Eukaryon, Vol. 1, 65-80, January 2005, Lake Forest College

Expression and Characterization Studies of α-Synuclein to Model Parkinson's disease in Yeast

Brandon E. Johnson[*]
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Summary

Parkinson's disease (PD) is a fatal, incurable, neurodegenerative disease that affects approximately 4 million people worldwide. In this disease, dopaminergic neurons selectively die causing symptoms of postural rigidity, slowed motor movement, and resting tremors. Recently, the α-synuclein gene has been linked to both familial and sporadic forms of PD. α-Synuclein protein misfolds, aggregates and ultimately leads to cell death in dopaminergic neurons, but molecular mechanisms that cause misfolding and cell death are not fully understood. α-Synuclein overexpression causes PD-like pathology and symptoms in fruit flies and mice (two common model organisms). Till date, no yeast model for PD has been reported. Given the present ability to genetically and biochemically manipulate yeast, we believe that this model system will facilitate understanding of α-synuclein misfolding and aggregation processes. This senior thesis project has four aims: to express α-synuclein in yeast, characterize its effect on yeast growth and metabolism, describe protein solubility, and characterize protein stability in yeast. Wild type and three α-synuclein mutant genes were put into yeast. Both wild type and mutant α-synuclein protein were expressed to similar levels in yeast, but all forms appear larger in size than predicted on protein gels suggesting added structural modifications. Neither wild type nor mutant α-synuclein expression grossly affected cell growth, but some mutants may have affected adenine metabolism. While all forms of α-synuclein were purified from yeast under native and denaturing conditions, some α-synuclein was resistant to solubilization, and mutants more frequently showed multiple monomeric forms. Also, mutants appeared more stable in yeast compared to wild type α-synuclein. We have developed one of the first yeast models that recapitulate protein misfolding and aggregation in PD. In the future, this model will allow us to genetically dissect factors that accelerate or inhibit α- synuclein misfolding.

Introduction

The Human Brain and Neurodegenerative Illnesses

The human brain tightly regulates our perceptions of the external environment as well as our physical and emotional behavior. Through its billions of cells it houses our memories and our ability to learn, and it mysteriously underlies our human consciousness. Irreparable cell death in this powerful organ loosens its regulation of physiological processes, and distorts perceptions of reality. The effects of a diseased brain can permeate the entire body and disrupt the balance of the human mind.

Neurodegenerative diseases (NDDs), including Alzheimer's disease, prion diseases, amyotrophic lateral sclerosis, and Parkinson's disease, are a diverse family of chronic, progressive illnesses of the nervous system with no known corrective treatments (Price et al., 1998). NDDs can be sporadic (most cases), genetic, or infectious (Shoulson, 1998). NDDs span all cultures and affect millions of people (Shoulson, 1998). Because of their high prevalence, morbidity and mortality, NDDs raise significant medical and sociological concerns.

Neuropathologically, NDDs are characterized by cellular abnormalities in specific regions of the central nervous system. The affected cells are discrete for each NDD, and each affected area confers a clinical phenotype of that particular illness (Sherman & Goldberg, 2001). For example, Alzheimer's disease, the most common NDD, is associated with the atrophy of brain regions and cells essential for memory and cognition, including the neocortex, amygdala, and hippocampus (Morrison et al., 1997). Cell death in these regions causes the familiar symptoms of memory loss, disorientation, deficits in problem solving and dementia (Morrison et al., 1997). Similarly, the behavioral symptoms of all other NDDs are caused by specific neuronal death and weakened signaling pathways. Though the affected regions of the brain have been mapped in most NDDs, the cellular mechanisms that are involved in the initiation and progression of these illnesses are not fully understood (Sherman & Goldberg, 2001).

At the molecular level, however, a common pathological theme has emerged: each known NDD is associated with the aggregation of one or more specific misfolded proteins within the nervous system (Sherman & Goldberg, 2001). All proteins must exist in a specific shape to be biologically active. While a protein's shape is governed by its amino acid structure, chaperone proteins often assist the folding processes within the dense cellular environment. Chaperones facilitate correct protein folding, maintain protein shape, and restore proper structure to misfolded proteins (Horwich et al., 1999). If the correct shape cannot be restored or if a protein's shape is damaged over time, the protein is tagged and degraded by the ubiquitin/proteosome complex (Hochstrasser, 1996). Sometimes, proteins acquire a misfolded shape that cannot be corrected by chaperones or removed efficiently by the degradation pathway. Aggregation of these misfolded proteins is presumed to cause neuronal death, underlying several NDDs (Taylor et al., 2002). This thesis focuses specifically on PD and the protein misfolding processes associated with its neurpathology, as detailed next.

Parkinson's disease

[*] The report of the investigation undertaken as a Senior Thesis, to carry one course of credit in the Department of Biology

This NDD affects 4 million people worldwide (Masliah et al., 2001). Its prevalence and cost to human society

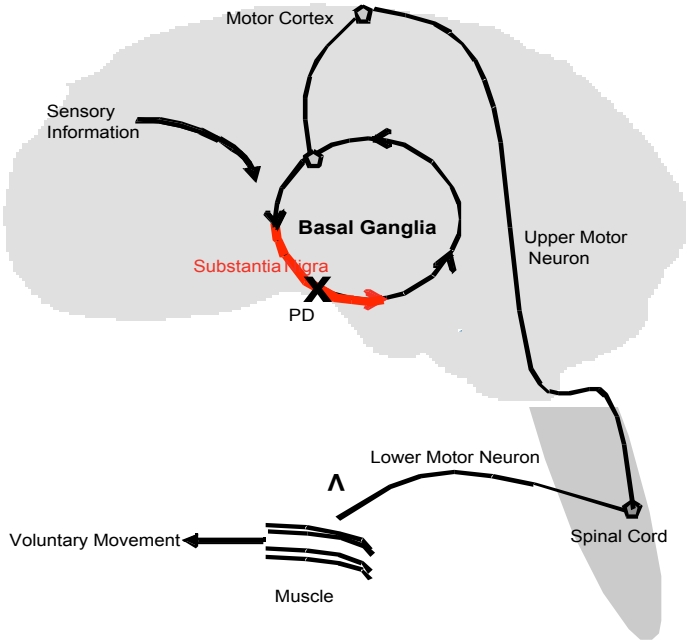

Figure 1. Neuronal Structures Regulating Voluntary Movement: Role of Basal Ganglia
Neurons in the basal ganglia integrate sensory and homeostatic information and signal the upper motor neurons in the frontal cortex to gate the initiation and termination of voluntary movement. These neurons pass the movement information to the lower motor neurons in the spinal cord, which innervate the muscles. The substantia nigra is an important group of cells in the basal ganglia. Cell death in the substantia nigra reduces the quality and precision of signaling to the motor cortex, resulting in hypokinesia (slowed movement). This is a schematic representation, not designed to reflect all anatomical connections.

necessitates research into its molecular basis and possible treatments. Behavioral symptoms of Parkinson's disease include muscular rigidity, postural instability, resting tremors, difficulties in initiating movement, and slowed motor function (Parkinson, 1817). The basal ganglia integrate sensory information and send signals to the primary motor cortex to regulate the initiation of voluntary movement (Goedbert, 2001). PD patients show severely reduced levels of the neurotransmitter dopamine (Ehringer & Hornykiewicz, 1960) and significant loss of dopamine producing cells in the substantia nigra, a midbrain structure within the basal ganglia (figure 1; Goedbert, 2001). The loss of substantia nigra neurons reduces signaling from the basal ganglia to the motor cortex, decreasing overall voluntary movement (Ehringer & Hornykiewicz, 1960).

Two Forms of PD

Sporadic PD: This dominant form of PD accounts for 95% of all cases (Goedbert, 2001), and affects one percent of all people over 60 years of age (Barbosa et al., 1997). Sporadic PD is linked to the loss of cellular processes during aging or exposure to poorly understood environmental conditions (Betarbet et al., 2001), including exposure to contaminated well water, herbicides, and industrial chemicals (figure 2; Olanow & Tatton, 1999). According to one hypothesis, these toxins may randomly mutate genes in dopamine-producing cells. Over a lifetime, somatic mutations in substantia nigra neurons may accumulate to significantly reduce dopamine levels and cause PD symptoms (Seidler et al., 1996). An infectious agent may also cause sporadic PD. Patients suffering from the infectious NDD encephalitis lethargica show PD-like symptoms (Takahashi & Yamada, 1999). Patients show neuronal damage confined to the substantia nigra, where the neurovirulent influenza A virus is also localized. A variety of other bacterial and viral agents have been reported to induce encephalitis and subsequent PD-like symptoms (Kohbata & Shimokawa, 1993), but repeated attempts to find an infectious agent consistent with authentic PD development have failed (Stoessl, 1999).

In the lab, animals have been designed to recapitulate PD pathology by exposing them to drugs and toxins. Rat models of sporadic PD include intravenous exposure to rotenone, a common pesticide (Betarbet et al., 2001), and 1-methyl-4-phenyl-1-2-3-6-tetrahydropyridine (MPTP; Singer et al., 1987), a byproduct of methamphetamine synthesis. Both molecules are inhibitors of mitochondrial complex I in rats (Betarbet et al., 2001, Singer et al, 1987), which plays an important role in ATP synthesis, and protects the cell against reactive oxygen species (ROS). When complex I is inactivated, cellular concentrations of ROS increase, causing protein and DNA damage (Langston et al., 1983; Singer et al. 1987). In rats, chronic exposure to rotenone and MPTP causes the selective degeneration of dopaminergic neurons, formation of inclusions in substantia nigra neurons, and induction of PD-like symptoms (Singer et al., 1987; Betarbet et al., 2001).

Dopamine synthesis also produces high amounts of ROS, increasing the likelihood of oxidative stress in dopamine-producing neurons (Olanow & Tatton, 1999). Substantia nigra neurons from PD patients show DNA, protein, and lipid oxidation (Jenner and Olanow, 1996). Oxidant stress may directly cause cell death, or it may modify proteins that then misfold, aggregate, and interfere with essential cell functions (figure 2; Giasson et al., 1999).

Familial PD: This form only constitutes 5% of all PD cases (Goedbert, 2001), yet the study of involved genes provides new insights into pathogenesis of all forms and guides development of new model systems.

At least three mutant genes cause separate forms of familial PD. Mutations in the α-synuclein (Polymeropolous et al., 1997) and ubiquitin carboxy-terminal hydrolase L-1 (UCHL-1) genes (Leroy et al.,1998) cause autosomal-dominant forms of PD. A mutation in parkin causes an autosomal-recessive form of PD (Kitada et al., 1998). The α-synuclein protein is abundantly expressed in the substantia nigra where it regulates dopamine levels (Jenko et al., 1998). Parkin and UCHL-1 are enzymes of the ubiquitin-proteosome pathway that label misfolded proteins for degradation (Kitada et al., 1998; Leroy et al., 1998). Four additional loci may be involved in the disease pathway, indicating additional contributing genes (Taylor et al., 2002). This thesis focuses on α-synuclein.

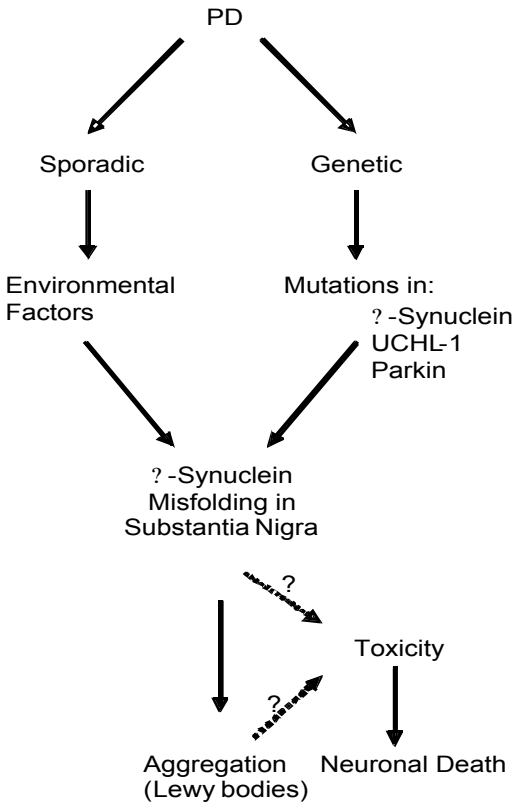

Figure 2. Proposed Molecular Mechanisms of Parkinson's Disease
Poorly understood environmental factors, and gene mutations in α-synuclein and ubiquitin ligases cause α-synuclein protein to misfold and aggregate. While misfolded α-synuclein may be toxic, the structural form causing cell death is not well understood.

