demonstrate that no difference exists between α -synuclein phenotypes in haploid, BY4741 and TSY623, or diploid, BY4743, strains (Sharma et al., 2006). Several other studies found similar results when one copy of α -synuclein is expressed but, two-copy expression is observed to be toxic to yeast except A30P (Outeiro et al., 2003; Dixon et al., 2005; Zabrocki et al., 2005).

Summary of α -Synuclein Studies in Budding Yeast

Numerous detailed growth analyses of α -synuclein-A53T expression in budding yeast found that it was toxic at elevated expression levels and/or when cells

had mutant proteasomes, and was capable of causing proteasomal dysfunction (Outeiro et al., 2003; Dixon et al., 2005; Zabrocki et al., 2005; Sharma et al., 2006). The hypothesis that toxicity is the result of impairment of α -synuclein degradation is further supported by the finding that when fibrillized, a significant portion of the protein is tagged with ubiquitin (Outeiro et al., 2003). In addition, A53T and WT plasma membrane localization may result from traffic through the ER-Golgi secretory pathway (Dixon et al., 2005). In contrast to A53T, A30P is not toxic at two copy or slightly elevated expression levels, and does not appear to pass through the secretory pathway (Outeiro et al., 2003; Dixon et al., 2005). Similarly, both A30P and A53T inhibit the ability of the proteasome to degrade other cellular proteins (Outeiro et al., 2003). Though few studies of A30P/A53T in yeast have been performed, our lab shows it to be non-toxic at heterologous multi-copy expression levels and to display a phenotype between that of A30P and A53T (Sharma et al., 2006).

Properties of Recently Discovered α -Synuclein-E46K

In 2004, Zarranz et al. discovered a new autosomal dominant familial PD mutant, α -synuclein-E46K, in a Spanish family. Unlike A30P and A53T, little knowledge exists of this novel mutation. Of the four published studies on E46K, three have characterized its role in aggregation and lipid binding (Choi et al., 2004; Greenbaum et al., 2005; Pandey et al., 2006). The fourth one examined the clinical manifestations of E46K and found patients to have abnormal sleep in addition to motor deficits (Zarranz et al., 2005).

Immediately following the discovery of E46K, Choi et al. conducted the first study of the mutation (2004). They analyzed the effects of E46K on α -synuclein phospholipid binding, *in vitro*, and compared them to A30P and A53T. Each mutant and WT were incubated with liposomes that resemble the membranes of synaptic vesicles to measure the absolute binding of α -synuclein to the membranes. Compared to WT, E46K increased lipid binding by 56%. In contrast, A30P

and A53T reduced binding by 56% and 9 to 12% respectively. Furthermore, E46K increased the binding of α -synuclein to liposomes regardless of their size. Thus, E46K increases the binding affinity of α -synuclein for liposomes/membranes (Choi et al., 2004).

Both A30P and A53T are linked to an increase in fibril concentration, but A53T also accelerates the rate at which those fibrils are formed unlike A30P (Conway et al., 1998). Similar to A53T, E46K also increases the rate of fibril formation (Choi et al., 2004; Greenbaum et al., 2005). Two *in vitro* studies demonstrated that as α -synuclein concentration increases, E46K increases the rate of fibril formation compared to WT (Choi et al., 2004 and Greenbaum et al., 2005). In addition, Pandey et al. evaluated E46K effects on aggregation, *in vivo*, using human catecholaminergic neuroblastoma cells (2006). Their experiments revealed E46K to form larger and more concentrated fibrils compared to WT, A30P, and A53T (Pandey et al., 2006). From these studies, it can be concluded that E46K confers a higher propensity for α -synuclein aggregation than the other familial mutants (Choi et al., 2004; Greenbaum et al., 2005; Pandey et al., 2006).

Hypotheses and Aims

Hypothesis: The E46K mutation alters the conformation of α -synuclein in a potentially toxic manner that results in increased α -synuclein misfolding and alters its plasma membrane localization.

Little is known about the novel α -synuclein-E46K mutation, but previous research found it to increase α -synuclein aggregation both *in vitro* and *in vivo*, and increase lipid binding affinity *in vitro*. (Choi et al., 2004; Greenbaum et al., 2005; Pandey et al., 2006). Therefore, the purpose of this thesis was to characterize α -synuclein-E46K in budding yeast, *Saccharomyces cerevisiae*. We hypothesized that the E46K mutation alters the conformation of α -synuclein in a potentially toxic manner that results in increased α -synuclein misfolding and alters its plasma membrane localization. Our first aim was to determine if E46K and E46K variations are toxic to yeast. Secondly, we wanted to examine the effects of E46K on α -synuclein membrane localization and aggregation. Our final aim was to evaluate the effects of E46K on α -synuclein expression. To assess whether E46K effects are strain and/or ploidy-specific, these analyses were performed on several well studied haploid yeast strains, and a diploid strain. We found E46K toxicity to exhibit both strain and ploidy-specific toxicity, and α -synuclein membrane localization to be correlated with toxicity. The results of this study will significantly contribute to the body of knowledge on this new mutation, and be the first analysis of E46K in yeast.

Methods

Yeast Strains: The wild-type budding yeast strains BY4741, BY4742, BY4743, W303, and TSY623 were used (Open Biosystems).

Vectors: The pYES2.1/V5-His-TOPO DNA vector (Invitrogen) was used to express all forms of α -synuclein and green fluorescent protein (GFP) in the yeast.

α -Synuclein Variations: Human α -synuclein wild-type, A30P, A30P/A53T, E46K, A30P/E46K, E46K/A53T, and

A30P/E46K/A53T with C-terminus GFP tags in the pYES2.1/V5-His-TOPO plasmid were used.

α -Synuclein Expression: A tightly regulated galactose inducible promoter (GAL1) within the pYES2.1/V5-His-TOPO plasmid was used to control α -synuclein and GFP expression. Yeast were grown in either Synthetic Complete (SC)-Uracil raffinose (2%) or SC-Uracil glucose (2%) to allow growth but suppress α -synuclein expression. To allow for expression, yeast were grown in either SC-Uracil raffinose (2%) or SC-Uracil glucose (2%) overnight, centrifuged at 2500 rpm at 4 °C for 5 min, and then washed with water. The glucose or raffinose-free culture was then used to inoculate a volume of SC-Uracil galactose (2%), resulting in α -synuclein expression. The galactose culture was then incubated at 30 °C and 200 rpm shaking.

Site-Directed Mutagenesis: α -synuclein mutant A30P/E46K was synthesized previously in our lab by Sina Vahedi. The α -synuclein mutants E46K/A53T, A30P/E46K/A53T all with C-terminus GFP were synthesized using the Gene Taylor Site-Directed Mutagenesis System and Taq High Fidelity polymerase (Invitrogen; Figures 4 and 5). Both the E46K forward and reverse primers bound within the α -synuclein gene in the pYES2.1/V5-His-TOPO plasmid at 5'-ATGTAGGCTCCAAAACAAGAAGGGAGTGGTGC-3' and 5'-CTTGGTTTTGGAGCCTACATAGAGAACACC-3' (Qiagen). The gene was mutated using either an α -synuclein-A30P, α -synuclein-A53T, or α -synuclein-A30P/A53T template all with C-terminus GFP tags in pYES2.1/V5-His-TOPO vectors.

Agarose gel electrophoresis of the DNA mutagenesis products revealed bands near 6000 base-pairs corresponding to those expected for α -synuclein with a GFP tag. The initial DNA mutagenesis thermocycled product was transformed into One Shot MAX Efficiency DH5 α -T1 Competent Cells (Invitrogen). The mutated plasmid was then isolated from those cells using the Qiagen Mini-Prep kit. A portion of this DNA and α -synuclein-E46K was sent to the University of Chicago Cancer Research Center DNA Sequencing Facility which confirmed the desired mutations (Figure 5).

Transformation of Yeast Strains: The yeast strains BY4741, BY4742, BY4743, TSY623, W303, MHY810, and MHY803 were transformed with one of the following α -synuclein mutants: A30P/E46K, E46K/A53T, and A30P/E46K/A53T all with C-terminus GFP tags in the pYES2.1/V5-His-TOPO vector (Table 1). The sequenced α -synuclein-E46K with C-terminus GFP plasmid was transformed into BY4741. The A30P/A53T mutant with C-terminus GFP was transformed into W303. Transformations were confirmed by whole-cell yeast polymerase chain reaction (PCR).

