Eukaryon, Vol. 1, 102-108, January 2005, Lake Forest College

α-Synuclein Toxicity and Localization in Yeast that Lack Superoxide Dismutases

Katrina Brandis* and Ruja Shrestha
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Summary

Parkinson's disease is a neurodegenerative disease that is caused by the death of dopaminergic neurons in the substantia nigra. α-Synuclein aggregation and oxidative stress have each been linked to the incidence of this neuronal loss, but the mechanisms linking these events together remain unclear. This study focused on the effects of α-synuclein expression in yeast strains lacking Cu,Zn-SOD (*sod1*Δ) and Mn-SOD (*sod2*Δ), enzymes that metabolize oxygen radicals. The *sod2*Δ strain expressing α-synuclein was less viable than the parent strain, while *sod1*Δ showed minor toxicity compared to parent strain. To examine *sod2*Δ toxicity further, we used live cell fluorescent microscopy to detect GFP-tagged α-synuclein localization in *sod2*Δ; we found no change in α-synuclein distribution compared to the parent strain. Treatments of the parent strain with 2mM H_2O_2 showed no change in viability or α-synuclein localization. *Sod2*Δ strains expressing α-synuclein were less viable when treated with H_2O_2 compared to untreated cells. The increased toxicity in untreated *sod2*Δ may be caused by increased levels of damaging oxygen radicals due to lack of Mn-SOD activity; increased toxicity due to H_2O_2 treatment may be caused by even higher levels of oxygen radicals.

Introduction

Parkinson's Disease (PD) is a neurodegenerative disease characterized by resting tremors, muscle rigidity and loss of movement. These symptoms are caused by the death of dopaminergic neurons in the substantia nigra pars compacta (SNpc). These dying neurons contain aggregates of the α-synuclein protein, called Lewy bodies (LBs). LBs are not proven to directly kill SNpc neurons, but have only been linked to the incidence of neuronal death. This has been shown in mouse (Kirik, 2002), *Drosophila* (Feany, M & W Bender, 2000), and *C. elegans* (Lakso et al., 2003) models. In fact, recent studies have shown the aggregation of α-synuclein may be a protective mechanism against neuronal death (Tanaka et al., 2004).

Genetic PD cases are caused[*] by familial missense mutations in the α-synuclein (Polymeropoulos et al., 1997, Kruger et al., 1998, Zarranz et al., 2004), DJ-1 (Bonifati et al., 2002), parkin (Kitada et al., 1998), PINK1 (Valente et al., 2004) and PARK8 (Funayama et al., 2002) genes. α-Synuclein mutations occur either at the 53rd codon (from alanine to threonine; A53T) (Polymeropoulos et al., 1997) or at the 30th codon (from alanine to proline; A30P) (Kruger et al., 1998). These mutations lead to the misfolding of α-synuclein, whereas the more common sporadic form of PD results in the random misfolding of α-synuclein. A third missense mutation in α-synuclein, the E46K mutation (Zarranz et al., 2004), also leads to misfolding, and results in a form of dementia with Lewy Bodies. Sporadic PD is also linked to α-synuclein misfolding, but the mechanism by which this random misfolding occurs is unknown.

α-Synuclein is a presynaptic protein (Jakes et al., 1994) whose specific function is not yet known. The N-terminal region of the protein is known to bind lipid vesicles (Eliezer et al., 2001), and has increased α-helical structure when bound to phospholipids (Davidson, et al., 1998). Most recently, however, wild type α-synuclein has been shown to induce the polymerization of tubulin into microtubules, while mutant α-synuclein does not show the same capability (Alim et al., 2004). Other findings suggest α-synuclein to have a chaperone-like function since it has been shown to bind many proteins such as phospholipase D2 (Jenco et al., 1998) and synphilin-1 (Engelender et al, 1999), and because it has functional homology with the 14-3-3 cytoplasmic chaperone protein family (Ostrerova et al., 1999). The function of α-synuclein is still obscure due to its apparent involvement in many cellular roles.