Mutations in parkin imply that deficiencies of proteolytic enzymes are linked to the onset of the disease (Kitada et al., 1998). Parkin interacts with a family of structurally related proteins including α-synuclein, recognizes misfolded forms of these proteins, and ubiquitinates them for proteolytic digestion (figure 3; Kitada et al., 1998). Mutations in parkin disrupt its ability to recognize substrate proteins and label them for degradation (Shimura et al., 2000). UCHL-1 mutations are more rare in familial PD, but they also cause the loss of catalytic activity of ubiquitin transfer to α-synuclein (figure 3; Leroy et al., 1998). The UCH family of enzymes also cleaves polymers of ubiquitin into monomeric units (Hershko & Ciechanover, 1998), regenerating pools of free cytosolic ubiquitin. UCHL-1 mutations may decrease proper ubiquitin transfer and proteolytic degradation of α-synuclein (figure 3; Hershko & Ciechanover, 1998). Increased cytoplasmic concentrations of undigested α-synuclein may be cytotoxic or facilitate the formation of other toxic structures (Imai et al., 2000; Shimura et al., 2000).

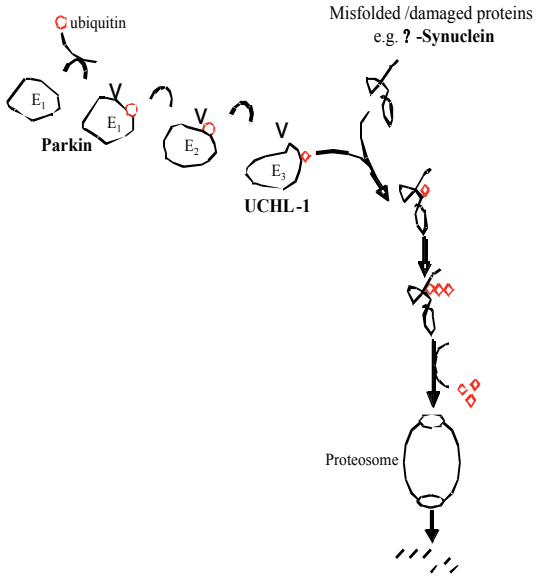

Figure 3. Current Model for Interactions Between α-Synuclein, Parkin, and UCHL-1 in familial PD
Misfolded cytoplasmic proteins are tagged and degraded by the ubiquitin-proteosome complex. Parkin and UCHL-1 are ubiquitin ligases that recognize misfolded α-synuclein as a substrate, and label it for degradation. Mutations in any one of these three genes inhibits proper α-synuclein degradation, and underlies the onset of familial PD (adapted from Cooper, 2000).

The synuclein family is comprised of three soluble axon-localized proteins, including α, β, and γ synucleins (Clayton & George, 1998). They contain a high degree of sequence conservation, and may serve as vesicle-associated proteins in neuronal axons (Clayton & George, 1998). β and γ-synucleins have not been implicated in NDDs, and β-synuclein may actually protect against α-synuclein toxicity (Hashimoto et al., 2001). α-Synuclein is a 14 kDa cytosolic protein abundantly expressed in a variety of neurons and non-neuronal cells (Duda et al., 1999). In dopaminergic neurons of the substantia nigra, α-synuclein is known to maintain dopamine pools and regulate synaptic connections (Clayton & George, 1998). Two point mutations A53T (Polymeropoulos et al., 1998) and A30P (Kruger et al., 1998) of the α-synuclein gene are correlated to separate forms of familial PD. These mutant forms of α-synuclein may allow the protein to misfold and aggregate, and initiate the disease state (figure 3; Polymeropoulos et al., 1998; Kruger et al., 1998). These mutant forms of α-synuclein are found in insoluble filaments and Lewy bodies from PD patients (Arima et al., 1998; Spillantini et al., 1998).

α-Synuclein properties in cell culture & test tubes

α-Synuclein misfolds and aggregates in dopaminergic neurons, a feature that correlates with the onset of all forms of PD (Goedbert, 2001). α-Synuclein aggregation proceeds through several structural intermediates in test tubes (Conway et al., 2001), but the toxic intermediates have not been identified (Conway et al., 2001). First, monomeric α-synuclein

polymerizes and forms spherical structures that facilitate its continued reactivity (Ding et al., 2002). These α-synuclein polymers then rapidly fuse to form small, insoluble intermediates (Lee & Lee, 2002). These small intermediates link together to form large fibrils (Conway et al., 2000). Mammalian cell culture studies show that amino acids within these fibrils are targeted for degradation by the ubiquitin/proteosome complex (Hershko & Ciechanover, 1998). However these large fibrils cannot be broken down, and sequester ubiquitin and parkin during the formation of Lewy bodies (Trojanowski et al., 1998, Abeliovich et al., 2000).

While Lewy bodies are often found in conjunction with cell death, some forms of familial PD do not contain Lewy body pathology (Kitada et al., 1998), indicating that an intermediate α-synuclein aggregate may be the toxic form. However, Lewy bodies may interfere with protein degradation by reducing parkin availability. When parkin levels decrease, non-digested target proteins may reach toxic concentrations (Shimura et al., 2000). For example, synphilin-1 protein is a substrate for parkin (Chung et al., 2001). In cultured cells, synphilin-1 forms insoluble aggregates with α-synuclein in the absence of parkin (Chung et al., 2001; Engelender et al., 1999). However, aggregates do not form under the same conditions when synphilin is absent (Chung et al., 2001), showing that high concentrations of synphilin-1 are sufficient for α-synuclein aggregation.

Bacterial expression studies have compared the aggregation rates of wild type and mutant α-synuclein forms (Conway et. al. 1998, Giasson et. al. 1999, Conway et. al. 2000, Serpell et. al. 2000). All forms polymerize at different rates. At high concentrations wild type and mutant (A30P and A53T) forms polymerize more rapidly than wild type *in vitro* (Conway et al., 2000). After polymerization, insoluble α-synuclein forms fibrils. A30P α-synuclein forms fibrils more slowly than WT protein (Serpell et. al.1999, Conway et al., 2000), while A53T forms fibrils more quickly (Conway et al., 1998). These studies show that both mutant forms facilitate polymerization, but do not necessarily accelerate fibril formation. Because both mutant forms accelerate the disease state (Giasson & Lee, 2001), α-synuclein polymers may be the toxic structure (Conway et al., 2000), whereas subsequent structures like fibrils may be non-toxic endpoints. Moreover, dopamine inhibits α-synuclein fibril formation *in vitro* and stabilizes α-synuclein polymers (Conway et. al., 2001). Therefore, dopamine may actually stabilize the toxic α-synuclein polymers, thereby confining neuronal death to dopaminergic neurons in the substantia nigra.

Remaining Questions

The mechanisms of α-synuclein misfolding in sporadic PD are still unknown. The structure and function of α-synuclein is central to the study of this disease. To date, the toxic form of α-synuclein has not been identified, and how it participates in the disease state has not been fully determined. These questions may be better addressed when the factors that cause α-synuclein misfolding are identified. Likewise, the interaction of misfolded α-synuclein with members of the proteolytic pathway is not well understood. α-Synuclein aggregates may hinder the activity of the proteosome by sequestering proteolytic enzymes, or it may form toxic structures when bound to these proteins. Because cell death is largely limited to dopaminergic neurons, the role of dopamine in PD pathology needs to be established *in vivo*.

New Animal Models for PD

To begin to address these questions, several mice models of PD expressing α-synuclein have been developed in the past three years. Overexpression of human wild-type α-synuclein causes PD-like pathology and behavior in mice (Masliah et al., 2000). Severity of symptoms and cytoplamic aggregates increase with α-synuclein abundance (Masliah et al. 2001). While both wild type and mutant forms of α-synuclein cause pathology, mutant α-synuclein enhances toxicity when expressed to similar levels (Van der Putten et al., 2000). A recent mouse model of human A53T α-synuclein expression shows late onset PD-like behavior, and the cytoplasmic inclusions closely resemble authentic filamentous fibrils from PD patients (Giasson et al., 2002). These models show that α-synuclein over-expression is sufficient for PD in mice, and the A53T mutant accelerates the disease state (Giasson et al., 2002).

Like mice and humans, fruit flies have dopaminergic neurons that coordinate motor movement. A fruit fly model of PD expressing human α-synuclein shows aggregates indicative of human pathology (Feany & Bender, 2000). This model system has already proven useful for exploring possible regulatory factors. Chaperone proteins refold cellular proteins into their correct, functional shape (as explained on page 2). Expression of the chaperone protein Hsp70 in conjunction with human α-synuclein in flies prevents dopaminergic cell death (Auluck et al., 2002). Conversely the inhibition of fly chaperone proteins accelerates cell death and PD symptoms (Auluck et al., 2002). If accelerated protein refolding is sufficient for rescuing cells from PD pathology, misfolded α-synuclein may be the direct cause of cell death in PD (as opposed to being a side product of another toxic process).

Yeast as a model organism for studying human disease

Saccharomyces cerevisiae (baker's yeast) is an attractive model organism because the ease of working with a unicellular organism is matched with the presence of a wide range of evolutionary conserved eukaryotic genes. Despite over a billion years of evolutionary separation, yeast contain many of the same regulatory processes found in humans including the ubiquitin/proteosome pathway and chaperone proteins. This simple, but powerful system has been used to study basic tenets of the eukaryotic cell cycle (Elledge et al., 1996), endocytosis (Dulic et al., 1991), cellular export and import (Guerin et al., 1978), and the central dogma of genetic flow of information (DNA to RNA to protein; Paushkin et al., 1997). Several human diseases have been investigated in yeast, including the function of the human ras proto-oncogene in cancer (Gibbs & Marshal, 1989) and NDD pathology. Importantly, yeast studies have probed specific protein interactions and protein misfolding in several NDDs such as amyotrophic lateral sclerosis (Kunst et al., 1997), prion diseases (Ma & Lindquist, 1999), and Huntington's disease (Krobitsch et al., 2000). Manipulation of proteosome and chaperone proteins successfully modulated the misfolding of the huntingtin protein in yeast (Krobitsch et al., 2000, Muchowski et al., 2000; Meriin et al., 2002; Muchowski et al., 2002). The interplay of eukaryotic chaperone proteins, proteolytic enzymes and misfolded proteins in several NDDs including PD may be successfully modeled in this simple and easily manipulated model system.

Specific aims of this thesis

Currently, no yeast model for PD has been established. With significant initial successes of NDD research in yeast discussed above we believe that such a model will facilitate our understanding of how α-synuclein misfolds. We can probe the structural characteristics and compare differences between wild type and mutant forms of α-synuclein expressed in yeast. Because chaperone proteins and the ubiquitin/proteosome complex have been extensively studied in yeast, their ability to properly fold different forms of α-synuclein may be thoroughly investigated.

The specific aims of this thesis were to develop a yeast α-synuclein expression model, determine if α-synuclein aggregates in yeast, and assess if misfolding differences exist between wild type and mutant forms. Yeast do not contain a protein similar to human α-synuclein, so we put wild type and three mutant forms of human α-synuclein (A53T, A30P, A53T-A30P) into yeast. All four forms of α-synuclein protein were made in yeast, and all forms appear to be structurally modified. All forms of α-synuclein have been purified and evidence from multiple assays supports that α-synuclein may be misfolding in yeast. Ongoing and future experiments to understand protein shape and solubility will compare the molecular characteristics and behavior of wild type and mutant α-synuclein.

Results

Wild type and mutant α-synuclein yeast vectors created and amplified in *E. coli*

Our first goal was to subclone α-synuclein into a vector (figure 5a; pYES2.1/V5-His-TOPO; Invitrogen) chosen for the following features. First, the vector contains the pUC and 2μ origins for high copy replication and long-term storage in bacteria and yeast. Secondly, the vector contains an ampicillin-resistance gene and a *URA3* gene. These genes allow selection of vector-containing bacteria and yeast in the presence of ampicillin (a drug that kills bacteria) and in media lacking uracil (a yeast nutrient required for making RNA), respectively. Thirdly, it contains the galactose-sensitive *GAL1* promoter next to the subcloned transgene that allows the experimenter to induce expression of the transgene at will by growing yeast in galactose. Lastly, this vector adds two small epitopes to the protein expressed from the transgene. The added V5 epitope (14 amino acids long) allows detection of protein via the use of an anti-V5 antibody. The second added poly-histidine epitope (six amino acids long) allows one-step purification of protein, using nickel column chromatography.