Western Blotting: Cells were grown in SC-Uracil raffinose (2%) overnight, centrifuged at 2500 rpm at 4 °C for 5 min then washed with water. They were then transferred to SC-Uracil galactose (2%) media. In most cases, cells from an ongoing O.D. 600 nm growth analysis were counted at 24 hours after α -synuclein expression, and 2.5×10^7 of those cells were isolated through centrifugation at room temperature and 14,000 rpm in order to make cell lysates. The cells were lysed using small glass beads and electrophoresis

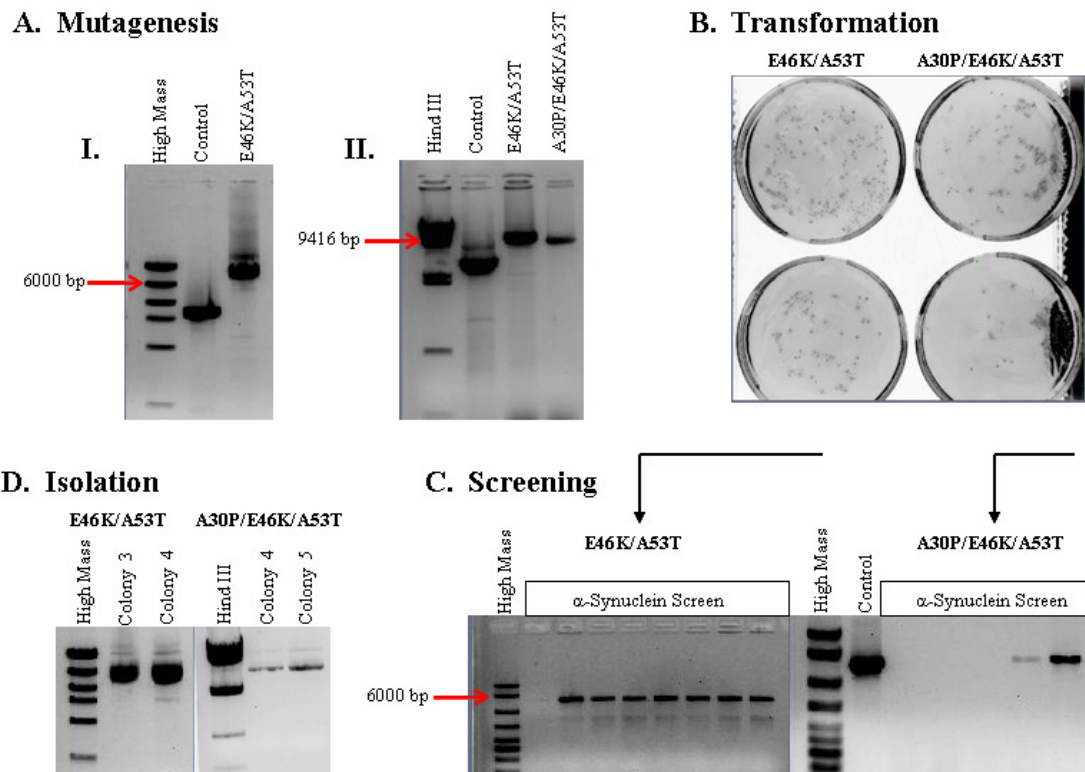


Figure 4: E46K/A53T and A30P/E46K/A53T α -Synuclein Mutagenesis.

- Thermocycled Mutagenesis Products:** Thermocycled mutagenesis DNA products on 1% agarose gels from the E46K/A53T and A30P/E46K/A53T mutagenesis reactions using the pYES2 parent plasmid with either α -synuclein-A53T or A30P/A53T genes as the template. Gel I has two lanes with the E46K/A53T mutation at ~6000 bps on the High Mass® ladder. Gel II has three lanes with A30P/E46K/A53T and a second mutagenesis reaction product for E46K/A53T, with both below 9416 bps on the Hind III® mass ladder. In both gels the control reaction consisted of a mutation within the LacZ gene.
- Transformation of *E. coli*:** Thermocycled products from **A** were transformed into *E. coli* and plated in two volumes on LB + ampicillin plates (selective media) in order to circularize the linear pYES2 plasmid. The pYES2 plasmid contained an ampicillin resistance gene.
- PCR Screening for Plasmid:** Clean sequencing results require plasmid obtained from a single ancestor. Individual colonies from both E46K combination mutants were screened for the pYES2 plasmid using PCR and primers specific for α -synuclein with C-terminal GFP. Isolated α -synuclein-pYES2 was run as a control for the triple mutant but not for the double. All positive screens are indicated by black bands at ~6000 bps on 1% agarose gels.
- Plasmid Isolation:** DNA must be isolated from the cell to sequence. Colonies 3 and 4 from E46K/A53T and 4 and 5 from A30P/E46K/A53T that positively screened for the plasmid in **C** were grown in liquid LB + ampicillin. The plasmid was then isolated to be sent for sequencing at the University of Chicago. These are 1% agarose gels of the plasmid isolation product.

solubilizing buffer (ESB), which was composed of numerous protease inhibitors and Sodium Dodecyl Sulfate (SDS) detergent. The lysates were then stored at -20 °C. Tris-glycine gels (Invitrogen) were used to separate the proteins within the cell lysates. Protein mass was determined using 10 μ L of See Blue mass ladder (Invitrogen). From each cell lysate, 20 μ L was transferred into the gel and electrophoresed at 130 V. Running buffer diluted to 1X, (29.0 g Tris-Base, 144.0 g Glycine, 10.0 g SDS, 1 L H₂O, and pH 8.7) and 1X transfer buffer (18.2 g Tris-Base, 90.0 g Glycine, 500 mL H₂O, and pH 8.3) were used. Protein was transferred to PVDF membranes using the Semi-Dry Transfer apparatus (Biorad). Blotting was performed using primary and secondary Anti-V5/Alkaline Phosphatase (Invitrogen) to bind to α -synuclein, and primary Anti-Phosphoglycerate Kinase (PGK) from the

Western Breeze kit (Invitrogen). Coomassie staining was done by electrophoresing cell lysates on a Tris-Glycine gel (as described above) and then washing the gel in fixing solution with slight agitation for two hours. The fixing solution was removed and coomassie staining solution was added for one hour. Fixing solution was then added for 5 min, removed, and replaced with de-staining solution overnight. The gel was then photographed and dried.

Growth Curve Analysis: Optical density at 600 nm wavelength was used to assess cell growth during α -synuclein expression. Cells were grown overnight in 5 mL of SC-Uracil raffinose (2%) or SC-Uracil glucose (2%) at 30 °C and 200 rpm shaking. Cultures were counted to determine cell density using a

A. AAACCCGGATCGGCTACTAGCAGCTGTAATACGACTCACTATAGGGAATATTAAGCTCGCCCTTGGCGCCAAAGC
 TTGCCATGGATGTATTTCATGAAAGGACTTTCAAAGGCCAAGGAGGGAGTTGTGGCTGCTGCTGAGAAAACCAA
 ACAGGGTGTGGCAGAAAGCAGCAGGAAAGACAAAAGAGGGTGTCTCTATGTAGGCTCCAAAACCAAGAAAGG
 AGTGGTGCATGGTGTGACAACAGTGGCTGAGAAAGCCAAAGAGCAAGTGACAAATGTTGGAGGAGCAGTGGT
 GACGGGTGTGACAGCAGTAGCCCAAGAGCAGTGGAGGGAGCAGGGAGCATTGCGAGCTGCTGGCTTTGTG
 AAAAAGGACCAAGTTGGGCAAGAATGAAGAAAGGAGCCCCACAGGAAGGAATTCTGGAAAGATATGCCTGTGGAT
 CCTGACAATGAGGCTTATGAAATGCCTTCTGAGGAAGGGTATCAAGA CTACGAACCTGAAGGCCAAGGGCAAT
 TCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAATGGCTAGCAAAGGAGAA

B. CCCGGACGGACTCTAGCAGCTGTAATACGACTCACTATAGGGAATATTAAGCTCGCCCTTGGCGCCAAAGCTTG
 CCATGGATGTATTTCATGAAAGGACTTTCAAAGGCCAAGGAGGGAGTTGTGGCTGCTGCTGAGAAAACCAAAC
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 GACGGGTGTGACAGCAGTAGCCCAAGAGCAGTGGAGGGAGCAGGGAGCATTGCGAGCAGCCACTGGCTTTGT
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 AATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAATGGCTAGCAAAGGAGAAAGAACTTTTCA
 TCGGAGTTGTCCCAATTCTTGTGAATTAGATGGTGTATGTAA

Figure 5: α -synuclein-E46K/A53T and A30P/E46K/A53T Mutagenesis DNA Product Sequences.

The DNA sequence of the E46K/A53T mutagenesis product is shown in **A**. The A53T mutation is shown in blue and the E46K mutation in red. The A30P/E46K/A53T DNA sequence is shown in **B**, and the A30P mutation is in yellow, E46K in red, and A53T in blue. No other mutations within the α -synuclein gene are present.

hemocytometer, centrifuged at 2500 rpm for 5 min at 4 °C, and washed with water. A flask of 35 mL SC-Uracil galactose (2%) was inoculated to a cell density of 2.0×10^6 cells/mL and incubated at 30 °C and 200 rpm shaking for 48 hours. At 0, 3, 6, 12, 18, 24, 36, and 48 hours of α -synuclein expression, 1 mL from each culture was pipetted into a cuvet in duplicate. Each cuvet was then analyzed using a Hitachi U-2000 spectrophotometer to determine culture density. The readings from each time-point were averaged and plotted on a graph of absorbance versus time.

Growth Analysis through Cell Spotting: Cells were grown in SC-Uracil raffinose (2%) or SC-Uracil glucose (2%) overnight. The cell density of each culture was determined after which, they were centrifuged at 2500 rpm and 4 °C for 5 min, and then washed with water. From each culture, 2.0×10^7 cells were removed and pelleted through centrifugation at room temperature and 14,000 rpm, and then the pellets were re-suspended in 1 mL of water. Each culture of density 2.0×10^7 cells/mL had 100 μ L transferred to a 96-well microtiter plate. These cells were then serially diluted 5-fold across the plate. Using the multi-pronged frogger, a small volume (< 5 μ L) of culture contained on the tip of each prong was spotted onto three SC-Uracil galactose (2%) and three SC-Uracil glucose (2%) plates. Each plate was then incubated at 30 °C for 36 to 48 hours after which, they were scanned or photographed and stored at 4 °C.