Oxidative stress has been implicated as one of the many factors that cause sporadic PD. The death of dopaminergic neurons has been shown to increase when complex I of the mitochondria is deficient (Kweon et al., 2004), and this deficiency has been observed in PD (Mizuno et al., 1989). Moreover, the inhibition of complex I leads to more ROS, which can damage the electron-transport chain and lead to further production of ROS (Dauer, W & S Przedborski S). A connection between α-synuclein misfolding and oxidative stress has yet to be fully established, although recently, certain proteins have been shown to form a complex with aggregated α-synuclein *in vivo* in the presence of rotenone, an oxidative and PD inducing agent (Zhou et al., 2004). In addition, a yeast genetic screen of *S. Cerevisiae* has identified a number of proteins that when knocked out, increase the toxicity of α-synuclein to yeast cells (Willingham et al, 2003). One of these proteins, manganese-superoxide dismutase (Mn-SOD) is embedded in the inner membrane space of the mitochondria (Slot et al, 1986) and reduces the level of superoxide radical formed during mitochondrial respiration. Interestingly, copper,zinc-SOD, a cytosolic SOD, does not have an effect on α-synuclein toxicity in the yeast genetic screen (Willingham et al, 2003). This study further examines the effect of Cu,Zn-SOD and Mn-SOD on α-synuclein toxicity.

A recently published model of α-synuclein expression in yeast (Outeiro and Lindquist, 2003) showed that α-synuclein localized to the plasma membrane, formed intracellular inclusions, inhibited phospholipase D, increased lipid droplet formation, and influenced vesicle trafficking in wild type cells. We have also established a similar model (Johnson, 2003; Sharma, 2004) which showed that wild-type (WT) and mutant forms of α-synuclein do not cause toxicity to yeast cells. Furthermore, GFP-tagged WT, A53T, and double mutant (DB) α-synuclein localize to the membrane, while A30P fluoresces diffusely in the cytoplasm. WT, A53T and DB α-synuclein also showed formation of foci in the cytoplasm. Cells treated for a short term with hydrogen peroxide, an oxidizing agent, showed a redistribution of α-synuclein to the vacuole, while a long-term treatment showed α-synuclein exclusion from the vacuole in the form

* This paper was written based on original scholarship conducted in BIO 324 Advanced Cell Biology, by Dr. Shubik DebBurman.

of foci. In this study, α-synuclein toxicity, localization, and expression were evaluated in yeast knockouts of Cu,Zn-SOD (*sod*1Δ) and Mn-SOD (*sod*2Δ).

Results

α-Synuclein expression in parent *sod1*Δ and *sod2*Δ strains

Western analysis was conducted with lysates made from *sod1*Δ and *sod2*Δ and the isogenic wild type parent strain each expressing the four forms of α-Synuclein in order to determine the relative levels of α-Synuclein expression among the strains. Expression was induced for 24 hours, and lysates were run on two gels, one for Coomassie staining (Figure 1A) and one for Western transfer and blotting (Figure 1B). The Coomassie stain showed that equal amounts of protein were loaded into each lane. The transferred membrane was blotted with Anti-V5 AP 1° Antibody to detect the V5 epitope that is tagged to the C-terminus of the α-synuclein protein. The α-synuclein band was visualized directly below the 64 kDa marker in the ladder, because α-Synuclein +GFP has a molecular weight of 62 kDa. Expression of α-synuclein in the parent strain was similar among the WT and mutant forms. This has been previously shown in other wild-type strains of yeast (Sharma, 2004). *Sod1*Δ showed similar levels of WT, A53T, and DB α-synuclein expression (Figure 1B, lanes 5-8), but A30P appeared to have no detectable expression. In sod2Δ, WT and mutant forms of α-synuclein had similar levels of expression.

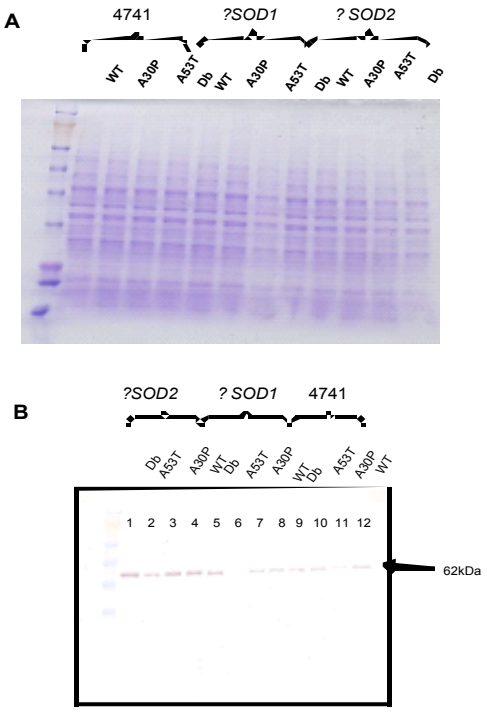


Figure 1. Expression of α-synuclein in parent, *sod1*Δ, and *sod2*Δ strains
Protein was expressed for 24 hours in galactose media. A) Coomassie control staining of all protein in lysates. Lanes are as designated by labels (reversed from lanes in the blot). B) Western blot of α-synuclein protein was detected with anti V5 AP 1° antibody. α-synuclein-GFP fusion protein migrateed to 62kDA. Lanes 1-4 are from *sod2*Δ , lanes 5-8 are from *sod1*Δ, and lanes 9-12 are from the parent strain (4741). Lanes 1,5,9 = DB α-synuclein. Lanes 2,6,10=A53T. Lanes 3,7,11=A30P. Lanes 4,8,12=WT.