PCR-amplified wild type and A53T α-synuclein were subcloned into this vector, which were respectively named pWTα-syn and pA53Tα-syn. These vectors were transformed into chemically competent *E. coli,* and cells that contained the vector were selected for growth in ampicillin-containing media (figure 5b). The vectors were purified from bacteria and screened for the presence of the transgene through restriction digestion (data not shown), forward orientation of transgene through PCR with respect to the Gal1 promoter (figure 5c), and sequence confirmation of transgene through DNA sequencing (data not shown).

WT and A53T α-synuclein transformed and expressed in yeast

Our next goal was to transform purified pWTα-syn and pA53Tα-syn into a haploid strain of *Saccharomyces cerevisiae* (*MATα ade-2-1-1 his3-*Δ200 leu2-3, 112 ura3-52) lacking a chromosomal copy of URA3 (a gene required for the synthesis of uracil). Cells containing pWTα-syn and pA53Tα-syn were selected for growth in media lacking uracil (figure 5d), and screened for the presence of α-synuclein using whole cell colony PCR (figure 5e).

Next, we induced the expression of α-synuclein protein in these yeast and determined optimal yield conditions. Yeast containing pWTα-syn and pA53Tα-syn were grown in uracil-lacking media with either galactose or glucose as the sugar source. Galactose binds to the GAL1 promoter and induces α-synuclein expression, whereas glucose inhibits this process. Cells were drawn from the culture at 0 h, 3 h, 6 h, 12 h, 24 h, 36 h, and 48 h after induction. The expression of α-synuclein was determined by Western blot analysis, using the anti-V5 antibody. The predicted size of α-synuclein is 19 kDa (including the 5 kDa epitope tags). As expected, yeast that grew in glucose media did not express α-synuclein over this time course (figure 6). But, when induced with galactose, a 28 kDa band appeared from both pWTα-syn and pA53Tα-syn -containing cells (figure 6). Protein was maximally expressed in both types of cells by 24 h. We assigned the 28 kDa band to be α-synuclein, presumably post-translationally modified by a mechanism yet to be determined. To confirm that these bands were truly α-synuclein, a Western blot containing pWTα-syn yeast lysate was probed with an anti-α-synuclein monoclonal antibody (figure 6c). The single band closely matched the migration of α-synuclein as determined from previous anti-V5 antibody probed blots (6a & b).

Two additional mutants (A30P and A30P/A53T) created and expressed in yeast

We then created the A30P α-synuclein mutant in order to compare protein folding characteristics between A30P and A53T, the two known mutations of α-synuclein found in PD patients. We also combined the two point mutations in the same transgene to assess if the double mutant would accelerate protein misfolding and be more toxic. To create these two mutations, a site-directed base pair substitution was made at codon 30 of α-synuclein onto the parent pWTα-syn and pA53Tα-syn vectors (as described in methods). The resulting vectors were designated pA30Pα-syn and pA30P/A53Tα-syn, respectively. The mutation was confirmed by DNA sequencing (data not shown). These vectors were then transformed into yeast, and protein expression was induced and probed by Western analysis, as done before. Just as before, a 28 kDa band appeared in cells induced with galactose, but not glucose (figure 7). Once again, maximal protein expression occurred by 24 h, but interestingly a minor, higher-running band at ~34 kDa was detected in A30P-expressing cells. Also, a weak, lower band at ~16 kDa was seen in both mutant forms, presumably degradation products.

To demonstrate that α-synuclein expression required the α-synuclein transgene, we also grew yeast cells that simply contained the parent pYES2 vector (without α-synuclein transgene) with glucose or galactose. As shown in figure 8a, these cells do not express α-synuclein under either condition, while cells that contain α-synuclein-specific pYES2 vectors express the protein only in the presence of galactose. To demonstrate that glucose treated (non-induced) cells contained the same amount of total cellular protein as galactose treated (induced) cells, we probed a duplicate blot with an antibody against RSP5, an

abundant house-keeping protein required for yeast survival (Hein et al., 1995). As expected, RSP5 is expressed to similar levels in all cells, irrespective of α-synuclein expression or sugar source (figure 8b). In these blots protein samples were prepared from the same number of yeast cells, which was confirmed by the overall staining profile of the RSP5 antibody in all lanes of figure 8b (because the RSP5 antibody titer is high, this antibody was used in crude serum form and resulted in the high background staining; Linda Hicke, personal communication). Independently,

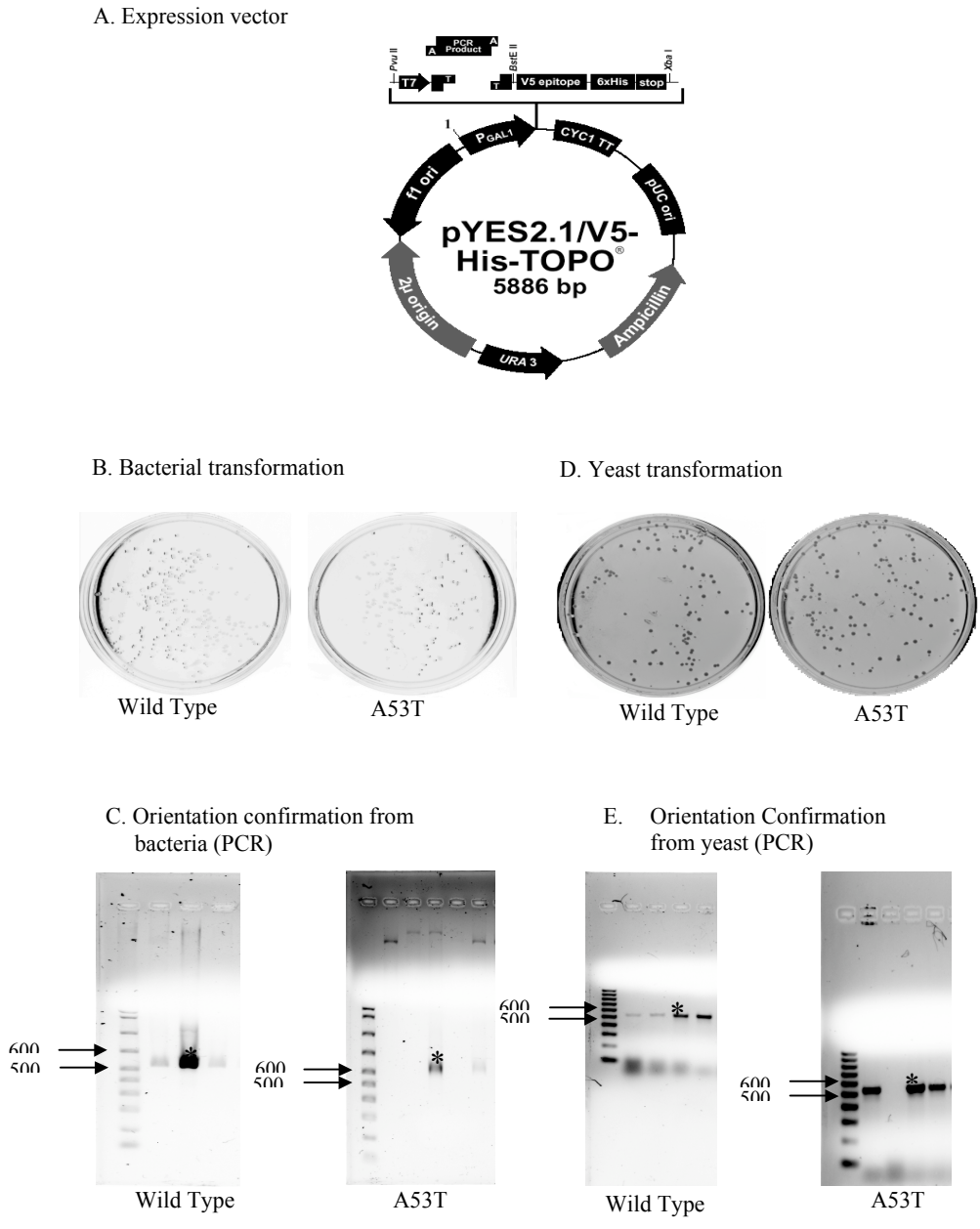

Figure 5. Transformation of Wild Type and A53T α-Synuclein into Bacteria and Yeast
A: pYES2.1/V5-His-TOPO vector map. The vector contains the pUC and 2(origins for replication in bacteria and yeast; ampicillin resistance and URA3 genes to allow selective growth of vector-containing bacteria and yeast; the GAL1 promoter to allow galactose induced expression of the transgene; and the V5 and 6x-His sequences that provide an antigen for immunostaining, and a poly-histidine tag for nickel column protein purification. B: PCR-amplified wild type and A53T (-synuclein, subcloned into the pYES2.1/V5-His-TOPO vector were transformed into E. coli. Only cells containing the expression vector should survive. Cells were plated on LB-ampicillin selective media. C: PCR amplification of WT and A53T (-synuclein from E. coli. The primer pair amplified a 550 bp (*) product if (-synuclein were subcloned into the expression vector in the forward orientation. D: WT and A53T (-synuclein transformed into S. cerevisiae. Cells were plated on uracil-lacking media, and only cells containing the expression vector should survive. E: Yeast colony PCR amplification of WT and A53T (-synuclein with the same primer pair used in B. The primer pair amplified a 550bp (*) product if (-synuclein was in the forward orientation. For B & D, 1% agarose gels resolved DNA. The left lane is a size standard.

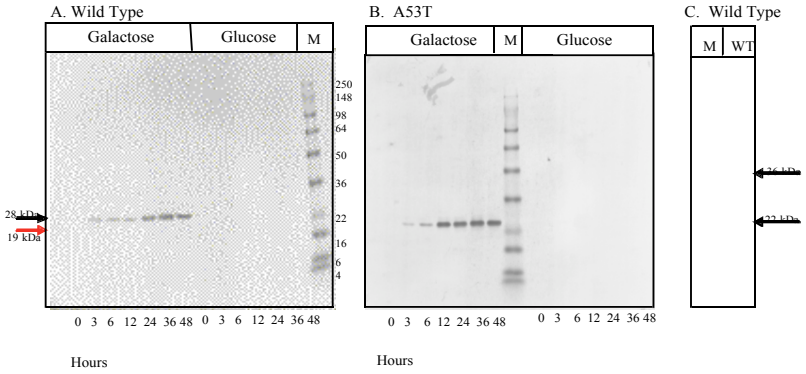

Figure 6. Western Blot Analysis of Timecourse Expression of Wild Type and A53T α-Synuclein
Induced Expression of (**A**) wild type and (**B**) A53T α-synuclein in yeast grown in uracil-lacking media containing galactose (right-hand side of blot) and glucose (left-hand side of blot). Yeast cells were removed from culture at successive intervals, and equivalent cell lysate concentrations were loaded into each well. Blots were probed with anti-V5 antibody that specifically bound to the V5 epitope tag of (-synuclein. For A & B, (-synuclein size was compared to a protein ladder (M). (-Synuclein migrated to approximately 28 kDa. Red arrow indicates size of fragment predicted by amino acid sequence (19kDa: 14 kDa (-synuclein + 5 kDa tag). C: Wild type cell lysate probed with anti- (-synuclein antibody.

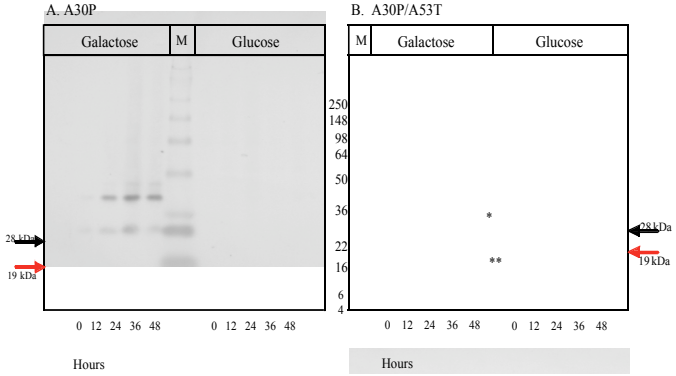

Figure 7. Western Blot Analysis of Timecourse Expression of A30P and A30P/A53T α-Synuclein
Induced Expression of (A) A30P and (B) A30P/A53T α-synuclein in yeast grown in uracil-lacking media containing galactose (right-hand side of blot) and glucose (left-hand side of blot). Yeast cells were removed from culture at 12 h intervals, and equivalent cell lysate concentrations were loaded into each well. Blots were probed with anti-V5 antibody that specifically bound to the V5 epitope tag of α-synuclein. For A & B, α-synuclein size was compared to a protein ladder (M). α-Synuclein migrated to approximately 28 kDa. Red arrow indicates size of fragment predicted by amino acid sequence (19kDa: 14 kDa (-synuclein + 5 kDa tag). Higher (*, ~34 kDa) and lower (**, ~16 kDa) migrating bands.

equivalent protein loading was confirmed in time course experiments previously shown in figures 6 & 7, and in the experiment shown in figure 8 by Coomassie staining (data not shown).