Time-Course Microscopy: In order to analyze α -synuclein properties during expression, cultures from a concurrently running O.D. 600 nm growth analysis were microscopically imaged at 18, 24, 36, and 48 hours of α -synuclein expression. All images were taken using a Nikon TE2000-U fluorescent microscope and Metamorph® 6.0 software. From the GFP, wild-type α -synuclein, A30P, A53T, A30P/A53T, E46K,

A30P/E46K, E46K/A53T, A30P/E46K/A53T cultures (all with C-terminus GFP tags), 1 mL was removed and centrifuged at room temperature and 14,000 rpm for 6 min. The media was poured off, and the pellet re-suspended in the remaining media. Then, 10 μ L of each culture was pipetted onto a glass slide and covered with a number-1 cover slip. At 18 and 24 hour time-points in BY4741, E46K, A30P/E46K, E46K/A53T, and A30P/E46K/A53T cultures were not as dense as the others. In order to establish equal density for microscopy, a second 1 mL of culture from E46K and associated mutants was added to their respective pellets, centrifuged, and re-suspended. Imaging was performed by obtaining both differential interference contrast (DIC) and fluorescence images of the same cells. This procedure was repeated enough times as was needed to image more than 500 cells from each culture. If less than 500 cells were counted, those cultures are indicated by *.

Statistical Analyses: Statistical significance was established using a T-Test comparing the means of pYES2 and E46K or E46K variations at 18 and 24 hours of α -synuclein expression.

Results

α -Synuclein-E46K is Toxic to Haploid Strain BY4741

Our first goal was to determine the effects of E46K expression on budding yeast growth, the α -synuclein mutants E46K, A30P/E46K, E46K/A53T, or A30P/E46K/A53T were expressed using a galactose-inducible promoter in the wild-type haploid budding yeast strain BY4741. This is one of the most commonly studied of all yeast strains and has a completely sequenced genome. O.D. 600 nm growth analysis over a 48-hour α -synuclein expression time-course was performed (Figure 6-A). The growths of cultures expressing E46K or E46K variations were compared

<u>Construct</u>	<u>Expression Vector</u>	<u>Strain</u>
E46K a-synuclein-GFP	pYES2	BY4741 BY4742
A30P/E46K a-synuclein-GFP	pYES2	BY4741 BY4742 BY4743 TSY623 W303 MHY803 MHY810
E46K/A53T a-synuclein-GFP	pYES2	BY4741 BY4742 BY4743 TSY623 W303 MHY803 MHY810
A30P/E46K/A53T a-synuclein-GFP	pYES2	BY4741 BY4742 BY4743 TSY623 W303 MHY803 MHY810
Parent Plasmid	pYES2	BY4742
GFP	pYES2	BY4742
WT a-synuclein-GFP	pYES2	BY4742
A30P a-synuclein-GFP	pYES2	BY4742
A53T a-synuclein-GFP	pYES2	BY4742
A30P/A53T a-synuclein-GFP	pYES2	BY4742

Table 1: Transformed *Saccharomyces cerevisiae* Strains. Newly and previously synthesized α -synuclein DNA constructs within the pYES2 vector were transformed into *Saccharomyces cerevisiae*. On the left is the DNA construct, pYES2 expression vector in the center, and the right column indicates the strain that was transformed with the DNA construct within the same row.

with pYES2 and GFP controls, and WT, A30P, A53T, and A30P/A53T α -synuclein isoforms. During the first 10 hours of α -synuclein expression, no differences in growth were observed. However, at 18 and 24 hours after induction E46K and E46K variations significantly impaired growth compared to pYES2 and GFP controls,

WT, A30P, A53T, and A30P/A53T ($P_{18hrs} \leq 0.00068$, $P_{24hrs} \leq 0.0016$). Though E46K and E46K variations were toxic, E46K was significantly more toxic than A30P/E46K, E46K/A53T, and A30P/E46K/A53T at 18 and 24 hours in BY4741 ($P_{18hrs} \leq 0.0096$, $P_{24hrs} \leq 0.0085$). By 48 hours of α -synuclein expression, all

BY4741 cultures had returned to equal density. We use "toxicity" to denote growth impairment.

Cell spotting was used as a second means for assessing E46K and E46K variation BY4741 growth impairment. Separate cultures containing pYES2 and GFP controls, and WT, A30P, A53T, A30P/A53T, E46K, and E46K variations were serially diluted 5 times and spotted, in equal volume, onto SC-Uracil galactose plates to induce α -synuclein expression and SC-Uracil glucose plates as a control (Figure 6-B). Similar to O.D. 600 nm analysis, E46K and E46K variations were toxic to BY4741 compared to the other familial mutants and controls.

Expression of α -Synuclein-E46K is Decreased in BY4741

By 48 hours of α -synuclein expression in BY4741, E46K toxicity was no longer observed and all cultures had returned to equal density. To determine the effects of E46K on α -synuclein expression during toxicity, western analysis of BY4741 was conducted at 24 hours after α -synuclein induction using an α -synuclein specific V5 antibody, and Anti-PGK as a loading control (Figure 6-C). α -Synuclein expression was similar for WT, A30P, A53T, and A30P/A53T. In contrast, expression of E46K and E46K variations was decreased. Interestingly, E46K expression was lower than the E46K variations.

Toxicity is Correlated with Membrane Localization in BY4741

Because in vitro studies found E46K to increase phospholipid binding, we wanted test if α -synuclein localizes to the plasma membrane in yeast (Choi et al., 2004). Live-cell intracellular localization of α -synuclein in BY4741 at 18, 24, 36, and 48 hours after induction was investigated using C-terminal GFP tagged α -synuclein constructs and fluorescence microscopy (Figure 6-D). At least 500 cells from control GFP, WT, A30P, A53T, and E46K cultures were quantified at these time-points for either 1) Diffuse fluorescence, 2) Plasma membrane localization, or 3) Aggregation. At all time-points, GFP remained cytoplasmically diffuse with modest aggregation at 48 hours of expression. A30P was diffuse throughout the time course as well. In contrast, WT, A53T, and E46K were membrane bound.

E46K is Not Toxic to Haploids TSY623 and W303

In order to determine if E46K toxicity is strain-specific, O.D. 600 nm and cell spotting growth analyses were performed identical to those for BY4741 on the commonly employed and well studied haploid strains TSY623 and W303. Both growth analyses revealed no signs of toxicity for any of the α -synuclein mutants and controls (Figure 7-A and B; Figure 8-A and B). Western analysis using the α -synuclein specific V5 antibody and Anti-PGK as a loading control at 24 hours post α -synuclein induction demonstrated equal expression between mutants and controls in W303 (Figure 8-C). In TSY623, α -synuclein expression was decreased for GFP, A30P, A30P/E46K, E46K/A53T, and A30P/E46K/A53T (Figure 7-C). Unexpectedly, a distinct band was visible in the pYES2 lane near 50 kDa on the ladder, slightly lower than the ~ 54 kDa observed for GFP tagged α -synuclein. Repeated western analysis in TSY623 produced an identical result (data not shown).

α -Synuclein Translocates to Intracellular Compartments in TSY623

No toxicity was observed in TSY623 therefore, we wanted to compare the toxic and non-toxic α -synuclein phenotypes. Analysis of α -synuclein intracellular localization was performed using live-cell GFP fluorescence microscopy at 18, 24, 36, and 48 hours after α -synuclein induction in TSY623. At least 500 cells per time-point were quantitatively analyzed for either 1) Diffuse fluorescence, 2) Plasma membrane localization, 3) Aggregation, or 4) Intracellular compartmental fluorescence (Figure 7-D). At 18 hours of α -synuclein expression, GFP and A30P exhibited diffuse cytoplasmic fluorescence and WT, A53T, and E46K localized to the plasma membrane. However, at 24 hours post-induction membrane localization was nearly lost in WT, A53T, and E46K, and intracellular compartmental fluorescence was observed. In all GFP and α -synuclein cultures, compartmental diffuse fluorescence became the predominant phenotype through 24, 36, and 48 hour time-points.

Diploidy Abolishes Toxicity in BY4743

BY4741 mates with the nearly genetically identical BY4742 to form diploid BY4743. To investigate the effects diploidy on E46K toxicity, O.D. 600 nm and cell spotting growth analyses were performed on BY4743 as described previously (Figure 9-A and B). Surprisingly, doubling of the genome eliminated any signs of toxicity in both OD. 600 nm and cell spotting growth assays. However, western analysis at 24 hours post-induction did not reveal a uniform level of expression across all mutants and controls (Figure 9-C). Expression was reduced in A30P, A30P/A53T, E46K, A30P/E46K, and E46K/A53T compared to GFP, WT, A53T, and A30P/E46K/A53T.

E46K Toxicity is Haploid-Specific

Because E46K toxicity was observed in haploid BY4741 but not in the closely related diploid BY4743, identical growth and protein expression assays were performed on the BY4741 opposite mating-type BY4742 to determine if E46K toxicity is mating-type or ploidy-specific (Figure 10-A and B). If E46K is toxic to BY4742 then toxicity is ploidy-specific. However, if E46K is not toxic then toxicity is mating-type specific. Interestingly, growth curve and cell spotting analyses found A30P/E46K to be significantly toxic to BY4742 ($P_{18hrs} = 0.00001$, $P_{24hrs} = 0.000004$).