α-Synuclein more toxic to yeast without SODs

An optical density assay was conducted to establish growth curves of strains expressing α-synuclein. Whether the parent strain expressed α-synuclein, GFP or PP, growth rates were the same (Figure 2A), indicating that α-synuclein is not toxic to the parent strain. The *sod*1Δ strain had similar growth rates except for when A30P α-synuclein was expressed, but showed a slight lag in growth compared to other forms of α-synuclein and GFP and PP controls (Figure 2B). Conversely, *sod*2Δ cells expressing α-synuclein show decreased rates of growth compared to GFP and PP controls (Figure 2C). Moreover, *sod*2Δ cells expressing A30P, A53T, and A30P/A53T α-synuclein show decreased rates of growth compared to cells expressing WT α-synuclein. The glucose controls (4741 GLU, *sod1* GLU, and *sod2* GLU) were cells transformed with WT _-synuclein, but grown in glucose (no α-synuclein expression). This control shows a higher rate of growth, which is due to the difference in which yeast cells metabolize glucose as a sugar source compared to galactose, and not due lack of α-synuclein, GFP, or PP expression.

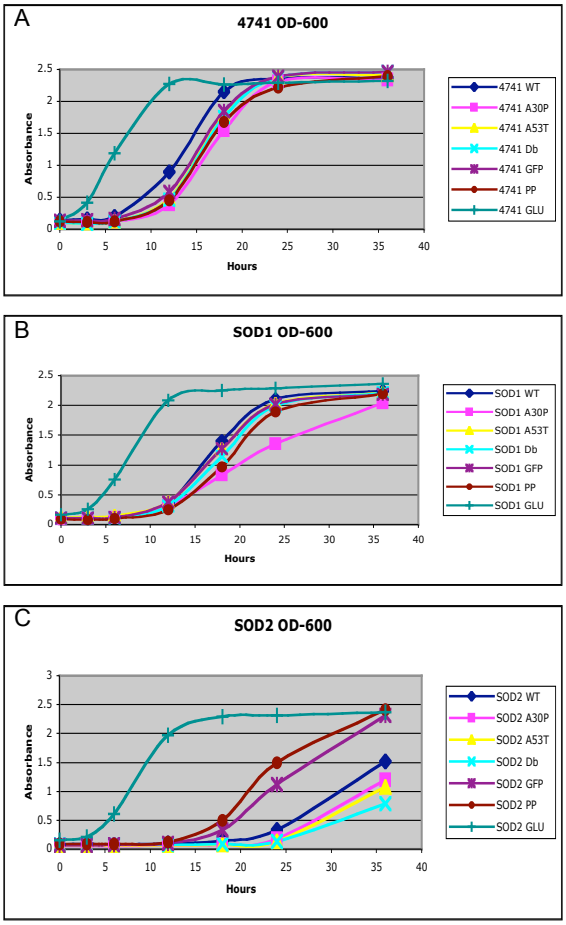


Figure 3. Cell growth curves using optical density.
(A) *Sod2*Δ cells expressing WT α-synuclein showed decreased growth rate compared to parent (4741) and *sod1*Δ strains. (B) *sod1*Δ and *sod2*Δ cells expressing A30P showed decreased growth rates compared to the parent strain expressing A30P. (C) *sod2*Δ cells expressing A53T showed decreased growth rate compared to *sod1*Δ and parent strains expressing A53T. (D) *sod2*Δ cells expressing Db showed decreased growth rate compared to *sod1*Δ and parent strains expressing Db. E,F,G) Parent, *sod1*Δ and *sod2*Δ strains expressing GFP, PP, or with no expression (grown in glucose) show similar growth rates.