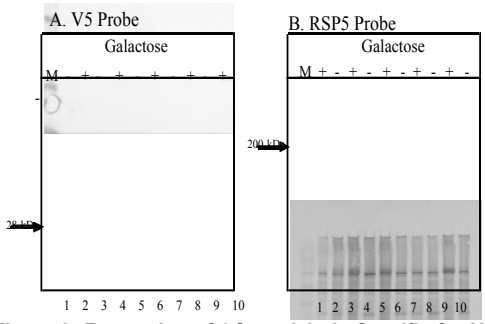

Figure 8. Expression of (-Synuclein is Specific for Yeast Carrying Transgene and Does not Affect Overall Protein Expression
Yeast transformed with control vector, wild type and mutant forms of (-synuclein were grown in uracil-lacking media containing galactose (+) or glucose (-) for 24 h. Duplicate Western blots of whole cell lysates, probed with (A) Anti-V5 Ab & (B) Anti-RSP5 Ab. Lanes: (1-2) pYES2; (3-4) WT; (5-6) A30P; (7-8) A53T; (9-10) A53T/A30P. RSP5 is a 200kDa protein that is unaffected by (-synuclein expression.

α-Synuclein does not drastically affect cell growth, but may affect adenine metabolism

Previous studies have shown that (-synuclein is toxic to nerve cells (Masliah et al., 2001a), so we asked if (-synuclein affected yeast cell growth. We compared a 48 h growth curve of yeast cells that expressed (-synuclein (wild type or mutant), cells that contained parent pYES2 vector, and cells that expressed the non-toxic protein β-galactosidase. Cells were grown in either glucose or galactose. Cell density of the cultures was determined by counting cells with a hemocytometer under a microscope at 0 h, 3 h, 6 h, 9 h, 12 h, 15 h, 24 h, 30 h, 36 h, and 48 h. First, cells grown in galactose demonstrated a delayed log phase of growth with respect to cells grown in glucose media, presumably due to metabolic transition time needed by yeast cells when exposed to an alternate sugar source (figure 9a; compare dashed lines to solid lines). More interestingly, little or no difference was observed between cell growth patterns under induced conditions, irrespective of α-synuclein expression (figure 9a). Therefore, α-synuclein appears not to drastically affect cell growth.

However, yeast culture coloration differences were observed between expressed forms of α-synuclein. When exogenous adenine levels are limited,

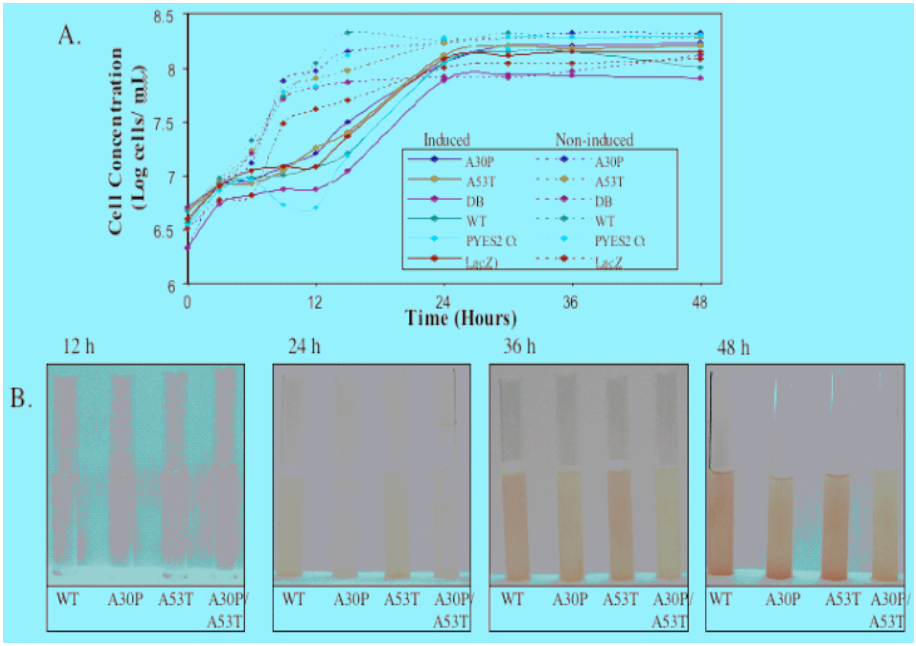

Figure 9. Expression of α-synuclein does not affect yeast growth, but may affect metabolism.
α-synuclein were grown in uracil-lacking media, containing galactose(---> or glucose). 5 mL of cells were periodically removed from culture, and counted using a hemocytometer chamber under a microscope. Growth was compared to cells transformed with pYES and LacZ under the same conditions. B: Changes in culture media color were assessed over a 48 h time course at 30ϒC in uracil-lacking media containing galactose. Photographs were taken with a digital camera.

yeast initiate adenine biosynthesis. The host yeast strain used in this study lacks a chromosomal copy of the ade2 gene necessary for adenine synthesis. Instead of forming adenine, these yeast only synthesize the intermediate metabolite phosphoribosylaminoimidazole (AIR) that is transported to the yeast vacuole for degradation (Chaudhuri et al., 1996). In the vacuole, AIR gets structurally modified and turns red (Chaudhuri et al., 1996). Here we show that the form of expressed α-synuclein affected the shade of media coloration over a 48 h time course. Media containing wild type and A53T expressing α-synuclein turned a dark shade of red over 48 h (figure 9b). Media containing A30P-expressing cells were lighter than wild type, and A30P/A53T-expressing cells became least red of all forms (figure 9b). These differences may indicate differences between wild type and mutant yeast metabolism or trafficking.

α-Synuclein purified from yeast under denaturing and native conditions

We purified α-synuclein from yeast to conduct future *in vitro* biochemical experiments. All forms of α-synuclein have a propensity to aggregate in human neurons, and α-synuclein expressed in bacterial cells require chemical denaturants for purification (Serpell et. al. 2000, Conway et. al. 1998, Giasson et. al. 1999, Conway et. al. 2000b). Therefore, we first purified wild type α-synuclein from yeast under denaturing conditions. Some protein remained insoluble after cell lysis and centrifugation at 5000 x g (figure 10a, lanes 1&2), whereas the majority of the protein remained in the soluble portion of the lysate (figure 10a, lane 3). No significant amount of protein was washed from the column prior to elution (figure 10a, lanes 4-9). α-Synuclein protein was eluted from the nickel column during the first three 1 mL elution fractions (figure 10b, lanes 1-3).

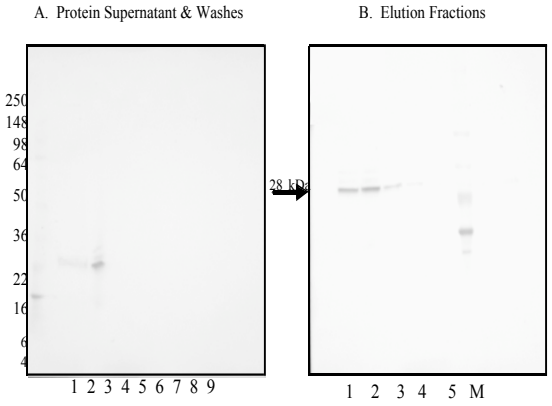

Figure 10 : Purification of Wild Type α-Synuclein.
A: α-synuclein remaining in the cell pellet after cell lysis and centrifugation (1-2), soluble α-synuclein in supernatant prior to purification (3), protein washed from the purification column prior to elution (4-9). B: α-synuclein eluted from column (1-4), protein marker (M).

We then repeated the same purification procedure for the mutant forms under denaturing conditions. Total cell lysates for the wild type and each mutant were probed for the presence of α-synuclein (figure 11a, lane 1). A majority of α-synuclein remained in the insoluble fraction of the cell lysate (figure 11a, lane 2). We observed no significant differences in the amount of insoluble α-synuclein between wild type and mutant forms (compare figure 11a, lane 2). Similar amounts of α-synuclein protein were eluted from the purification

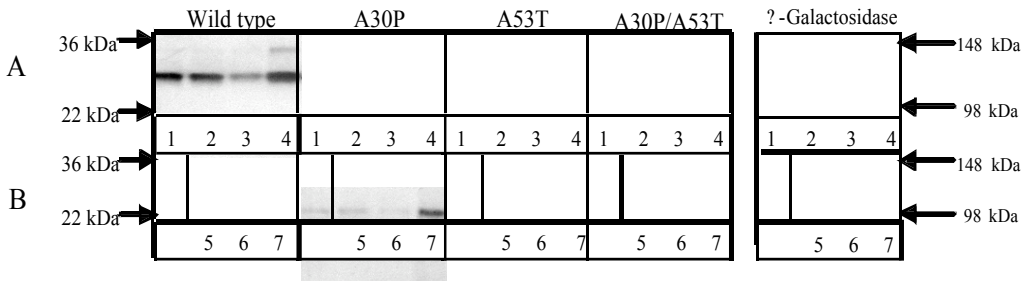

Figure 11. α-Synuclein protein purification under native and denaturing conditions
Western analysis of α-synuclein purified from yeast under denaturing conditions (A): Total cell lysate (lane 1); insoluble fraction of cell lysate (lane 2); purified α-synuclein from soluble fraction of cell lysate (lane 3); 10-fold concentration of purified α-synuclein (lane 4). Protein purified under native conditions (B): Total cell lysate (lane 1); insoluble fraction of the cell lysate (lane 5); purified α-synuclein from soluble fraction of cell lysate (lane 6); 10-fold concentration of purified α-synuclein (lane 7). All blots were probed with anti-V5 Ab.

column for each wild type and mutant form (compare figure 11a, lane3), and eluted protein was concentrated ten fold (figure 11a, lane 4). Notably, in addition to the prominent band at ~28 kDa, Western analysis showed an additional α-synuclein band running to ~34 kDa for all forms with highest intensity occurring in concentrated A30P and A30P/A53T protein.

Next, we asked if wild type and the three mutant α-synuclein forms could be purified under native conditions. We hypothesized that mutant proteins would be more difficult to purify under these conditions due to their greater tendencies to aggregate (Conway et al., 1998, Giasson et al. 1999) and fall out of solution. α-Synuclein abundance was measured in total cell lysates (Figure 11b, lane 1), and in the insoluble lysate fraction after 5000 x g centrifugation (Figure 11b, lane 2). The amount of α-synuclein remaining in the cell pellet was consistent between wild type and mutant forms under native and denaturing conditions (Compare figure 11a and 11b). All α-synuclein forms were subsequently purified from the soluble cell fraction of the cell lysate (figure 11b, lane 3) using nickel column chromatography, and were concentrated (figure 11b, lane 4). An additional band ran to ~34 kDa in the concentrated A30P sample, similar to the Western profile of this mutant form concentrated under denaturing conditions (compare figure 11a and 11b). We also bead-beat cell lysates before centrifugation to further ensure lysis had indeed occurred, and this step had no impact on protein solubility (data not shown). Cell lysis with bead-beating was sufficient to recover β-galactosidase in the soluble fraction at 5000 x g (figure 11). We determined the amount of concentrated protein purified under native and denaturing conditions (table 1). More wild type protein was quantified than all three mutant forms, with purified A30P/A53T showing lowest yield.

Table 1. Purified α-synuclein protein concentrations

α-Synuclein	Concentration (native)	Concentration (denatured)
Wild type	153 μg/mL	54 μg/mL
A30P	55 μg/mL	41 μg/mL
A53T	53 μg/mL	36 μg/mL
A30P/A53T	13 μg/mL	24 μg/mL

α-Synuclein remains in yeast after loss of induction
We asked if α-synuclein stability in yeast required a continuous supply of newly synthesized protein, and how quickly would the protein be degraded in the absence of induction. We first grew yeast containing α-synuclein to saturation (figure 12, - lane), and then induced α-synuclein expression in galactose for 24 h. Pelleted cells were transferred back to glucose, and the presence of α-synuclein was periodically determined over the next 60 h. Western analysis showed that no wild type α-synuclein remained in the cell media 12h after loss of induction (figure 12, 12 -60 h). All mutant forms of α-synuclein remained in cell media for 60 h after loss of induction. In addition to a 28 kDa band, up to two more bands running to ~34 kDa were most prominent in A30P/A53T lysates. Lower running α-synuclein bands (~6-16 kDa) presumably were partial α-synuclein degradation products.