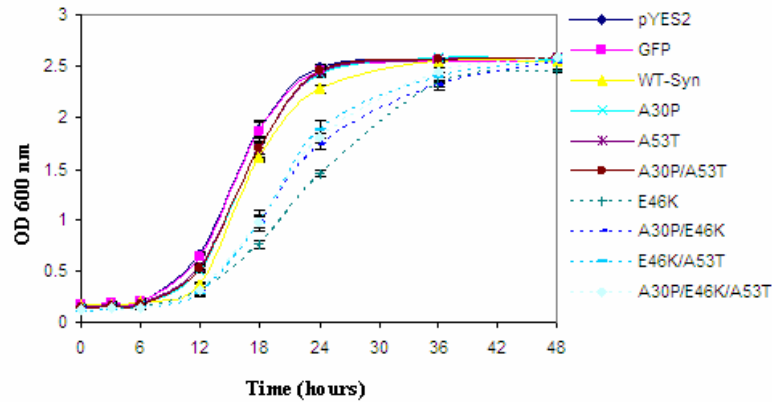
Decreased Expression is Linked with Toxicity in BY4742

To determine if reduced α -synuclein-E46K expression was characteristic of haploid cells resistant to E46K toxicity, western analysis of BY4742 was conducted at 24 hours after α -synuclein induction using V5 and PGK antibodies (Figure 10-C). As in BY4741, BY4742 exhibited a decrease in expression for the toxic A30P/E46K mutant, while all non-toxic mutants and controls were expressed consistently.

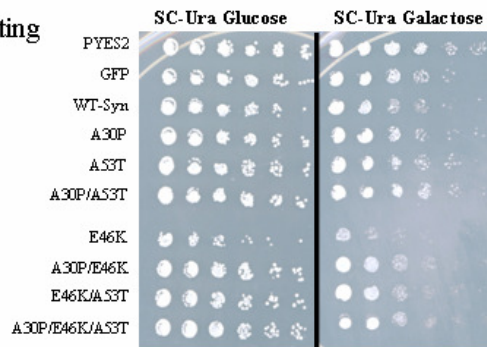
Discussion

The mechanism by which α -synuclein causes PD remains to be discovered. However, many previous studies have begun to characterize α -synuclein, and suggest that protofibrillar intermediates rather than fibrils are the toxic agent (Conway et al., 2000; Ding et al., 2002; Volles et al., 2001; Lansbury et al., 2003). The toxic protofibril hypothesis is strongly supported by

A. Growth Curve



B. Cell Spotting



C. Protein Expression

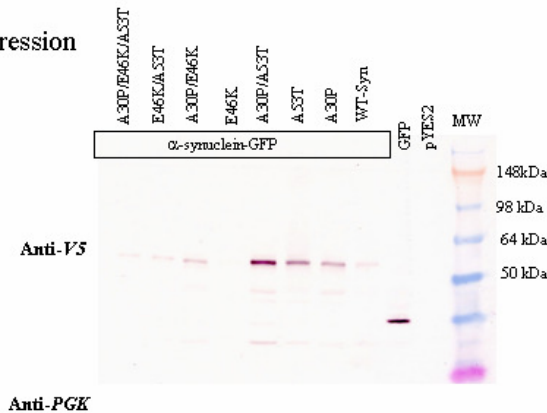


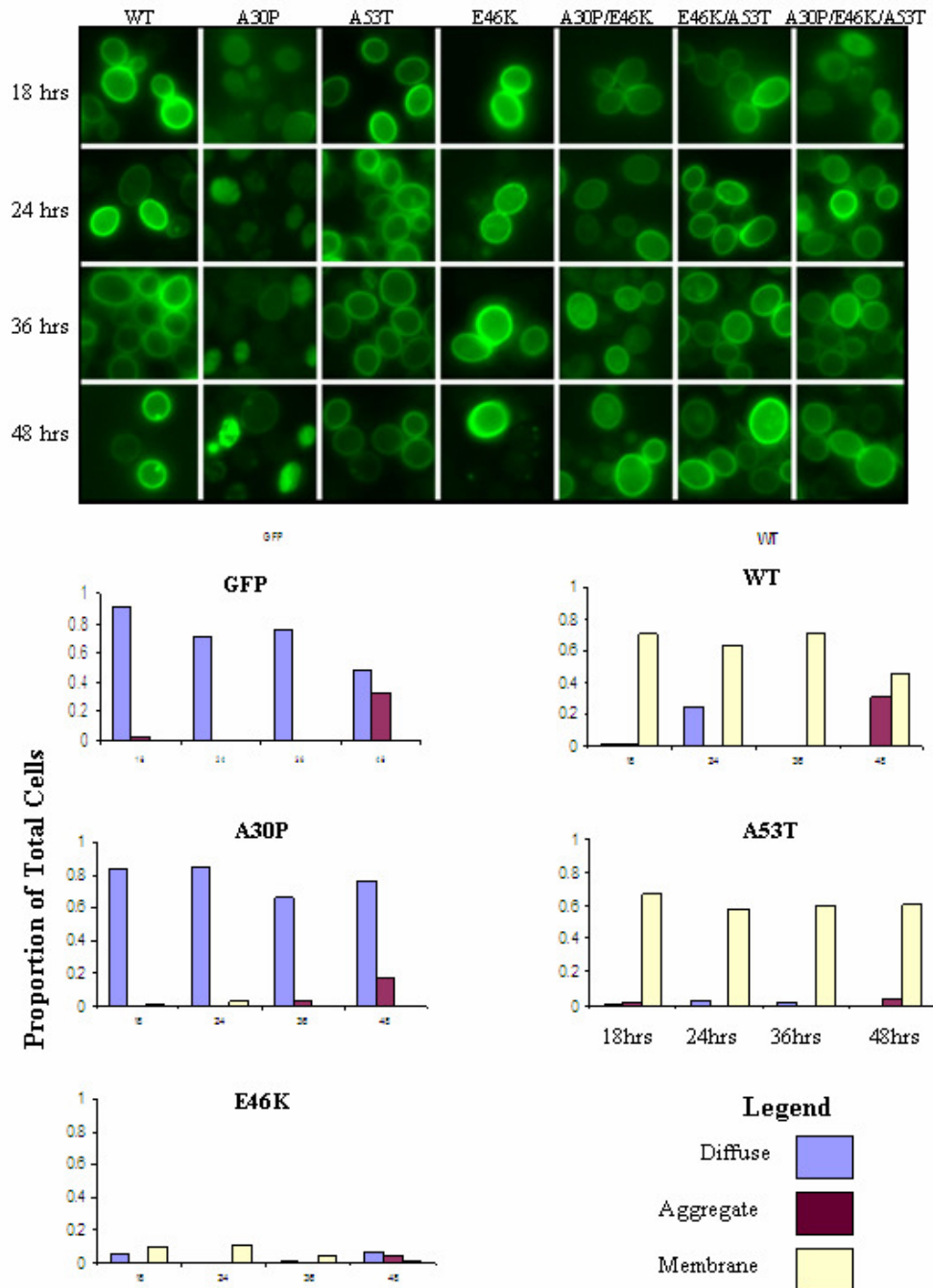
Figure 6: E46K is Toxic to Haploid BY4741.

A. Growth Curve Analysis: The growth curve for BY4741 during 48 hours of α -synuclein expression for WT, A30P, A53T, A30P/A53T, E46K, A30P/E46K, E46K/A53T, or A30P/E46K/A53T, and pYES2 and GFP controls is shown. Culture density is indicated by absorbance on the y-axis and time after α -synuclein induction on the x-axis. Significant growth impairment is observed for E46K, A30P/E46K, E46K/A53T, and A30P/E46K/A53T at 18 and 24 hours (P18hrs \leq 0.00068, P24hrs \leq 0.0016). Though all E46K and E46K variations are toxic to BY4741, E46K is significantly more toxic at 18 and 24 hours than A30P/E46K, E46K/A53T, and A30P/E46K/A53T (P18hrs \leq 0.0096, P24hrs \leq 0.0085). By 48 hours, all cultures had returned to equal density. Each of the points on the curves represents the mean from three trials. Standard Error of the Mean (S.E.M.) bars for a 95% confidence interval are given for each point.

B. Cell Spotting: BY4741 cells were serially diluted and spotted onto both α -synuclein inducing and suppressing media to evaluate the effects of α -synuclein expression on growth. The solid media plate with non- α -synuclein inducing SC-Uracil glucose media is on the left and α -synuclein inducing SC-Uracil galactose media on the right. E46K and E46K variations are toxic when expressed, as indicated by the decrease in growth on SC-Uracil galactose media compared to the other familial mutants and controls.