In comparing the expression of WT α-synuclein among all three strains, *sod*2Δ cells showed distinct toxicity compared to the parent strain and *sod*1Δ cells (Figure 3A). This same pattern was also shown in *sod*2Δ cells expressing A53T (Figure 3C) and Db α-synuclein (Figure 3D). *Sod*2Δ

cells expressing GFP (Figure 3E) or PP (Figure 3F), or cells with no expression in glucose (Figure 3G) did not show the lag in growth observed with α-synuclein expression, which indicates that it is α-synuclein that caused toxicity in *sod*2Δ. *Sod*1Δ and *sod*2Δ cells expressing A30P α-synuclein showed decreased growth rates compared to the parent strain (Figure 3B), with *sod*2Δ showing more pronounced toxicity. *Sod*1Δ cells expressing other forms of α-synuclein showed *slight* lag in growth compared to the parent strain as well. Due to the marked difference in toxicity in *sod*2Δ cells, *sod*2Δ was further examined in other assays. In order to observe cell viability in a different assay, spotting was conducted. Cells were grown in raffinose, equalized, diluted by 5-fold, and were spotted onto repressing (glucose) and inducing (galactose) media. The plates were incubated for 3 days. The glucose control plates showed that cell density within each dilution was equal in that each column showed similar cell growth (Figure 4). The galactose plates showed that *sod*2Δ cells expressing α-synuclein had less growth than the parent strain expressing α-synuclein, which is consistent with the toxicity observed in the optical density assay. This was most clearly observed in the 5th lane (marked *). *Sod*2Δ cells expressing GFP or PP showed similar growth to the parent strain expressing GFP or PP, which was also consistent with the growth rates observed with the GFP and PP controls in the optical density assay.

α-Synuclein localization unchanged in the absence of SOD

Due to the toxicity of α-synuclein in *sod*2Δ cells, we conducted live cell fluorescent microscopy to determine if α-synuclein is localized differently in these dying cells compared to the parent strain or *sod1*Δ. α-Synuclein was expressed for 24 hours in galactose media. In the parent strain and *sod1*Δ, WT and A53T α-synuclein localized to the plasma membrane, while A30P fluoresced diffusely in the cytoplasm in the parent strain (Figure 5). Db α- synuclein showed a mixed phenotype of halos and diffuse fluorescence in the parent strain. A30P and Db α- synuclein are also shown to be excluded from the vacuole, which is the dark circle seen in some of the cells. These findings are consistent with previous α-synuclein localization