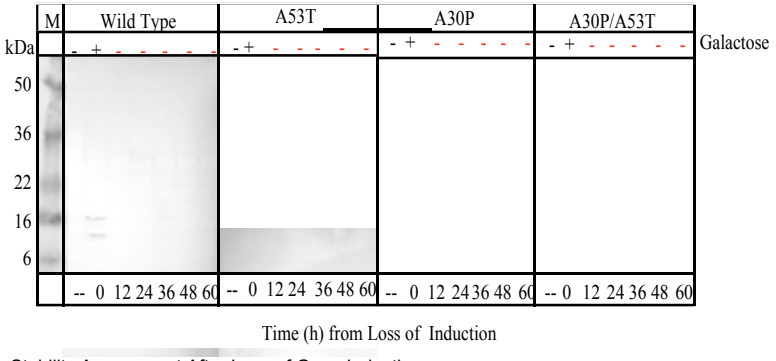

Figure 12. α–synuclein Stability Assessment After Loss of Gene Induction.
Cells expressing all forms of α-synuclein were grown in glucose-containing media for 24 h and cell lysates were probed for (-synuclein expression (-). Cells were transferred to galactose-containing media for an additional 24 h and cell lysates were probed for (-synuclein expression (0). Finally cells were returned to glucose-containing media (-). 200 (L cell samples were removed from glucose containing media at 12 h intervals and probed for (-synuclein expression (12-60). Molecular marker (M).

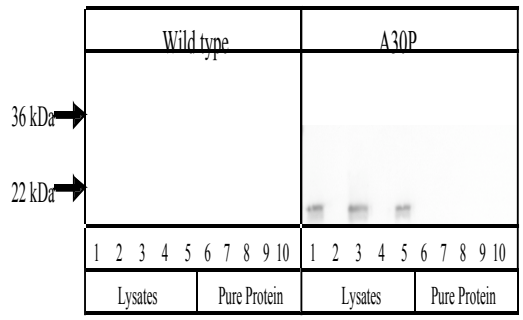

Figure 13. High Speed Centrifugation Pellets Wild Type and A30P (-Synuclein.
Cell lysates containing (-synuclein (1) were centrifuged at 11,000 x g or 110,600 x g for 30 min; soluble (-synuclein (2 & 4, respectively) was separated from the pelleted fraction (3 & 5, respectively). Purified (-synuclein (6) was centrifuged at 11,000 x g or 110,600 x g; the soluble fractions (7 & 9, respectively) and insoluble fraction (8 & 10, respectively) were probed for (-synuclein.

(-Synuclein pellets at low centrifugal force
Previous *in vitro* studies show that mutant forms of α-synuclein tend to aggregate more rapidly than wild type α-synuclein (Conway et al., 2000a). We probed for protein solubility differences among wild type and A30P α-synuclein by a preliminary differential centrifugation assay. Cell lysates and purified protein were centrifuged at two speeds. For cell lysates, both wild type and mutant α-synuclein were totally separated into the pelleted fraction at low force (11, 000 x g; figure 13, compare lanes 2 & 3). Likewise, at higher force (110,600 x g) both forms of α-synuclein also pelleted (figure 13, compare lanes 4 & 5). Surprisingly, α-synuclein purified and concentrated one week earlier was not detected by Western analysis. While this part of the experiment failed, it suggests that purified α-synuclein only remains soluble for a short period of time. To capture differences in solubility, we may need to centrifuge below 11,000 x g and use freshly purified protein.

Discussion

A major challenge in PD research has been finding effective ways to reverse or prevent protein aggregation and cell death associated with this disease. Development of diverse model systems will accelerate such discoveries. Baker's yeast has served well to model aspects of other NDDs (Kunst et al., 1997, Ma & Lindquist, 1999, Krobitsch et al., 2000). We created a yeast system for α-synuclein expression and evaluated its ability to model protein misfolding associated with PD. This study has four notable findings worth discussing. First, we show that all forms of recombinant human α-synuclein expressed in yeast appear larger in size than predicted on protein gels. Secondly, none of these α-synuclein forms adversely affects yeast growth, but mutant forms may have altered metabolism. Thirdly, while all forms were purified from yeast under native and denaturing conditions, mutant forms more often show multiple sizes on protein gels more prominently than wild type protein. Lastly, preliminary results suggest that mutant α-synuclein is more slowly degraded in yeast than wild type protein. With this model we can begin to analyze how α-synuclein interacts with partners proteins, and genetically screen factors that may inhibit α-synuclein misfolding.

Altered migration of α-synuclein suggests post-translational modification
Our first major finding is that α-synuclein from yeast lysates migrates to approximately 28kDa in denaturing protein gels in contrast to the predicted mass of 19 kDa (14 kDa protein + 5 kDa epitope tags). We also often, but not always, find faint bands between 30-34 kDa from cell lysates. Interestingly, we did see α-synuclein run to the predicted 19 kDa (figure 12; A30P/A53T, 0 h), but only once. Published sizes of human and recombinant α-synuclein have been reported in several models (*E. coli*, mice, and rats) as summarized in Table 2. In both human patients and in *E. coli* the expected 14 kDa size was only seen in three reports (Conway et al., 2000 b; Uversky et al., 2001; Hasegawa et al., 2002). In others α-synuclein models size varied between 16-19 kDa without any obvious added protein epitopes contributing to their reported sizes (Spillantini et al., 1998; Okochi at al., 2000; Shimura et al., 2001; Ellis et al., 2001; Kahle et al., 2000; Sharon et al., 2001; Masliah et al., 2001; Bianco et al., 2002; Biere et al., 2000). Our observed 28 kDa α-synuclein band is consistent with these reports of aberrant synuclein migration. However, no clear explanations have been provided for the majority of increased sizes in previous studies with the following exceptions: Okochi et al. 2000 report a band at 25 kDa in addition to 19 kDa due to serine phosphorylation (addition of phosphate groups); Shimura et al. 2001 report α-synuclein running to 22 kDa in addition to 16 kDa caused by glycosylation (addition of sugars); and Sharon et al. 2001 show α-synuclein running to 36 kDa due to lipidation (addition of lipids). While we do not know why synuclein migrates to 28 kDa in our study, it may be due to one or more of these three forms of post-translational modifications. Currently, we are conducting experiments to examine N-linked and O-linked glycosidases and phosphatases (enzymes that cleave sugars and phosphates, respectively) on purified and cell lysate forms of α-synuclein.
Additonally, studies summarized in Table 2 show high running synuclein dimers and other aggregates (Uversky et al., 2001; Sharon et al., 2001; Masliah et al., 2001). These variations were attributed to temperature, time, and pH dependant conditions (Giasson et al., 1999). We have not observed any α-synuclein bands running higher than 34 kDa on denaturing gels (to be a dimer α-synuclein would have to run to 38 kDa). Either α-synuclein may not be aggregating to dimers and higher order structures to significant amounts in yeast, or aggregates do form but get separated during denaturation steps before loading proteins on gels for
electrophoresis analysis. Future experiments with purified protein will examine temperature, time, or pH dependant aggregation.

Previous studies have not reported any significant differences between wild type and mutant α-synuclein migration on protein gels. Similarly we see wild type and mutant forms
migrating to the same extent on protein gels, except that the higher running bands (~30-34 kDa) are more intense for mutant protein (discussed below).
Subtle metabolic differences may indicate α-synuclein toxicity otherwise not apparent by yeast survivability

Our next result indicates that yeast growth rates are relatively unaffected by α-synuclein expression. The yeast genome does not have an α-synuclein gene, and the presence of this foreign protein does not appear to be lethal. Overexpression of α-

synuclein in other model organisms (fruit flies and mice) that do naturally make this protein (they have an α-synuclein gene) show that α-synuclein misfolds and forms insoluble fibrils in dopamine-producing neurons, causing cell death (Masliah et al., 2000; Van der Putten et al., 2000; Giasson et al., 2002; Feany & Bender, 2000). Moreover fruit flies and mice that overexpress α-synuclein closely resemble the pathology and symptoms of human PD (Feany and Bender 2000, Giasson et al 2002). Fruit flies and mice share many cellular processes with human cells that allow PD pathology and symptoms to be closely recapitulated. However, yeast do not age, degenerate or have a programmed cell death pathway like human cells and may not die as easily from toxic forms of α-synuclein. Other endpoints to measuring cellular toxicity need to be further investigated. We are currently confirming effects of α-synuclein on yeast growth by spotting serially diluted yeast on glucose and galactose yeast culture plates.

Serendipitously, we noted differences in media coloration (associated with adenine metabolism) among yeast expressing wild type and mutant forms of α-synuclein, when examined over a 48 h time course. One possibility is that A30P and A30P/A53T α-synuclein is inhibiting the transport of the adenine precursor metabolite AIR to the yeast vacuole. If so, how can yeast growth remain unaffected if adenine synthesis is slowed? In our experiments yeast were grown in media that contains all amino and nucleic acids (except uracil). Yeast metabolism may be affected by α-synuclein, but growth in this rich medium may mask any potential toxicity. If adenine biosynthesis pathways are differentially affected in yeast expressing mutant α-synuclein (this was easy to examine), it stands to reason that other process may be affected. Alternatively, these differences may be due to slowed metabolic processes in these cells that cannot be captured in growth rates in enriched media. Instead, yeast can be grown in media deficient in essential yeast nutrients to determine if α-synuclein interferes with yeast metabolism.

Several yeast model systems have been successfully used to study protein misfolding characteristics of NDDs without affecting yeast survivability. Yeast models of Huntington's disease show that transgenic expression of wild type or disease causing forms of huntingtin aggregate, but are not toxic to yeast (Krobitsch & Lindquist, 1997). Cytoplasmic aggregation of the human prion protein in yeast recapitulates many protein structure aspects of human prion diseases, but does not appear to be lethal (Ma & Lindquist, 1999). Yeast expression of a mutant form of human superoxide dismutase that causes ALS has been used to identify some of its partner proteins, but does not affect yeast growth on rich media (Kunst et al., 1997). To determine if factors causing ALS in humans are toxic to yeast, growth analyses were conducted under stressful conditions. Yeast growth rates were inhibited when grown on medium lacking amino and nucleic acids, showing that genetically modified yeast were unable to biosynthetically produce necessary nutrients (Nishida et al., 1994). Affected yeast cultured in a 100% oxygen atmosphere did not grow, indicating that yeast were unable to detoxify high concentrations of respiration byproducts (ROS; Nishida et al., 1994). These assays may be used in our research to comparatively examine α-synuclein toxicity in yeast.

α-Synuclein sediments *in vitro* and may form several monomeric structures

Three lines of evidence support the insoluble nature of α-synuclein protein in yeast, difficulty in maximizing purification yield, and preserving long-term solubility. First, a significant portion of α-synuclein sediments from cell lysates at 5000 x g during the first step of protein purification. This pelleting is universally found when purified wild type or mutant protein under denaturing or native lysis conditions. Two possibilities may explain this phenomenon. α-Synuclein may form insoluble structures in yeast that are separated into the cell pellet fraction at low centrifugation. In initial support of this, α-synuclein sedimented after treatment under stringent denaturing conditions (6 M guanidinium buffer). Alternatively, α-synuclein may not be released from intact cell as a result of incomplete cell lysis. To test this latter possibility we conducted two experiments. In addition to lysing cells in the presence of lyticase, we bead-beat cells for 3 min at high speed, and centrifuged the lysate at 11,000 x g. At this slightly higher speed all protein was fractioned into the cell pellet. We also assessed the fractionation of recombinant β-galactosidase, a known soluble protein under the same conditions of centrifugation. This alternative was tested by purification of the soluble, cytoplasmic protein β-galactosidase from yeast. All β-galactosidase remained soluble under denaturing and native conditions, demonstrating that cells were lysed well enough to fully extract soluble protein. Secondly, lower yields of purified mutant α-synuclein were recovered than wild type protein, suggesting that mutant forms may be less soluble. Thirdly, after purifying soluble α-synuclein and confirming its presence on Western blots, we were unable to visualize it on a Western blot one week later during a subsequent experiment (compare presence of protein in figure 11 to absence of protein in figure 13).