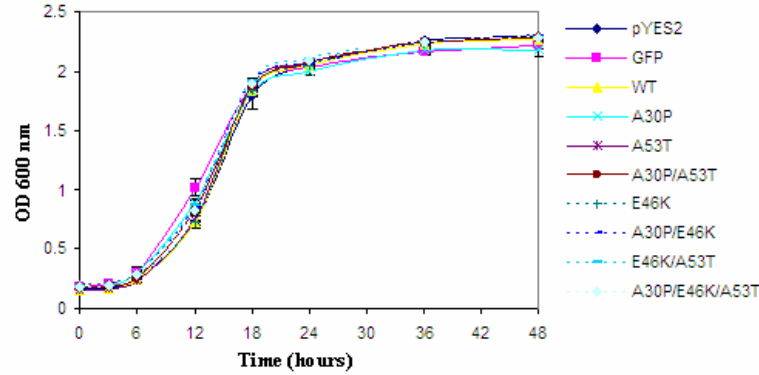
C. Protein Expression: At 24 hours of α -synuclein expression, western analysis was performed. Expression was quantified using an antibody specific for the V5 tag on α -synuclein. α -synuclein bands are present at ~54 kDa. There is a decrease in E46K, A30P/E46K, E46K/A53T, and A30P/E46K/A53T expression compared to WT, A30P, A53T, and A30P/A53T. E46K expression is also less than A30P/E46K, E46K/A53T, and A30P/E46K/A53T. An antibody for PGK was used as a loading control.

D. Live Cell Microscopy and Quantification of Phenotypes

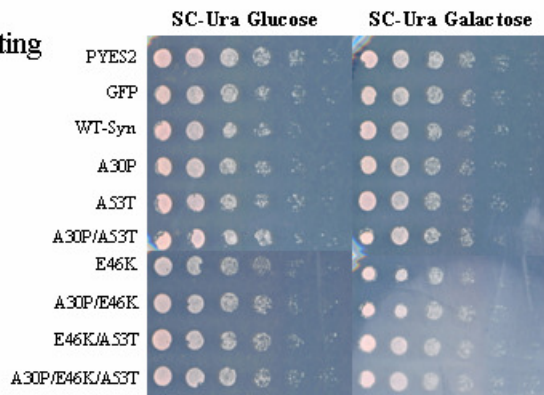


D. Live Cell Microscopy and Quantification of Phenotypes: All α -synuclein constructs were C-terminally tagged with GFP; GFP was also expressed alone as a control. At 18, 24, 36, and 48 hours after α -synuclein induction, at least 500 cells were imaged for GFP fluorescence at 1000x magnification from GFP, WT, A30P, A53T, A30P/A53T, E46K, A30P/E46K, E46K/A53T, and A30P/E46K/A53T cultures. At each time-point, the imaged cells from GFP, WT, A30P, A53T, and E46K cultures were quantified as having either 1) Diffuse Fluorescence (blue), 2) Aggregation (maroon), 3) Plasma Membrane Localization (yellow). The proportion of the total number of cells exhibiting a particular fluorescence phenotype is plotted for each familial mutant or control at the designated time-points. At all time-points, WT, A53T, and E46K were membrane localized while A30P and GFP remained diffuse in the cytoplasm.

A. Growth Curve



B. Cell Spotting



C. Protein Expression

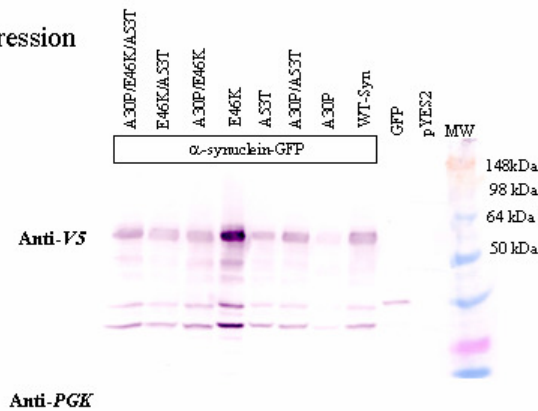


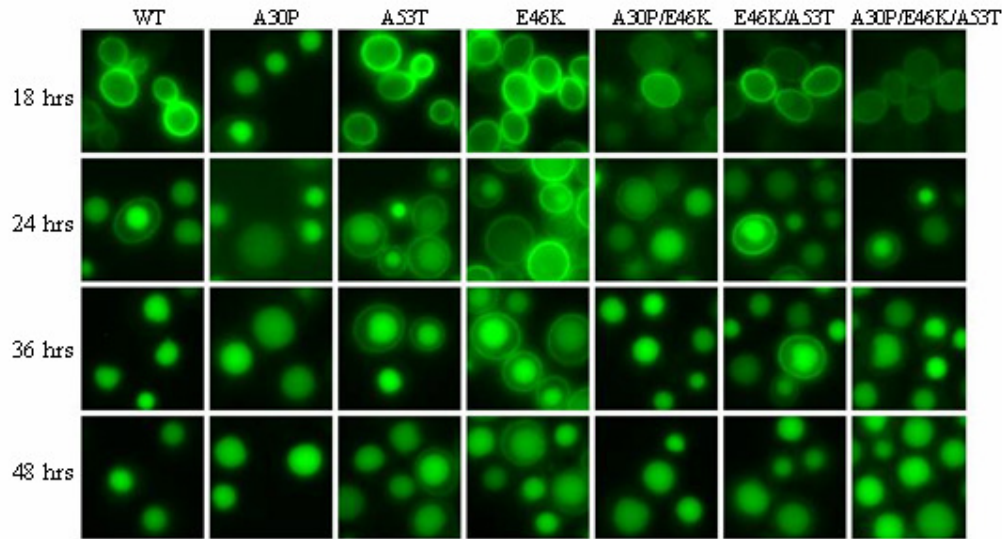
Figure 7: Characterization of E46K in Haploid TSY623.

A. Growth Curve Analysis: The growth curve for TSY623 during 48 hours of α -synuclein expression for WT, A30P, A53T, A30P/A53T, E46K, A30P/E46K, E46K/A53T, or A30P/E46K/A53T, and pYES2 and GFP controls is shown. Culture density is indicated by absorbance on the y-axis and time after α -synuclein induction on the x-axis. Each of the points on the curves represents the mean from three trials. Standard Error of the Mean (S.E.M.) bars for a 95% confidence interval are given for each point. No growth impairment is observed.

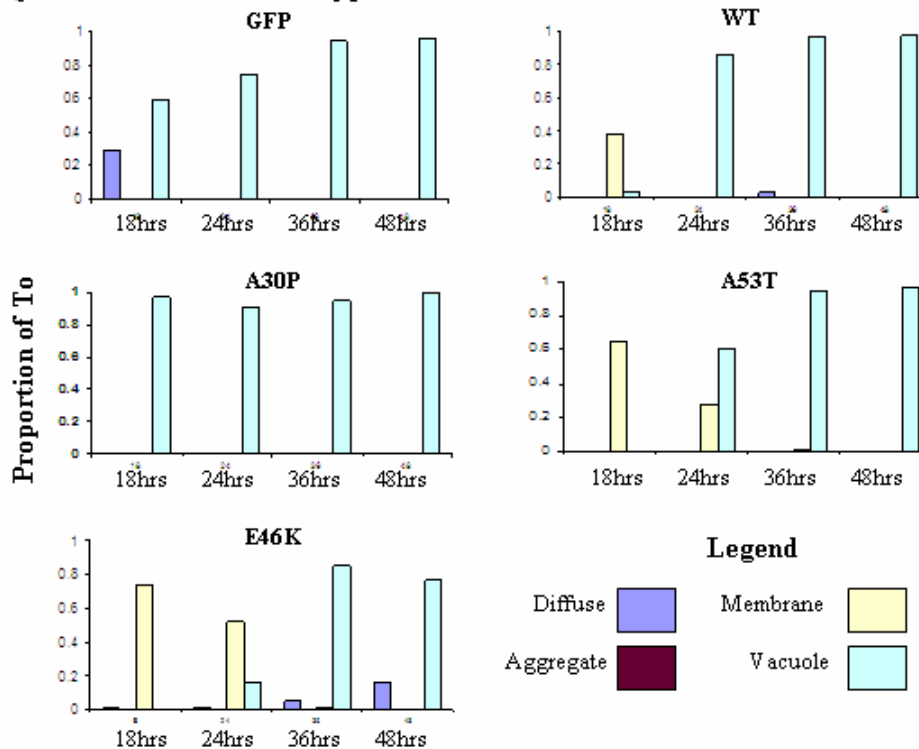
B. Cell Spotting: TSY623 cells were serially diluted and spotted onto both α -synuclein inducing and suppressing media to evaluate the effects of α -synuclein expression on growth. The solid media plate with non- α -synuclein inducing SC-Uracil glucose media is on the left and α -synuclein inducing SC-Uracil galactose media on the right. There is no observable difference in growth between familial mutants and controls in inducing media.

C. Protein Expression: At 24 hours of α -synuclein expression, western analysis was performed. Expression was quantified using an antibody specific for the V5 tag on α -synuclein. α -synuclein bands are present at ~54 kDa. Reduced expression is observed for GFP, A30P, A30P/E46K, E46K/A53T, and A30P/E46K/A53T. An antibody for PGK was used as a loading control.