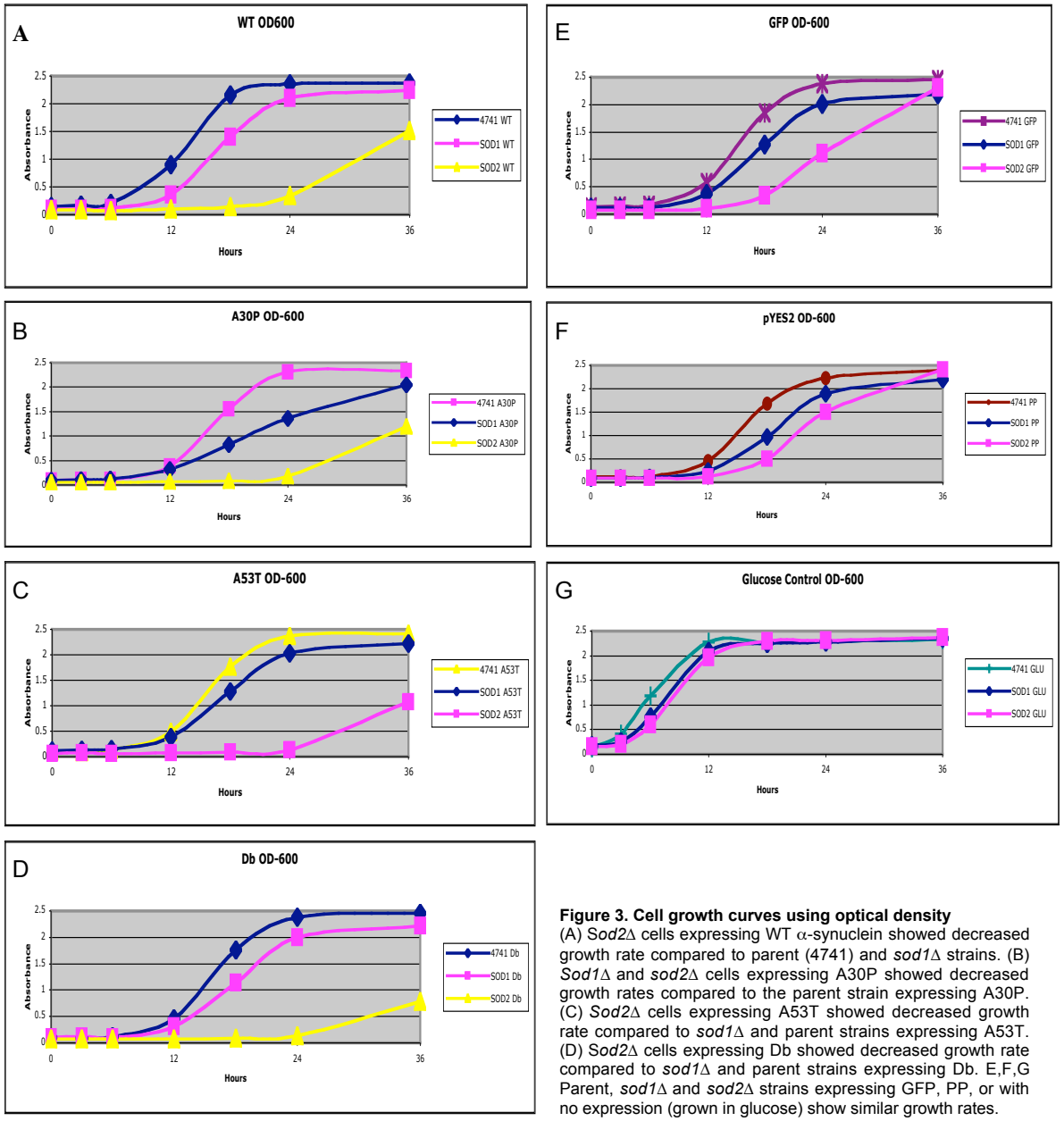


Figure 3. Cell growth curves using optical density
(A) *Sod*2Δ cells expressing WT α-synuclein showed decreased growth rate compared to parent (4741) and *sod1*Δ strains. (B) *Sod1*Δ and *sod*2Δ cells expressing A30P showed decreased growth rates compared to the parent strain expressing A30P. (C) *Sod*2Δ cells expressing A53T showed decreased growth rate compared to *sod1*Δ and parent strains expressing A53T. (D) *Sod*2Δ cells expressing Db showed decreased growth rate compared to *sod1*Δ and parent strains expressing Db. E,F,G Parent, *sod1*Δ and *sod*2Δ strains expressing GFP, PP, or with no expression (grown in glucose) show similar growth rates.

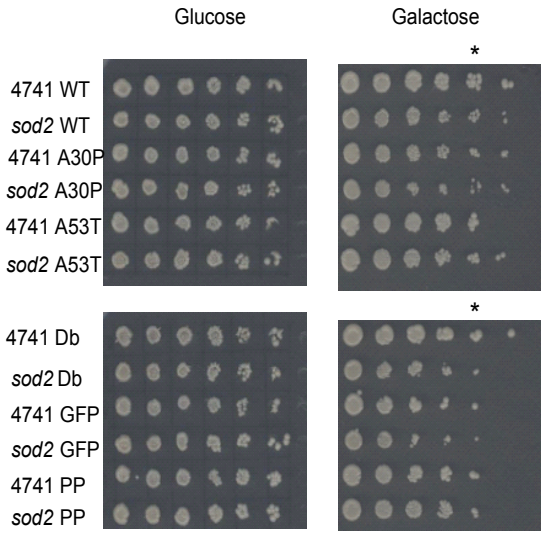


Figure 4. Growth analysis using spotting assay
Parent strain (4741) and *sod2*Δ cells were spotted on non-inducing (glucose) and inducing (galactose) media. Cells were grown for 3 days. *Sod2*Δ cells expressing all four types of α-synuclein showed decreased growth, best seen in lane 5 (denoted with asterisks) compared to the parent strain expressing α-synuclein.

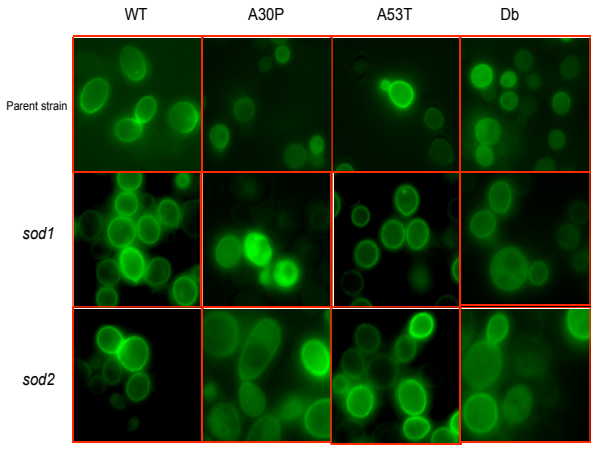


Figure 5.GFP microscopy of parent, *sod1*Δ and *sod2*Δ strains
Cells were grown in galactose media for 24 hours to induce α-synuclein expression. WT and A53T α-synuclein localized to the membrane in parent, *sod1*Δ and *sod2*Δ strains. In some cells, WT and A53T formed foci in all three strains. A30P and Db α-synuclein fluoresced diffusely in the cytoplasm with exclusion from the vacuole in the parent strain, *sod1*Δ and *sod2*Δ. A30P localization in *sod1*Δ was not as cytoplasmically diffuse as in parent and *sod2*Δ strains.

observed with other wild types strains in our lab (Sharma, 2004). Surprisingly, *sod2*Δ cells show similar α-synuclein localization as with the parent strain. WT, A53T, and Db α-synuclein are similarly localized to the plasma membrane, while diffuse fluorescence and exclusion from the vacuole is observed with A30P and A30P/A53T α-synuclein. This was unexpected in that *sod2*Δ shows α-synuclein toxicity but no change in α-synuclein localization.