As previously mentioned we often see up to three different structural forms of α-synuclein (between 28-34 kDa on protein gels; figure 11, lane 1). This finding is striking because such close running multiple bands on protein gels have not been reported for α-synuclein from *E. coli*, mice, or humans (Table 2). Higher running bands (between 30-34 kDa) are most consistently seen with mutant forms and/or when purified protein is concentrated. Interestingly, mutant α-synuclein tends to aggregate more than wild type protein as seen in other animal models (Giasson et al., 2002; Feany & Bender, 2000). Also, when synuclein is concentrated, it tends to aggregate, as seen in *E. coli* studies (Conway et al., 2000 b; Uversky et al., 2001). We speculate that the higher running bands we observe in yeast are associated with aggregation.

Because these bands run too low to be dimeric structures, we hypothesize that these bands indicate structurally distinct and stable monomers. Several studies have identified structural intermediates that may be part of the unfolding pathway for α-synuclein aggregation (figure 14a, reviewed in Goldburg & Lansbury, 2000). But less is known about the native state of α-synuclein, the types of stable monomeric structures it may adopt, and the role of potential post-translational in acquiring these states (figure 14b). Future experiments with native gels may better resolve these monomeric structures and allow us to visualize α-synuclein dimers or polymers that may be forming in yeast.

Mutant α-synuclein may form stable aggregates *in vivo*

Wild type α-synuclein protein is undetectable within 12 h of loss of gene induction, but mutant forms remain in yeast even 60 hours after α-synuclein expression is suppressed. These results suggest that wild type protein is more easily degraded whereas the mutants are more resistant. A continuous supply of newly synthesized protein does not seem necessary for the increased stability of mutant α-synuclein, coinciding with the SDS-resistant bands resolved on protein gels. This indicates that the mutant forms of α-synuclein may be forming aggregates that recapitulate properties of PD-causing structures in human neurons and in model organisms (Okochi et al., 2000; Van der Putten et al., 2000; Giasson et al., 2002; Feany & Bender, 2000). The loss of induction assay was only conducted once, and must be repeated to confirm this finding. These data also show inconsistent tapering of α-synuclein abundance during loss of induction, probably due to improper protein loading although cell lysates were prepared from the same number of cells. To control for loading in future experiments, duplicate Western blots would be probed with an antibody against a continuously expressed house-keeping protein, and loss of induction will be compared to soluble β-galactosidase. Lastly, this result will be refined by conducting a pulse-chase experiment that will measure the lifetime of newly synthesized α-synuclein in yeast using radioactive-labeled protein.

Future Investigation

In addition to several experiments suggested throughout this discussion, protease digestion experiments are being developed to characterize differences between wild type and mutant α-synuclein tertiary structures. α-Synuclein misfolding has been correlated with mitochondria damage in PD patients, and these effects can easily be studied in this yeast system. Perhaps we can get better yield if we express α-synuclein to lower levels. A copper-binding promoter can more finely control α-synuclein induction, and α-synuclein concentration dependant aggregation and mitochondria damage assays may then be developed. Yeast contain highly conserved eukaryotic chaperone and ubiquitin/proteosome proteins that regulate protein misfolding and degradation. Co-immunoprecipitation assays may show if α-synuclein complexes with these proteins or is ubiquitinated. Immunofluorescence will show α-synuclein localization in fixed cells, and green fluorescent protein (GFP)-tagged α-synuclein will be used to visualize α-synuclein in living yeast. The effects of overexpressed or gene knock-outs of ubiquitin/proteosome enzymes and chaperone proteins in this yeast system will be evaluated by identifying changes in α-synuclein localization with the GFP expression system.

Conclusion

To our best knowledge, this is the first study to show wild type and mutant forms of α-synuclein expressed in yeast. Initial evaluation of this expression system indicates that α-synuclein may aggregate in yeast and in test tubes. Preliminary results indicate that mutant α-synuclein forms affect yeast metabolism, are more stable in yeast compared to wild type, and have a greater tendency to aggregate *in vitro*. This model does recapitulate many properties reminiscent of α-synuclein behavior in PD patients and will be a useful model to identify factors that may inhibit or correct α-synuclein misfolding.

Experimental Procedures

Transformation into *E. coli*

Gene Amplification: Wild type and A53T α-synuclein cDNA-containing vectors were a gift from Chris Ross (Johns Hopkins University). These vectors were extracted from *E. coli* using a standard Qiagen miniprep protocol. Wild type and A53T α-synuclein cDNA was amplified using polymerase chain reaction (PCR). The following α-synuclein-specific primer sequences were synthesized to allow for subsequent subcloning into the pYES2.1 TOPO TA vector.

Forward Primer 1 (FP1): 5'AAAAAAGAGCTCATGGATGTATTCATGAAAGGA3'

Reverse Primer 1 (RP1): 5'GCCTTCAGGTTCGTAGTCTTGATACCCTTC3'

The forward primer contained a 5' overhang with a unique *Sac* I restriction site. cDNA was amplified with the following thermocycle profile: 30 cycles at 95° C for 30s, 55° C for 30 s, 72° C for 30 s; 72° C 5 min. Confirmation of PCR product with an expected size of 442 bp was analyzed by electrophoresis on a 1% agarose gel.

Sub-cloning and transformation of α-synuclein into E. coli: The PCR product from the preceding section was subcloned into the pYES2.1/V5-His-TOPO expression plasmid as described by the Invitrogen pYES2.1 TOPO TA cloning kit (K4150-01). The plasmid was then transformed into chemically competent *E. coli* (F'{lacI^{q}Tn*10*(Tet^R)}*mcr*AΔ(*mrrhsd*RMS*mcr*BCΦ80*lac*ZΔM15Δ*lacX74recA1deoRaraD139Δ(araleu)7697galUgalKrps*L(Str^R)*end*A1*nup*G). The cells were grown on LB-ampicillin plates to select for colonies containing the vector. Twenty colonies from each plate culture were randomly selected and grown in liquid LB-ampicillin media. Vector DNA from each of these cultures was extracted by Qiagen miniprep.

Analysis of gene orientation

Three tests were conducted to confirm the presence of vector DNA, the forward orientation of α-synuclein cDNA with respect to the Gal1 promoter, and the exact sequence of α-synuclein cDNA in the vector. First, a restriction digest reaction was completed on all twenty colonies. Purified vector from the twenty screened colonies was incubated with *Sac* I and *Xba* I restriction enzymes for 24 hours. The *Sac* I restriction site was previously engineered into the forward primer used to PCR-amplify α-synuclein cDNA. The *Xba* I restriction site was located on the vector 96 bp downstream from the α-synuclein cDNA insert. The results of the digest were analyzed by gel electrophoresis. An expected size of 547 bp band indicated the presence of α-synuclein in the vector in the forward orientation. Secondly, α-synuclein cDNA was amplified using the same PCR cycle profile from the *gene amplification* section. A forward primer (GAL1 FP: 5'AATATACCTCTATACTTTAACGTC3') designed by Invitrogen to bind to the vector at the galactose promoter region upstream from the gene insert was used in this reaction. The PCR product with an expected size of 547 bp was analyzed by gel electrophoresis using a 1% gel. Thirdly, purified vector solutions containing wild type α-synuclein cDNA and A53T α-synuclein cDNA were sequenced at the University of Chicago sequencing facility, using the GAL1 FP. Bacterial cells containing the sequenced vector with the gene in the correct orientation were preserved at -80° C.

Site-Directed Mutagenesis

Methylation: A30P mutant α-synuclein and A30P/A53T α-synuclein cDNA were mutagenized from wild type and A53T α-synuclein, respectively. The protocol for this method was published in *Gene Tailor Site-Directed Mutagenesis System: 12397-014*. 1.6 μL methylation buffer, 1.6μL 10x SAM buffer, 1 μL DNA methylase, and 10.8 μL were added to 1μL purified plasmid DNA containing α-synuclein. The reactions were incubated at 37°C for one hour.

Mutagenesis reaction: A PCR reaction was used to amplify vector DNA with a new point mutation. Primers were designed

to make the specific A30P mutation, which is underlined (FP: 5'AACAAGGTGTGGCAGAAGCACCAGGAAAGAGAA3'

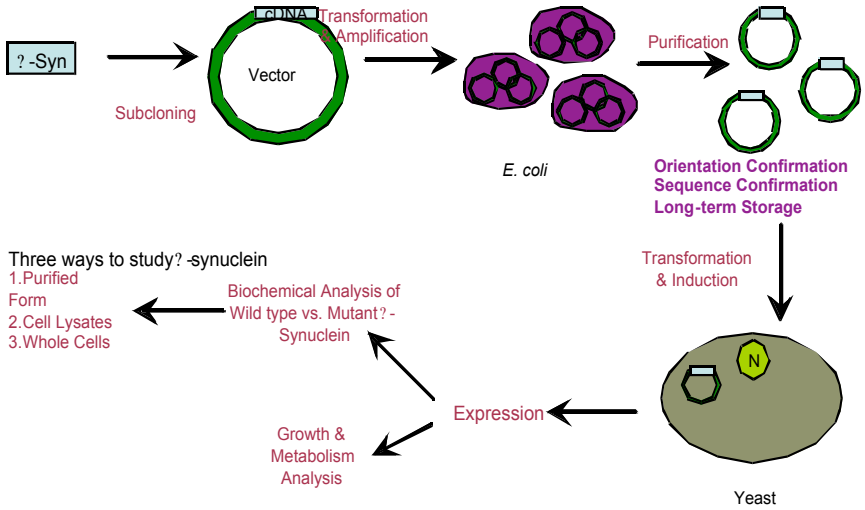

Figure 4: Proposed Schematic for α-Synuclein Expression and Characterization in Yeast. The α-synuclein cDNA was sublconed into a galactose inducible expression vector, amplified in bacteria, purified, and transformed into yeast. Expression of α-synuclein was induced in the presence of galactose. Growth and metabolism studies were determined in yeast expressing wild type and mutant forms of α-synuclein. Whole cells, lysates, and purified protein were used to conduct several tests including loss of induction and differential centrifugation assays.

RP: 5'TGCTTCTGCCACACCCTGTTTGGTTTTCTC3').
PCR cycle: 94° C for 1 min; 20 cycles of 94° C for 30 s, 55° C for 30 s, 68° C for 6:20 min; 68° C for 10 min.

Transformation: 2μL of the mutagenesis reaction was added to DH5α-T1 chemically competent cells and heat shocked at 42°C for 30 s. 200 μL SOC medium was added to the cells, and the mixtures were incubated at 37° C for 1 hr. Cells were plated onto LB-ampicillin plates. Background control cells transformed with freshly mutated Lac-Z-containing control vector were plated onto LB-ampicillin plates containing 0.5 mg/mL X-gal. β-galactosidase converts X-gal into a blue product that is easily seen in a bacteria colony. If the LacZ gene were mutated, bacteria would not produce the blue product. We determined the efficiency of mutagenesis by determining the proportion of colorless colonies in the control mutagenesis reaction. Over 90 % of the colonies remained colorless. Results of the mutagenesis were analyzed by DNA sequencing at the University of Chicago sequencing facility.

Transformation into Yeast
Transformation: The pYes2.1 vector containing α-synuclein was transformed into a haploid, URA-3 deficient strain of *Saccharomyces cerevisiae* (TSY623: *MATα ade2-101 his3-Δ200leu2-3,112 ura3-52*). The protocol for *High efficiency transformation* was followed (Burke, 2000). 5mL YEPD was inoculated with yeast and incubated overnight at 30° C. 50 mL YEPD was inoculated with the overnight culture to a concentration of 5x10^6 cells/mL and was incubated for 4 h. Cells were harvested, washed in sterile water, and resuspended in 250 μL 0.1 M lithium acetate. 50 μL of the yeast suspension was transferred to a microfuge tube. Transformation mix (240 μL 50% w/v PEG, 36 μL 1.0M LiAc, 25 μL 2.0 mg/ml single-stranded carrier DNA, 5 μL plasmid containing α-synuclein, 45 μL water) was added to the cell mixture. Cells were incubated at 30° C for 30 min, and heat-shocked at 42° C for 20 min. Cells were pelleted and resuspended in 1.0 mL water. Yeast cells were grown on synthetic-complete media lacking in uracil (SC-URA), selecting for yeast cells that contained the expression vector.

Whole yeast colony PCR: Yeast colony PCR was conducted to verify the presence of α-synuclein in the correct orientation with the expression plasmid. The gene was amplified once again using the vector-specific primer GAL1 FP, the α-synuclein-specific reverse primer, 2μL 10 x PCR buffer, 1.2μL 25 mM $MgCl_2$, 0.4μL 10 mM dNTPs, 15 pmol RP1, 15 pmol GAL1FP, 0.2μL Taq polymerase, and 10μL dH_20 added to a small yeast colony. The following PCR cycle profile was used: 94° C for 4 min; 35 cycles at 94° C for 1 min, 55° C for 1 min, 72° C for 2 min; final elongation 72° C for 10 min). The result was analyzed by gel electrophoresis. The PCR product with an expected size of 547 bp was analyzed by gel electrophoresis using a 1% agarose gel.