D. Live Cell Microscopy

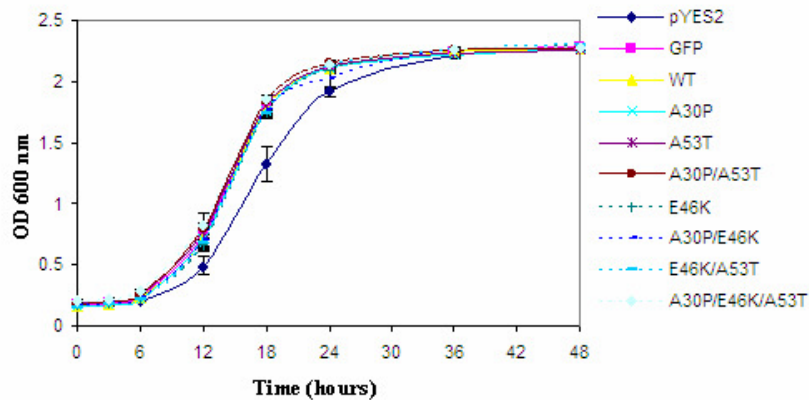


Quantification of Phenotypes

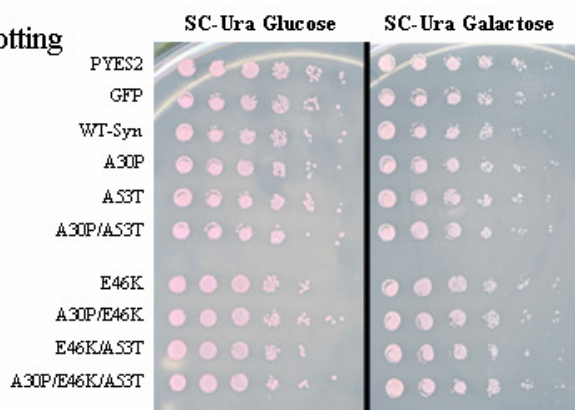


D. Live Cell Microscopy and Quantification of Phenotypes: All α -synuclein constructs were C-terminally tagged with GFP; GFP was also expressed alone as a control. At 18, 24, 36, and 48 hours after α -synuclein induction, at least 500 cells were imaged for GFP fluorescence at 1000x magnification from GFP, WT, A30P, A53T, A30P/A53T, E46K, A30P/E46K, E46K/A53T, or A30P/E46K/A53T cultures. At each time-point, the imaged cells from GFP, WT, A30P, A53T, and E46K cultures were quantified as having either 1) Diffuse Fluorescence (blue), 2) Aggregation (maroon), 3) Plasma Membrane Localization (yellow), 4) Vacuolar Fluorescence (light blue). The proportion of the total number of cells exhibiting a particular fluorescence phenotype is plotted for each familial mutant or control at the designated time-points. At 18 hours, WT, A53T, and E46K were membrane localized while A30P and GFP remained diffuse in the cytoplasm. Over time, membrane localization is lost for WT, A53T, and E46K, and all familial mutants and GFP predominantly exhibited vacuolar fluorescence by 24 hours.

A. Growth Curve



B. Cell Spotting



C. Protein Expression

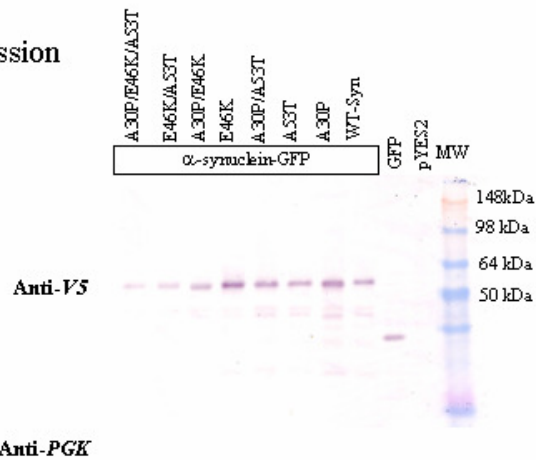


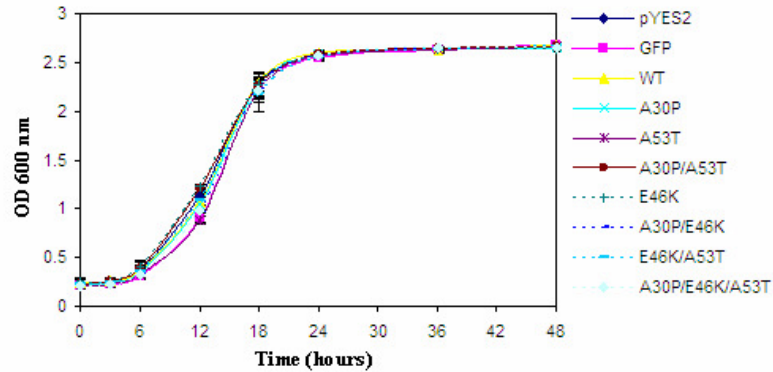
Figure 8: Characterization of E46K in Haploid W303.

A. Growth Curve Analysis: The growth curve for W303 during 48 hours of α -synuclein expression for WT, A30P, A53T, A30P/A53T, E46K, A30P/E46K, E46K/A53T, or A30P/E46K/A53T, and pYES2 and GFP controls is shown. Culture density is indicated by absorbance on the y-axis and time after α -synuclein induction on the x-axis. Each of the points on the curves represents the mean from three trials. Standard Error of the Mean (S.E.M.) bars for a 95% confidence interval are given for each point. No toxicity is observed.

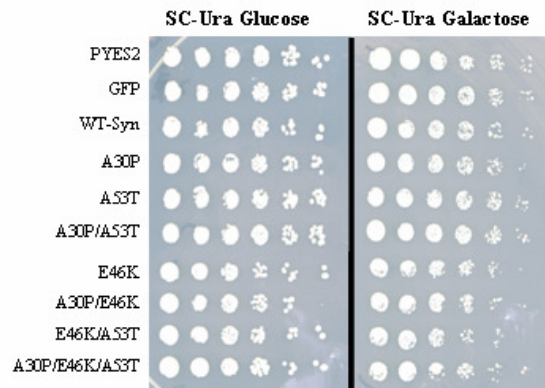
B. Cell Spotting: W303 cells were serially diluted and spotted onto both α -synuclein inducing and suppressing media to evaluate the effects of α -synuclein expression on growth. The solid media plate with non- α -synuclein inducing SC-Uracil glucose media is on the left and α -synuclein inducing SC-Uracil galactose media on the right. There is no observable difference in growth between familial mutants and controls in inducing media.

C. Protein Expression: At 24 hours of α -synuclein expression, western analysis was performed. Expression was obtained using an antibody specific for the V5 tag on α -synuclein. α -synuclein bands are present at ~54 kDa. Expression is consistent for familial mutants and controls. An antibody for PGK was used as a loading control.

A. Growth Curve



B. Cell Spotting



C. Protein Expression

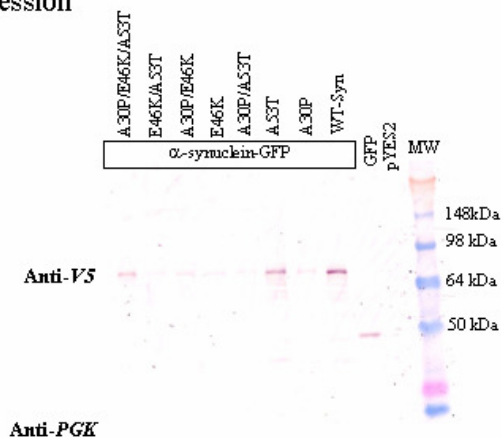


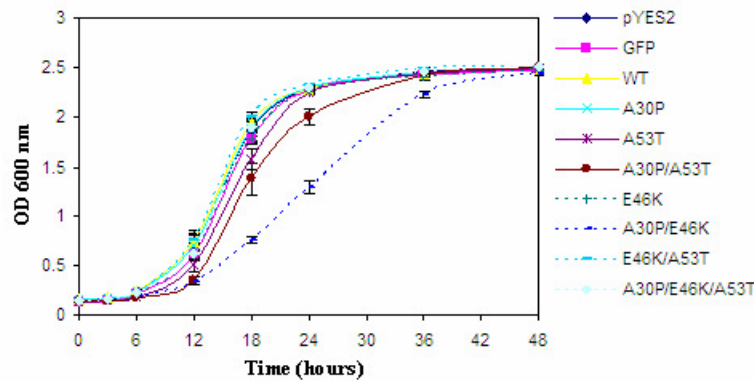
Figure 9: E46K is not toxic in Diploid BY4743.

A. Growth Curve Analysis: The growth curve for BY4743 during 48 hours of α -synuclein expression for WT, A30P, A53T, A30P/A53T, E46K, A30P/E46K, E46K/A53T, or A30P/E46K/A53T, and pYES2 and GFP controls is shown. Culture density is indicated by absorbance on the y-axis and time after α -synuclein induction on the x-axis. Each of the points on the curves represents the mean from three trials. Standard Error of the Mean (S.E.M.) bars for a 95% confidence interval are given for each point. No toxicity is observed.

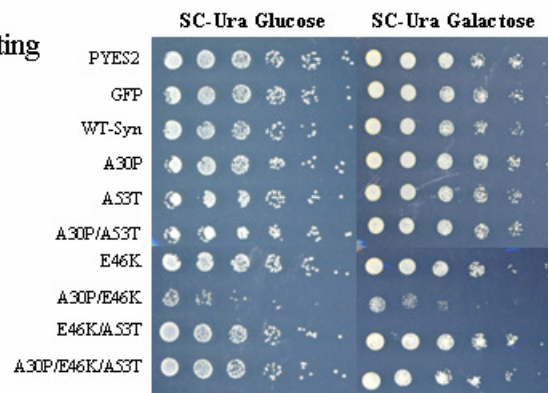
B. Cell Spotting: BY4743 cells were serially diluted and spotted onto both α -synuclein inducing and suppressing media to evaluate the effects of α -synuclein expression on growth. The solid media plate with non- α -synuclein inducing SC-Uracil glucose media is on the left and α -synuclein inducing SC-Uracil galactose media on the right. There is no observable difference in growth between familial mutants and controls in inducing media.