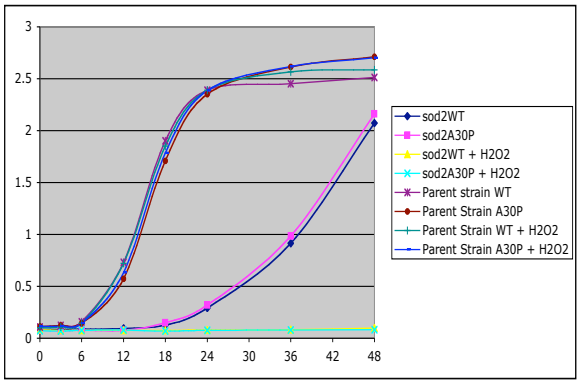
H_2O_2 does not affect yeast growth or α-synuclein localization when SOD is present

In order to determine if increased levels of oxidants cause toxicity or changes in α-synuclein localization, cells were treated with H_2O_2, and oxidizing agent. The parent strain expressing WT or A30P were grown in galactose with or without 2mM H_2O_2, and growth curves were established (figure 6A). H_2O_2 treatments did not have an effect on the growth rate of the parent strain expressing WT or A30P α-synuclein. The parent strain was then treated with 2mM H_2O_2 for a short-term (30 min) or long-term treatment (18hr), and cells were visualized by fluorescent microscopy to detect any changes in α-synuclein localization. The parent strain expressing WT α-synuclein did not show any changes in α-synuclein localization with any treatment of H_2O_2 (Figure 6B); the halo phenotype was conserved. The parent strain expressing A30P α-synuclein showed no changes in α-synuclein localization as well; diffuse cytoplasmic fluorescence and exclusion from the vacuole was conserved.

H_2O_2 lethal to yeast that express α-synuclein but lack SOD2

When *sod2*Δ expressing WT or A30P α-synuclein was treated with *H_2O_2*, *sod2*Δ cells exhibited prolonged toxicity compared to untreated cells. While untreated *sod2*Δ recovered after 24 hours of growth, cell density of treated cells stayed at baseline levels. This was expected in that *sod2*Δ cells are presumably oxidatively stressed with the lack of Mn-SOD. Because the cell density of treated *sod2*Δ did not increase, there were not enough cells to conduct GFP microscopy.

A



B

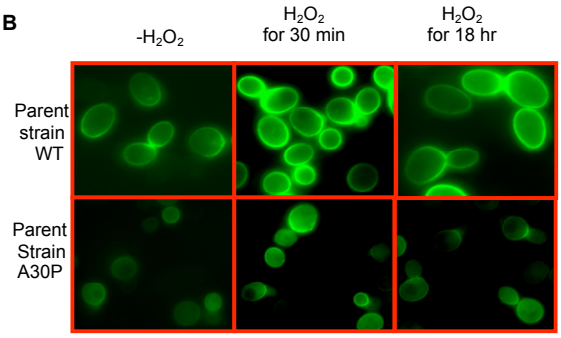


Figure 6.
A) Growth curves of cells treated with H_2O_2. Cells were grown in galactose or galactose containing 2mM H_2O_2 for 48 hours. The parent strain exhibited the same growth with or without H_2O_2.treatment. Treated sod2Δ exhibited no growth compared to untreated sod2Δ. At 24 hours of expression, sod2Δ began to recover growth, and almost reached saturation at 48 hours, while treated sod2Δ never recovered. B) GFP microscopy of the parent strain treated with H_2O_2. Cells were either induced in galactose for 18 hours and treated with 2mM H_2O_2 for 30 minutes, or were induced in galactose containing 2mM H_2O_2 for 18 hours.. The parent strain cells expressing WT α-synuclein showed membrane localization in the absence or presence of any treatment. Cells expressing A30P α-synuclein showed diffuse cytoplasmic fluorescence with exclusion from the vacuole in the absence or presence of any treatment.