Expression of α-Synuclein
Timecourse Expression: Since α-synuclein was subcloned next to the Gal1 promoter in one pYES2 vector in the presence of galactose and absence of glucose, the expression of α-synuclein should be induced. 50 mL of SC-URA with 2% galactose and 50 mL of SC-URA with 2% glucose (control) was inoculated with yeast containing the expression vector to a concentration of 5 x10^6 cells/mL. 5 mL of the culture was removed at 0 h, 3h, 6h, 12h, 24 h, 36 h, and 48 h. Cells were counted using a hemocytometer counting chamber.

Subsequent Expression: Expression of α-synuclein for all further experimentation was induced in SC-URA media for 24 h.

Western Blot Analysis
Preparation of Cell Lysates: Followed protocol of *Yeast protein extracts* (Burke, 2000). 2 ml of 50 mM Tris (pH 7.5), 10 mM NaN_3 (sodium azide) was added to yeast cell pellets containing approximately 2.5 x10^7 cells. The mixture was vortexed and repelleted in a 1.5 mL microfuge tube. The supernatant was pipetted from the tube. 30 μL Electrophoresis Sample Buffer (ESB) buffer (2% SDS, 80 mM Tris (pH6.8), 10% glycerol, 1.5% DTT, 1mg/mL bromophenol blue) containing a protease inhibitor cocktail (1 mM PMSF, 0.7μg/mL pepstation A, 0.5 μg/mL leupeptin, 10 μg/mL E64, 2 μg/mL aprotinin) was added to the cell pellet. The mixture was vortexed and heated at 100° C for 3 min. Approximately 0.1g of 0.5 mm glass beads were added to the cell mixture. The microfuge tube was vortexed for three minutes. 70 μL ESB was added, and the tube was centrifuged for 30 s.

SDS Gel Electrophoresis and Western Transfer: 15uL of cell homogenate was added to each lane of 4-20% SDS gel. The gel was run for 90 minutes at 130 volts. *Sea Blue* (Invitrogen) was used as the molecular standard. Protein gels were transferred to PVDF membrane (BioExpress) by electorphoresis for 90 minutes at 30 volts.

Protein Detection: PVDF membranes were rinsed with Tris-buffered saline (TBS) blocking buffer containing 3% Tween-20 for 30 minutes. The membrane was incubated with 10 mL primary antibody solution for at least one hour. The membrane was rinsed with TBS antibody wash solution. The membrane was incubated in alkaline phosphatase (AP) conjugated

secondary antibody solution for 30 minutes. The membrane was rinsed with TBS and deionized water. The secondary antibody was detected using BioRad 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) and p-nitro blue tetrazolium chloride (NBT) color developer (30 mg NBT dissolved in 1 mL 70% dimethylformamide, 15 mg BCIP dissolved in 1 mL 100% dimethylformamide, 100 mL Tris buffer). The membrane was exposed to the developing solution until bands appeared (30 s-5 min). The reaction was quenched with several rinses of deionized water.

Antibody Solutions: Antibodies were diluted in blocking buffer to 1x concentration. Antibodies used: Mouse anti-V5 IgG 5000x (Invitrogen 46-0705), used for the detection of the V5 epitope on α-synuclein protein; Goat Anti-mouse AP-conjugated secondary IgG 1x (*Western Breeze)*, used to detect of Anti-V5 primary IgG; rabbit anti-RSP5 polyclonal Ab 5000x, used for the detection of yeast house keeping protein RSP5 (gift from Linda Hicke, Northwestern University); Mouse anti-α-synuclein monoclonal IgG (Zymed) used to detect α-synuclein protein; goat anti-rabbit AP-conjugated secondary IgG 2500x (Santa Cruz biotech #6192), used to detect anti-RSP5 primary antibody.

Protein Purification under Denatured Conditions

α-Synuclein expression was induced in galactose medium for 24 h. Protein was purified using the *Invitrogen Probond Purification System* under denaturing conditions. A 50 mL yeast culture was centrifuged and the supernatant was decanted. 8 mL 6 M guanidinium lysis buffer (6 M guanidinium HCl, 20 mM $NaPO_4$ (pH 7.8), and 500 mM NaCl; 1 mM PMSF, 0.7μg/mL pepstation A, 0.5 μg/mL leupeptin, 10 μg/mL E64, 2 μg/mL aprotinin) was added to the cell pellet. Cells were lysed by vortex mixing for several minutes, and the soluble fraction was separated by centrifugation. The supernatant was transferred to a column containing nickel-coated resin. The protein containing the poly-histidine tag was allowed to bind over a 30 min incubation period. The resin was allowed to settle and the supernatant was removed. The column was washed with two portions of 6 mL denaturing binding buffer (8 M urea, 20mM $NaPO_4$ (pH 7.8), 500 mM NaCl). The column was rinsed with two portions of 6 mL denaturing wash buffer (8 M urea, 20 mM $NaPO_4$ (pH 7.8), 500 mM NaCl). The column was then washed with four portions of 8 mL native wash buffer (containing 20 mM imidazole, pH 8.0). 500 μL of each fraction decanted from the column was stored for western analysis. α-Synuclein was eluted from the column with 8 mL native elution buffer containing (250 mM imidazole) in 1 mL fractions. Fractions were analyzed for the presence of α-synuclein by Western analysis just described, and α-synuclein rich fractions were pooled, dialyzed, and concentrated.

Protein Purification Under Native Conditions

All procedures were the same as purification under denatured conditions with the following exceptions. Yeast cells were lysed in native binding buffer (50 mM $NaPO_4$, 0.5 M NaCl, 10 mM imidazole, and 10 mM lyticase, 1 mM PMSF, 0.7μg/mL pepstation A, 0.5 μg/mL leupeptin, 10 μg/mL E64, 2 μg/mL aprotinin). The cell supernatant was allowed to bind for 90 min. The column was only washed four times with 8 mL native wash buffer. Protein was eluted as previously described. An additional step was recently tested. 1 mL concentrated cell solutions were bead-beaten with 300 mg 0.5 mm glass beads on a Biospec mini-beadbeater for 3 minutes at highest speed after the addition of lysis buffer. Bead beating had no effect on solubilizing α-synuclein.

Protein Dialysis and Concentration

Pooled elutions fractions (6 mL) were dialyzed and concentrated using the Tube-O-Dialyzer (786-142; Geno Technologies). Pooled fractions were transferred to the dialysis tubes containing a semi-permeable membrane cap. Samples were placed membrane-side down in a 1000 mL beaker filled with deionized water. Small molecules (including purification buffers) passively moved across the membrane. Water was changed 10-12 h later. Protein samples were dialyzed for at least 24 h. To concentrate protein the dialysis tubes were placed membrane-side down onto a beaker half filled with a high molecular weight polymer that absorbed the water through the membrane. The membrane cap was cleaned periodically to maximize water transfer. Protein was then brought to 500 μL to standardize protein concentration analysis.

Protein concentrations were determined using the Bio-Rad Protein Assay. 5 dilutions of bovine serum albumin (BSA; 0.0 μg/mL, 2.0 μg/mL, 4.0 μg/mL, 6.0 μg/mL, and 8.0 μg/mL) were prepared in duplicate in 800μL deionized water to make a standard curve. Duplicate samples of 20 μL concentrated protein were diluted to 800 μL (40x) in deionized water. 200 μL dye reagent concentrate (Bio-Rad 500-006) was added to each sample. The samples were vortexed and incubated at room temperature for 30 min. Absorbances for each sample were measured at 595 nm, using the Hitachi U-2000 spectrophotometer. Protein concentrations were determined from the equation of the standard curve and the 40x dilution factor.

Differential Centrifugation

Yeast cells containing α-synuclein cDNA were grown in uracil-lacking media containing galactose for two days. 300 μL of cell suspension was drawn from the media and pelleted. Cells were bead-beaten in 1 mL deionized water for 3 min. 25 μL ESB was added to a 100 μL sample of fresh cell lysate and a 100 μL sample of freshly purifed protein. 100 μL samples of cell lysate and purified protein were centrifuged at 14,000 rpm (11,000 x g) for 30 min in an Eppendorf 5415C microfuge. Likewise, 100 μL samples of cell lysate and purified protein were centrifuged at 111,000 x g for 30 min in a Beckman Coullier airfuge (lab of David Mueller, Finch Medical School). The supernatants were aspirated and added to 25 μL ESB buffer. The lysate pellets were resuspended in 125 μL ESB. The presence of α-synuclein was detected in all samples by Western analysis.

Loss of Induction

Yeast cells containing α-synuclein cDNA were grown in 50 mL uracil-lacking media containing galactose for 24 h. 1 mL sample of cells was drawn from the culture for Western analysis. The remaining cells were pelleted, washed with 0.10 M Na_2PO_4 buffer (pH 7.4), and repelleted. Cells were then resuspended in uracil-lacking media containing glucose for 24 h. 1 mL of cells was drawn from the culture for Western analysis. The remaining cells were pelleted, washed with 0.10 M Na_2PO_4 buffer (pH 7.4), and repelleted. Cells were resuspended in 50 mL uracil-lacking media containing glucose. 1 mL samples of cells were drawn from culture every 12 h over the next 72 h. Cells from each sample were counted in a hemocytometer chamber, and equivalent amounts of cells from each sample were prepared for Western analysis.

Acknowledgements

I would like to thank Dr. Shubhik DebBurman for the time and energy he has invested to teach me how to think, write, and approach experiments like a scientist. He has been an outstanding advisor and mentor, and his continued support has inspired me to maximize my potential. I thank Nijee Sharma for supporting this research and its presentation. I would also like to thank my labmates Samantha England, Olga Mikhaylov, and Adam Hanig for all of their help. I thank my senior thesis classmates Angie Eakley, Christina Christ and Kirsten Hafkemeyer for giving me feedback and motivation. I thank my family, friends, and my girlfriend Lexi for continuously supporting my education and this thesis endeavor. Finally, I thank my thesis committee Karen Kirk, Bob Glassman, Tim Morton, and Judy Potashkin (Finch University of Health Sciences) for reading and evaluating this project.

References

Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg D., Ho, W., Castillo, P., Shinsky, N., Verdugo, J., Armanini, M., Ryan, A., Hynes, M., Phillips, H., Sulzer, D., Rosenthal, A. 2000. Mice lacking α-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25:239-252.

Auluck, P., Chan, E., Trojanowski, J., Lee, V., Bonini, N. 2002. Chaperone suppression of α-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 295: 865-868.

Arima, K., Ueda, K., Sunohara, N., Hirai, S., Izumiyama, Y., Kawai, H. 1998. Immunoelectron microscopic demonstration of NACP/α-synuclein-

epitopes on the filamentous components of Lewy bodies of sporadic Parkinson's disease. *Am. J. Chem.* 152: 879-884.

Barbosa, E., Limongi, J., Cummings, J. 1997. Parkinson's disease. *Psychiatr. Clin. North Am.* 20: 769-790.

Biere, A., Wood, S., Wypych, J., Stevenson, S., Jiang, Y., Narhi, L., Citron, M. 2000. Parkinson's disease-associated α-synuclein is more fibrillogenic than β- and γ- synuclein and cannot cross-seed its homologs. *J. Biol. Chem.* 275: 34574-34579.

Bianco C., Ridet, J., Schneider, B., Deglon, N., Aebischer, P. 2002. α-Synucleinopathy and selective dopaminergic neuron loss in rats lentiviral-based model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 99: 10813-10818.

Chaudheri, B., Ingavale, S., Bacchawat, A. 1996. Apd1, a gene required for red pigment formation in ade6 mutants of *Schizosaccharomyces pombe*, encodes an enzyme required for glutathione biosynthesis: a role for glutathione-conjugate pump. *Genetics* 145: 75-83.

Chung, K., Dawson, V., Dawson, T. 2001. The role of the ubiquitin-proteosomal pathway in Parkinson's disease and other neurodegenerative disorders. *Trends Neurosci.* 24: 7-14.

Clayton, D., George, J. 1998. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends. Neurosci.* 21: 249-254.

Conway, K., Harper, J., Lansbury, P. 1998. Accelerated *in vitro* fibril formation by a mutant α-synuclein linked to early-onset Parkinson's disease. *Nat. Med.* 4: 1318-1320.