C. Protein Expression: At 24 hours of α -synuclein expression, western analysis was performed. Expression was obtained using an antibody specific for the V5 tag on α -synuclein. α -synuclein bands are present at ~54 kDa. Reduced expression is observed for A30P, A30P/A53T, E46K, A30P/E46K, and E46K/A53T compared to GFP, WT, A53T, and A30P/E46K/A53T. An antibody for PGK was used as a loading control.

A. Growth Curve



B. Cell Spotting



C. Protein Expression

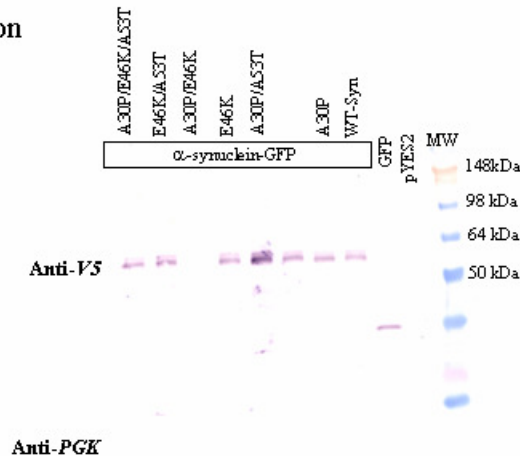


Figure 10: E46K is Required for Toxicity in Haploid BY4742.

A. Growth Curve Analysis: The growth curve for BY4742 during 48 hours of α -synuclein expression for WT, A30P, A53T, A30P/A53T, E46K, A30P/E46K, E46K/A53T, or A30P/E46K/A53T, and pYES2 and GFP controls is shown. Culture density is indicated by absorbance on the y-axis and time after α -synuclein induction on the x-axis. Each of the points on the curves represents the mean from three trials. A30P/E46K is significantly toxic at 18 and 24 hour of α -synuclein expression (P18hrs = 0.00001, P24hrs = 0.000004). Standard Error of the Mean (S.E.M.) bars for a 95% confidence interval are given for each point.

B. Cell Spotting: BY4742 cells were serially diluted and spotted onto both α -synuclein inducing and suppressing media to evaluate the effects of α -synuclein expression on growth. The solid media plate with non- α -synuclein inducing SC-Uracil glucose media is on the left and α -synuclein inducing SC-Uracil galactose media on the right. A30P/E46K is toxic when expressed, as indicated by the decrease in growth on SC-Uracil galactose media compared to the other familial mutants and controls.

C. Protein Expression: At 24 hours of α -synuclein expression, western analysis was performed. Expression was obtained using an antibody specific for the V5 tag on α -synuclein. α -synuclein bands are present at ~54 kDa. There is a decrease in A30P/E46K expression compared to the other familial mutants and controls. An antibody for PGK was used as a loading control.

the finding that protofibril formation remains the only similarity between the A30P and A53T familial mutants, and protofibrils bind and form pores in synthetic and mouse brain-derived membranes similar to those of bacteria toxins (Conway et al., 2000; Ding et al., 2002; Volles et al., 2001). Whether protofibrils exist within the E46K fibrillization pathway is unknown. The only known characteristics of this new mutant are that it increases α -synuclein aggregation both *in vitro* and *in vivo* relative to WT, and increases lipid binding *in vitro* compared to A30P, A53T, and WT (Choi et al., 2004; Greenbaum et al., 2005; Pandey et al., 2006).

In the present study, our budding yeast model was employed to characterize E46K in the commonly studied and naturally occurring haploid strains BY4741, BY4742, TSY623, and W303, and diploid strain BY4743 (Sharma et al., 2006). Specifically, we had three major aims: 1) Determine if E46K and E46K variations are toxic to yeast, 2) Examine the effects of E46K on α -synuclein membrane localization and aggregation, and 3) Evaluate the effects of E46K on α -synuclein expression. We hypothesized that the E46K mutation alters the conformation of α -synuclein in a potentially toxic manner that results in increased α -synuclein misfolding and alters its plasma membrane localization. To assess whether E46K effects are strain and/or ploidy-specific, these analyses were performed on several well studied haploid yeast strains, and a diploid strain.

E46K Toxicity is Strain and Ploidy-Specific

Our first finding was that E46K toxicity is specific to the naturally occurring and well studied haploids BY4741 and BY4742. There is little genetic variation between these two strains which, suggests that toxicity in one should be expected in the other. Our finding that only A30P/E46K is toxic to BY4742 indicates that the toxic species is not merely E46K itself, but rather that E46K must be present in order to induce toxicity. This, therefore, links E46K toxicity between BY4741 and BY4742. Because several other common haploid strains and diploid BY4743 were evaluated in an identical manner and no toxicity was observed, we can first conclude that toxicity is strain-specific.

The finding of strain-specific toxicity raises the important issue of rationalizing this observation. Due to the lack of E46K research, and the complete absence of it in yeast models, there is an array of possibilities, two of which will be introduced. Based on GFP fluorescence microscopy observations of α -synuclein in BY4741 and TSY623, it is feasible that each strain has a means for managing the expression of E46K. In TSY623, vacuolar isolation may prevent α -synuclein from performing toxic functions or inhibiting vital ones. Since these results do not implicate a similar mechanism in BY4741, the failure to degrade or isolate α -synuclein may be a cause of toxicity. A second possible explanation rests on the subtle genetic differences between strains. Perhaps the presence of chaperone, suppressor, or a variety of other enzymes integral to controlling cellular proteins differs between strains, and certain strain-specific proteins confer resistance to E46K toxicity.

E46K toxicity was not merely strain-specific but also haploid-specific. Thus, the genomic dosage affects the presence of toxicity. The BY4741, BY4742, and BY4743 evaluations provided evidence for the presence of toxicity-suppressing proteins that are not completely effective at single-copy expression levels.

However, at two-copy expression levels suppressor or regulatory proteins may be in high enough concentration to keep the cell below a toxic threshold concentration of α -synuclein.

In common between BY4741 and BY4742 is that the presence of E46K is required for toxicity thus, implying that the E46K mutation itself alters the conformation of α -synuclein in a toxicity inducing manner. If true, then why would E46K alone not be toxic in BY4742? The BY4741 and BY4742 genotypes are nearly identical but the cellular and cytoplasmic environments are certainly not. For example, there are differences in cell volume and expression levels of cellular proteins. The environment in which α -synuclein-E46K folds may lead to the protein acquiring different stable conformations in each strain but A30P/E46K in BY4742 and E46K in BY4741 are most similar. Therefore, we conclude that haploid specificity is likely due to the expression levels of specific suppressor or regulatory proteins that are present in BY4741, BY4742, and BY4743.

E46K Exhibits Strain-Specific Cellular Distribution

Our second finding was that quantitative live-cell GFP fluorescence microscopy of α -synuclein intracellular localization performed on haploid strain BY4741 produced results for GFP, WT, A30P, and A53T that are in agreement with those previously published by our lab (Sharma et al., 2006). No quantitative analysis of phenotypes was provided for the BY4741 portion of that previous study. This research establishes the first quantitative evidence for E46K membrane localization in BY4741 and budding yeast. Though there are no budding yeast models to compare this finding to, it does support the Choi et al. conclusion that E46K increases α -synuclein phospholipid binding affinity (2004). In contrast to our previously published data on TSY623, membrane localization for WT, A53T, and E46K is lost by 24 hours of α -synuclein expression, and intracompartamental fluorescence in what appears to be the vacuole predominates through 48 hours based on D.I.C. images. At no point in the BY4741 or TSY623 time-courses were aggregates the predominant phenotype in any familial mutants or controls. By 48 hours post-induction, modest aggregation was observed in many of the BY4741 cultures including GFP which, may be attributed to the accumulation of foreign protein after a long period of expression if not degraded. E46K aggregation was expected based on the Greenbaum et al. (2005) and Pandey et al. (2006) findings that E46K increases the propensity for α -synuclein to aggregate.