Discussion

SOD2 is required to withstand α-Synuclein expression in yeast

The optical density and spotting results shows that when the *sod2*Δ strain expresses any form of α-synuclein, it is less viable than the parent strain expressing α-synuclein. This was expected because *sod2*Δ was shown to be less viable when expressing WT α-synuclein in the yeast genetic screen (Willingham et al., 2003). The dysfunction or lack of Mn-SOD is widely known to increase the level of oxidants in a cell due to its inability to handle superoxide that is generated during oxidative phosphorylation. In speculating as to how the lack of Mn-SOD increases α-synuclein toxicity, there are two apparent scenarios (see figure 7 for proposed pathways to toxicity). First, these cells have certain cellular mechanisms or machinery, such as the proteasome or the vacuole, which handle the presence of rogue proteins such as α-synuclein. The lack of Mn-SOD increases oxidants in the cell, which may damage or affect the activity of the proteasome or vacuole, which can leave α-synuclein to its own devices, perhaps causing lethality. It has been shown that exposure of SH-SY5Y neuroblastoma cells to oxidants such as H_2O_2, paraquat, or $FeSO_4$ decreases proteasomal activity (Ding and Keller, 2001). If the increased levels of oxidants are damaging the proteasome, α-synuclein may not be degraded as quickly, which may lead to its toxic effects, whereas in the parent strain, α-synuclein is not toxic due to its normal rate of degradation.

Conversely, α-synuclein may be producing more oxidants itself. A study has shown that in human particularly the mutant forms, increases the level of oxygen radicals in neuroblastoma cells, the overexpression of α-synuclein, the cell (Junn E and MM Mouradian, 2002). Our results show greater toxicity in *sod2*Δ with expression of mutant α-synuclein than with the WT form. It is possible that in the parent strain, α-synuclein is somehow increasing the level oxygen radicals but is not causing any change in cell viability because Mn-SOD is managing oxidative stress. Because *sod2*Δ cells are already lack Mn-SOD, the addition of α-synuclein, especially mutant forms, may increase oxygen radicals and cause damage that leads to toxic effects. It is particularly surprising that lack of Cu,Zn-SOD does not result in toxicity in that it is widely known to be a cytoplasmic SOD. This poses many questions as to how α-synuclein either increases ROS that only Mn-SOD are able to regulate, or how the lack of Mn-SOD in particular increases cellular damage that enhances α-synuclein toxicity.

Cells that survive SOD toxicity surprisingly preserve a-synuclein membrane localization

Surprisingly, GFP microscopy results show that at 24 hours of expression, when toxicity is occurring, there is no difference between α-synuclein localization the *sod2*Δ and parent strains. We had expected to see a difference after seeing results showing toxicity because a change in α-synuclein localization could have been the driving force for toxicity. These results indicate that the absence of Mn-SOD increases α-synuclein toxicity without altering α-synuclein localization. This is particularly puzzling in that Mn-SOD is found in the mitochondrial matrix while α-synuclein is a cytoplasmic and membrane associated protein. It is also interesting to note that α-synuclein localization in *sod2*Δ is similar to that in *sod1*Δ, which shows slight toxicity compared to the parent strain.

A less apparent difference among α-synuclein localization in *sod1*Δ, *sod2*Δ and the parent strain may exist in that the protein may be aggregating to greater or lesser extents in each a particular cell type. Quantification of phenotypes could be conducted, particularly for the number of cells showing aggregates and the number of aggregates within each cell. It is possible that *sod2*Δ may have a significantly different amount of aggregation than *sod1*Δ or the parent strain, which may explain further the differences in viability.

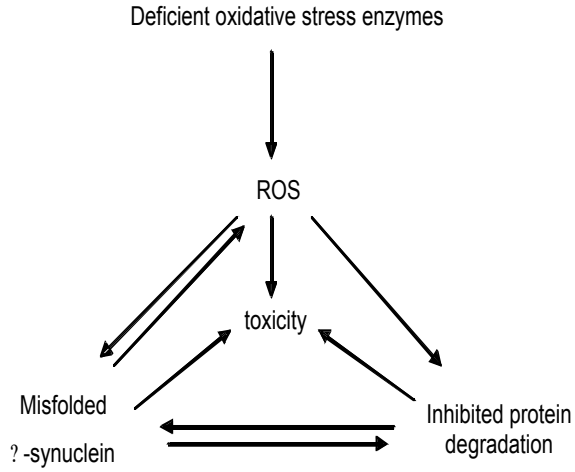


Figure 7. Hypothesized factors leading to toxicity
This model proposes that oxidative stress could affect the homeostasis of protein degradation and α-synuclein misfolding, or that α-synuclein could affect the levels of reactive oxygen species and/or protein degradation. All three occurrences could lead to the toxicity observed in this study.