Conway, K., Seung, L., Rochet, J., Ding, T., Williamson, R., Lansbury, P. 2000 a. Acceleration of oligmerization, not fibrillization, is a shared property of both α-synuclein mutations linked to early-onset Parkinson's disease: implications for pathenogenesis and therapy. *Proc. Natl. Acad. Sci. USA* 97: 571-576.

Conway, K., Harper, J., Lansbury, P. 2000 b. Fibrils formed *in vitro* from α-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry* 39: 2552-2563.

Conway, K., J. Rochet, R. Biegansky, Lansbury, P. 2001. Kinetic stabilization of the α-synuclein protofibril by a dopamine-α-synuclein adduct. *Science* 294: 1346-1350.

Ding, T., Lee, S., Rochet, J., Lansbury, P. 2002. Annular α-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. *Biochemistry*. 41: 10209-10217.

Duda, J., Shah, U., Arnold, S., Lee, V., Trojanowski, J. 1999. The expression of α-, β, and γ-synucleins in olfactory mucosa from patients with and without neurodegenerative diseases. *Exp. Neurol*. 160: 515-522.

Dulic, V., Egerton, M., Elguindi, I., Singer, B., Riezman, H. 1991. Yeast endocytosis assays. *Methods Enzymol* 194: 697-70.

Ehringer, H. & Hornykiewicz, O. 1960. Verteilung von noradrenalin und dopamine im gehrin des menschen und ir verhalten bei ekrankungen des extrapyranidalen. *Klin. Wschr.* 38 : 948; p.52

Ellis, C., Schwartzberg, P., Grider, T., Fink, D., Nussbaum, R. 2001. α-Synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases. *J. Biol. Chem.* 276: 3879-3884.

Elledge, S. 1996. Cell cycle checkpoints: Preventing an identity crisis. *Science.* 274: 1664-1672.

Engelender, S., Kaminsky, Z., Guo, X., Sharp, A., Amaaravi, R., Kleiderlein, J., Margolis, R., Troncoso, J., Lanahan, A., Ross, C. 1999. Synphilin-1 associates with α-synuclein and promotes the formation of cytosolic inclusions. *Nature* 22: 110-114.

Feany, M. & Bender, W. 2000. A *Drosophila* model of Parkinson's disease. *Nature* 23: 294-298.

Giasson, B., Duda, J., Murray, I., Chen, O., Souza, J., Hurtig, H., Ischeri, H., Trojanowski, J., Lee, V. 2000. Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synucleinopathy lesions. *Science* 290: 285-289.

Giasson, B., Duda, J., Quinn, S, Zhang, B., Trojanowski, J., Lee, V. 2002. Neuronal α-synucleinopathy with severe movement disorder in mice expressing A53T human α-synuclein. *Neuron* 34: 521-533.

Giasson, B., & Lee, V. Parkin and the molecular pathways of Parkinson's disease. 2001. *Neuron* 31: 885-888.

Giasson, B., Uryu, K., Trojanowski, J., Lee, V. 1999. Mutant and wild type human α-synucleins assemble into elongated filaments with distinct morphologies *in vitro. J. Biol. Chem.* 274: 7619-7622.

Gibbs, J., Marshall, M. 1989. The ras oncogene: An important regulatory element in lower eukaryotic organisms. *Microbiol. Rev*. 53:171-185.

Goedbert, M. 2001. α-Synuclein and neurodegenerative diseases. *Neurosci.* 2: 492-501.

Goldberg, M., Lansbury, P. 2000. Is there a cause and effect relationship between α-synuclein fibrillization and Parkinson's disease. *Nat. Cell Biol.* 2: 115-119.

Guerin, M., & Napias, C. 1978. Phosphate transport in yeast mitochondria: Purification and characterization of a mitoribosomal synthesis dependant proteolipid showing a high affinity for phosphate. *Biochemistry* 17: 2510-2516.

Hasegawa, M., Fujiwara, H., Nonaka, T., Wakabayashi, K., Mann, D., Iwatsubo, T. 2002. Phosphorylated α-synuclein is ubiquitinated in α-synucleinopathy lesions. *J. Biol. Chem.* 277: 49071-49076.

Hashimoto, M., Rockenstein, E., Mante, M., Mallory, M., Masliah, E. 2001. β-synuclein inhibits α-synuclein aggregation: a possible role as an anti-parkinsonian factor. *Neuron* 32: 213-223.

Hein, C., Springael, J., Volland, C., Haguenauer, R., Andre, B. 1995. NPII, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the rsp5 ubiquitin-protein ligase. *Mol. Microbiol* 18: 77-87.

Hershko, A. & Ciechanover, R. The ubiquitin system. 1998. *Annu. Rev. Biochem.* 67: 425-479.

Hochstrasser, M. 1996. Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.* 30: 405-409.

Horwich,A., Weber-Ban E., Finley, D. 1999. Chaperone rings in protein folding and degradation. *Proc. Natl. Acad. Sci. USA* 96: 11033-11040.

Imai, Y., Soda, M., Takahashi, R. 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem.* 275: 35661-35664.

Jenko, J., Rawlingson, A., Daniels, B., Morris, A. 1998. Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzyme by α-synuclein. *Biochemistry* 37: 4901-4909.

Jenner, P., Olanow, C. 1996. Oxidative stress and the pathenogenesis of Parkinson's disease. *Neurobiology* 47: 161-170.

Kahle, J., Neumann, M., Ozmen, L., Muller, V., Kretschmar, A., Haass, C. 2001. Selective insolubility of α-synuclein in human Lewy body diseases is recapitulated in a transgenic mouse model. *Am. J. Pathol.* 159: 2215-2225.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Yocochi, M., Mizuno, Y., Shimizu, N. 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605-606.

Kohbata, S. & Shimokawa, K. 1993. Circulating antibody to *Nocardia* in the serum of patients with Parkinson's disease. *Adv. Neurol.* 60: 355-357.

Krobitsch, S. & Lindquist, S. 2000. Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. *Proc. Natl. Acad. Sci. USA.* 97: 1589-1594.

Kunst, C., Mezey, E., Brownstein, M., Patterson, D. 1997. Mutations in SOD1 associated with amyotrophic lateral sclerosis cause novel protein interactions. *Nat. Genet.* 15:91-94.

Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J., Schöls, L., Riess O. 1998. Ala30Pro mutations in the gene encoding α-synuclein in Parkinson's disease. *Nat. Genet.* 18: 106-108.

Langston, J., ballard, P., Tetrud, J., Irwin, I. 1983. Chronic parkinsonism in humans due to a product of meperidine analog synthesis. *Science* 219: 979-980.

Lee, H. & Lee S. 2002. Characterization of cytoplasmic α-synuclein aggregates. Fibril formation is tightly linked to the inclusion-forming process in cells. *J. Biol. Chem.* 277: 48976-48983.

Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezzey, E., Harta, G., Brownstein, M., Gasser, T., Teinbach, P., Wilkinson, K., Polymeropoulos, M. 1998. The ubiquitin pathway in Parkinson's disease. *Nature* 395: 451-452.

Ma, J., Lindquist, S. 1999. *De novo* generation of a PrP^{sc}-like conformation in living cells. *Nat. Cell Biol.* 1: 358-361.

Masliah, E. Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Mucke, L. 2000. Dopaminergic loss and inclusion body formation in α-synuclein mice: implications for neurodegenerative disorders. *Science* 287:1265-1269.

Masliah E., Rockenstein, E., Veinbergs, I., Sagara, Y., Mallory, M., Mucke, L. 2001 b. β-Amyloid peptides enhance α-synuclein accumulation and neuronal deficits in a transgenic mouse model linking Alzheimer's disease

and Parkinson's disease. *Proc. Natl. Acad. Sci. USA.* 98: 12245-12250.

Meriin, A., Zhang, X., he, X., Newman, G., Chernoff, Y., Sherman, M. 2002. Huntington toxicity in yeast model depends on polyglutamine aggregation mediated by a prion-like protein Rnq-1. *J. Cell Biol.* 157: 997-1004.

Morrison, J., Hof, P. 1997. Life and death of neurons in the aging brain. *Science* 278: 412-419.

Muchowski, P., Ning, K., D'Souza, C., Fields, S. 2002. Requirements of an intact microtubule cytoskeleton for aggregation and inclusion body formation by a mutant huntingtin fragment. *Proc. Natl. Acad. Sci. USA* 99: 727-732.

Muchowski, P., Schaffar, G., Sittler, A., Wanker, E., Hayer-Hartl, M., Hartyl, F. 2000. Hsp70 and hsp 40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proc. Natl. Acad. Sci. USA* 97: 7841-7846.

Nishida, C., Grall, E., Valentine, J. 1994. Characterization of three yeast copper-zinc superoxide dismutase mutants analogous to those coded for in familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. USA* 91: 9906-9910.

Okochi, M., Walter, J., Koyama, A., Nakajo, S., Kahle, P., Haass, C. 2000. Constitutive phosphorylation of the Parkinson's disease associated α-synuclein. *J. Biol. Chem.* 275: 390-397.

Olanow, C. and W. Tatton. 1999. Etiology and pathenogenesis of Parkinson's disease. *Ann Rev. Neurosci* 22:123-144.

Parkinson, James. Essay on the Shaking Palsy. London; 1817, pp. 3,6,8 [Reprinted in Birmingham, AL: Classics of Medicine Library; 1986, pp. 3,6,8].

Paushkin, S., Kushnirov, V., Smirnov, V., Ter-Avanesyan, M. 1997. *In vitro* propagation of the prion-like state of yeast Sup35 protein. *Science* 277: 381-383.

Polymeropoulos, M., and 20 others. 1997. Mutations in the α-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045-2047.

Price, D., L., Sisodia, S, Borchelt, S.1998. Genetic neurodegenerative diseases: The human illness and transgenic models. *Science.* 282, 1079-1083.

Seidler A., Hellenbrand W., Robra P., Vieregge P., Nischan P., Joerg J., Oertel WH., Ulm G., Schneider E. 1996. Possible environmental, occupational, and other etiological factors for Parkinson's disease: A case-control study in Germany. *Neurology*. 46: 1275-1284.

Serpell, L., Berriman, J., Jakes, R., Goedert, M., Crowther, A. 2000. Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-conformation. *Proc. Natl. Acad. Sci. USA* 97: 4897-4902.

Sharon, R., Goldberg, M., Bar, I., Betensky, R., Shen, J., Selkoe, D. 2001. α-Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty-acid binding proteins. *Proc. Natl. Acad. Sci. USA* 98: 9110-9115.

Sherman, Michael Y., and A. Goldberg. 2001. Cellular defenses against unfolded proteins: A cell biologist thinks about neurodegenerative diseases. *Neuron* 29: 15-32.

Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., Suzuki, T. 2000. Familial Parkinson's disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet*. 25: 302-305.

Shimura, H., Schlossmacher, M., Hattori, N., Frosch, M., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, F., Selkoe, D. 2001. Ubiquitination of a new form of α-synuclein by parkin from human brain: Implications for Parkinson's disease. *Science* 293: 263-269.

Sherer, T., Betarbet, R., Greenamyre, J. 2001. Pathenogensis of Parkinson's disease. *Curr. Opin. Investig. Drugs* 2: 657-662.

Shoulson, I. 1998. Experimental therapeutics of neurodegenerative disorders: Unmet needs. *Science* 282, 1072-1074.

Singer, T., Castagnoli, N., Ramsay, R., Trevor, A. 1987. Biochemical events in the development of parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J. Neurochem.* 49: 1-8.

Spillantini, M., Schmidt, M., Lee, V., Trojanowski, J., Jakes, R., Goedbert M. 1998. α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 95: 6469-6473.

Stoessl, A. 1999. The etiology of Parkinson's disease. *Can. J. Neurol. Sci.* 26: S5-12.

Takahashi M., & Yamada T. 1999. Viral etiology for Parkinson's disease: a possible role of influenza A virus infection. *Jpn. J. Infect. Dis.* 52: 89-98.

Taylor, P., Hardy, J., Fischbeck, K. 2002. Toxic proteins in neurodegenerative diseases. *Science.* 296:1991-1995.

Trojanowski, J., Goedbert, M., Iwatsubo, T., Lee, V. 1998. Fatal attractions: Abnormal protein aggregation and neuron death in Parkinson's disease. *Cell Death. Differ.* 5: 832-837.

Uversky, V., Lee, H., Li, J., Fink, A., Lee, S. 2001. Stabilization of partially folded conformation during α-synuclein oligomerization in both purified and cytosolic preparations. *J. Biol. Chem.* 276: 43495-43498.

Van der Putten, H., and 13 others. 2000. Neuropathology in mice expressing α-synuclein. *J. Neurosci.* 20: 6021-6029.

.