E46K Toxicity is Dependent on Membrane Localization, Not Aggregation

Lastly, we found that critical analysis of the relationship between the fluorescence phenotypes and E46K or E46K variation toxicity in BY4741 leads us to conclude that toxicity is dependent on membrane localization, not aggregation. In TSY623, membrane localization is lost and no toxicity is observed. Based on the toxic protofibrillar hypothesis presented in the introduction, it is plausible that a membrane binding oligomeric α -synuclein species confers toxicity. Though this research provides no direct evidence of protofibrillar existence, the absence of visible aggregates during toxicity but presence of E46K membrane localization implies their involvement. Furthermore, membrane localization correlating with toxicity is also supported by

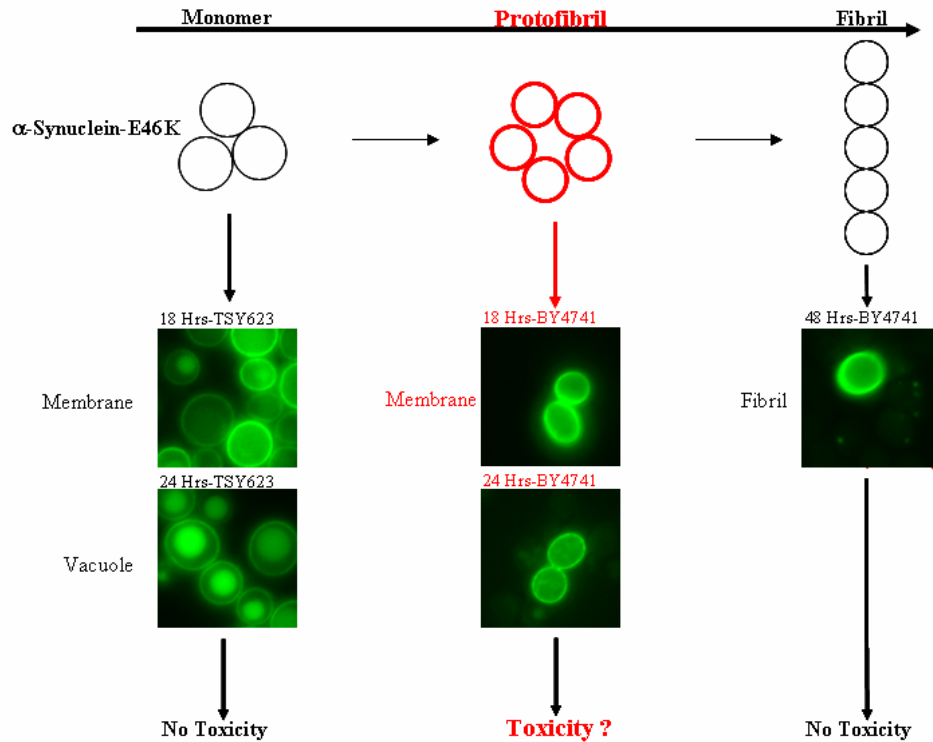


Figure 11: Does E46K Form Toxic Protofibrils in Yeast?

The transition of α -synuclein-E46K from monomer to fibril is depicted with the possible formation of protofibrils in red. In the monomeric α -synuclein lane, E46K is localized to the plasma membrane at 18 hours of α -synuclein expression, and within the vacuole at 24 hours in TSY623. No toxicity is observed with the perivacuolar phenotype in TSY623, and vacuolar localization may degrade the toxic α -synuclein species. The fibril lane shows the presence of E46K aggregates (red arrows) at 48 hours of α -synuclein expression in BY4741. No toxicity is observed with the fibrillar phenotype at this time-point in BY4741. The protofibril lane demonstrates the possibility that toxic protofibrils form at 18 and 24 hours post- α -synuclein induction in BY4741 and membrane localize to cause toxicity.

the Volles et al. (2001) and Ding et al. (2002) findings that protofibrils bind and form pores in synthetic membranes. Perhaps, membrane bound α -synuclein in BY4741 contains protofibrils that alter membrane permeability in a deleterious manner (Volles et al., 2001; Ding et al., 2002; Figure 11).

Membrane localization in WT, A53T, and E46K is lost in TSY623 by 24 hours of α -synuclein expression and no toxicity is observed. Perivacuolar localization of α -synuclein may protect TSY623 from E46K toxicity. Interestingly, Cuervo et al. demonstrated that A30P and A53T inhibited their own as well as other protein's translocation into the mammalian lysosome (2004). In budding yeast, the vacuole functions as an analogous structure to the lysosome, yet no such impairment is observed in TSY623. This contrasting result may be the product of different protein involvement in facilitating translocation of α -synuclein into the vacuole compared to the lysosome of human PC12 cells. Specifically, α -synuclein is transported into the lysosome of human cells by the chaperone protein hsp70 when it recognizes the VKKQ amino acid sequence in α -synuclein (Cuervo et al., 2004). In BY4741, no such vacuolar chaperone may exist and therefore, those cells are unable to degrade the toxic α -synuclein species via this mechanism while TSY623 can.

E46K Toxicity is Linked to Decreased α -Synuclein Expression

E46K and E46K variations are significantly toxic to BY4741 at 18 and 24 hours after α -synuclein induction. In BY4742, A30P/E46K is toxic at these times as well. Because western analysis of α -synuclein at 24 hours post-induction demonstrated decreased expression of all toxic species in both BY4741 and BY4742, and that toxicity is lost by 48 hours post-induction, it is plausible that cells expressing α -synuclein the least were selected for and allowed to replicate. Furthermore, E46K variations were significantly less toxic and slightly more expressed than E46K in BY4741 during toxicity. Thus, this serves as evidence for a scheme where α -synuclein expression decreases as toxicity increases. Since only A30P/E46K is toxic to BY4742, the degree of toxicity relative to α -synuclein expression cannot be elaborated on. These results from western analysis also imply that aggregation is not required for toxicity because E46K is expressed at such low concentration during the initial hours of induction that aggregates either do not form or are not observable. In addition, if aggregates are only observed in replicating cells then those clusters of α -synuclein may be in an inactive or protective state.

In TSY623 and BY4743 no toxicity was observed even though there were numerous instances of decreased α -synuclein expression. In TSY623 specifically, decreased expression of A30P and A30P/A53T was consistent with that of Sharma et al.

(2006). These findings potentially indicate that specific α -synuclein isoforms are toxic to BY4743 and TSY623 but that toxicity occurs before log phase growth and the cells expressing the deleterious isoform the least are selected for shortly after induction. A second possible explanation is that diploid BY4743 expresses proteins capable of degrading α -synuclein or suppressing its transcription in high enough concentration to quickly lower the cytoplasmic concentration of any toxic species. In TSY623, similar or analogous proteins expressed at single-copy levels are sufficient to lower the concentration of any toxic α -synuclein species below a certain threshold. Based on this logic, α -synuclein mutants with low expression may be toxic in those strains without altering the growth curves.

Inconsistencies

There are five prominent inconsistencies that remain to be accounted. First, and most importantly, the growth impairment during log phase in BY4741 yeast expressing E46K or E46K variations, and A30P/E46K in TSY623 is referred to as toxicity because log-phase growth is impaired. Toxicity is not used in a sense that implies the death of cells because a substance can be toxic without causing death. This research does not provide data indicating whether the cells are actually dying during toxicity or if they are growing at a slower rate. Second, our lab previously demonstrated that α -synuclein-WT and A53T localize to the plasma membrane in TSY623 at 24 hours after induction (Sharma et al., 2006). Our current results demonstrate that membrane localization is lost by 24 hours after induction in TSY623. If a time-course analysis were performed in the Sharma et al. study, it would allow for a more thorough and relevant comparison but the lack thereof warrants no further elaboration on the matter (2006). Third, A30P/E46K exhibited a slight decrease in growth in BY4742 on non-inducing SC-Uracil glucose media compared to the other mutants and controls. This cell spotting experiment was repeated several times, all producing similar results. A sporadic mutation within the pYES2 vector, such as one within the uracil synthesis gene, could reduce the ability of A30P/E46K to grow in non-inducing media that is depleted of uracil. Consequently, A30P/E46K toxicity may be slightly exaggerated on the growth curve and cell spotting assays. Fourth, significant growth impairment is observed in W303 for pYES2 ($P_{18hrs} = 0.01$). This could have resulted from a cell-counting error and is not observed in the cell spotting assay. Finally, a distinct band is visible within the pYES2 control lane on the TSY623 western blot that was probed with the α -synuclein-specific V5 antibody. This band is located at a lower molecular weight than the α -synuclein bands which, indicates that it may be the result of non-specific binding of the antibody. This experiment was repeated and produced an identical result. Re-transformation of TSY623 with pYES2 will need to be completed to provide a definitive explanation.

Future Research

This project will continue through August of 2008. Therefore, the future research to be discussed will actually be performed. Our first aim is to determine the rate of E46K degradation through western analysis at several time-points during the loss of α -synuclein induction. The degradation of α -synuclein is hypothesized to play a critical role in PD neuronal toxicity. Secondly, the presence of apoptosis during

toxicity will be investigated by staining them with dihydorrodamine (DHR-1, 2, 3) to determine if cells are dying or simply growing slower than the controls and other familial mutants. Third, oxidative stress is linked to PD and therefore, the effects of E46K on oxidative stress will be examined using the FUN® stain. Lastly, our laboratory previously established the first fission yeast (*Schizosaccharomyces pombe*) model for PD, and we intend to characterize E46K within it (Brandis et al., 2006).

Conclusion

Recently discovered α -synuclein-E46K increases aggregation both *in vivo* and *in vitro*, and increases its membrane binding affinity (Choi et al., 2004; Greenbaum et al., 2005; Pandey et al., 2006). In this study, we have characterized E46K in a variety of naturally occurring budding yeast strains. We demonstrate that E46K exhibits both haploid and strain-specific toxicity in yeast. Furthermore, the presence of the E46K mutation is required for toxicity, and toxicity is abolished in a diploid nearly genetically identical to the haploids E46K is toxic to. Toxicity is also linked with decreased α -synuclein expression. Finally, toxicity is dependent on membrane localization and not aggregation. This finding supports the toxic protofibrillar hypothesis. As numerous studies of A30P and A53T have provided valuable insight into the biological basis of PD, E46K is sure to reveal many characteristics of α -synuclein and its pathological function. This thesis has contributed a significant amount of cellular, molecular, and biochemical information to our understanding of the properties of E46K, and was the first to characterize it within yeast.

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