Oxidative damage & α-synuclein expression: Lethal Combination?

When the parent strain was treated with 2mM H_2O_2 for 48 hours, there was no difference in cell viability compared to untreated cells whether WT or A30P was expressed. This indicates that the parent strain is equipped with sufficient antioxidants to handle this degree of oxidative stress. When the culture that somehow overcome the damaging effects of the absence of Mn-SOD and the resulting level of oxidants that kill cells otherwise. The results of this study can only indicate that *sod2*Δ is mostly not equipped to handle this kind of oxidative stress when Mn-SOD is absent. Because a control without α-synuclein expression was not treated with H_2O_2 in the same manner, it *cannot* be assumed that α-synuclein is causing this greater toxicity. 2mM H_2O_2 may be enough stress to kill *sod2*Δ even without α-synuclein present. Whether the parent strain was treated for a short term (for 30 min after 18 hours of induction) or for a long term (18 hours from start of induction) with H_2O_2, α-synuclein localization was not altered. This was, again, a surprising result in that our lab has previously shown no toxicity with H_2O_2 treatment while WT α-synuclein relocalizes inside the vacuole with a short treatment and is excluded from the vacuole and forms aggregates with a long-term treatment. This may be due to a difference in the wild-type strains used in the separate experiments. Because *sod2*Δ treated with H_2O_2 shows complete lethality, there were no cells to evaluate with fluorescent microscopy to determine α-synuclein localization.

Conclusion

Our study has examined more in depth the toxicity observed with WT α-synuclein expression in *sod2*Δ first shown in the

yeast genetic screen (Outeiro et al, 2003) and not only found the same results, but found increased toxicity with mutant α-synuclein. These results lead to further inquiries of how Mn-SOD normally functions in the cell in a way that does not allow _-synuclein to be toxic. Identification of other key players, such as the proteasome or molecular chaperones, in this pathway leading to α-synuclein regulation will elucidate the relationship between Mn-SOD and α-synuclein, and may also shed light on the implications of oxidative stress and α-synuclein misfolding in PD. Further examination of the minor toxicity observed in *sod1*Δ may also aid in this discovery process, as could the examination of α-synuclein expression in knockouts of other oxidative stress enzymes such as catalase and glutathione peroxidase. A recent study has shown that polymorphisms in sod2 are associated with an increased risk of PD (Hattori, 2004), which supports the link between oxidative stress and the incidence of PD.

Experimental Procedures

Strains

Yeast strains were obtained from Invitrogen. The *S. Cerevisiae* parent strain used was YSC1048 (4741). Knockout strains were deleted for sod1, or Cu,Zn-SOD (strain YJR104C) and for sod2, or Mn-SOD (strain YHR008C).

Table 1. Transformed Yeast Strains Used in This Study

Constructs transformed in this study	Transformed strain
Wt ? -synuclein-GFP (CT)	*sod1*Δ
A30P ? -synuclein-GFP (CT)	"
A53T ? -synuclein-GFP (CT)	"
A30P/A53T ? -synuclein-GFP (CT)	"
GFP	"
Wt ? -synuclein-GFP (CT)	*sod2*Δ
A30P ? -synuclein-GFP (CT)	"
A53T ? -synuclein-GFP (CT)	"
A30P/A53T ? -synuclein-GFP (CT)	"
GFP	"
Constructs previously transformed	
Wt ? -synuclein-GFP (CT)	4741 wild type isogenic parent strain
A30P ? -synuclein-GFP (CT)	"
A53T ? -synuclein-GFP (CT)	"
A30P/A53T ? -synuclein-GFP (CT)	"
GFP	"

Transformation

(See Table 1 for construct, vector, and strain information) *S. Cerevisiae sod1* and *sod*2 knockout strains were grown in liquid YEPD media overnight at 30°C, 200rpm. Cell density was calculated, and 50ml YEPD was inoculated to a cell density of 5 x 10^6cells/ml and grown for 3-5 hours at 30°C, 200 rpm. Cells were washed with H_2O, then with .5ml 100mM lithium acetate (LiAc), and were finally re-suspended in 200ul 100mM LiAc. 25ul of this cell suspension was pelleted, and LiAc was removed. The following transformation mix was added to the pellet: 240ul PEG (50% w/v), 36ul 1.0M LiAc, 25ul of boiled single-stranded carrier DNA (2.0mg/ml), 5μl plasmid, and 45μl water. Six different plasmids were transformed into *S. Cerevisiae*, four containing a form of α-synuclein, one with GFP, and one closed vector (parent plasmid). Wild-type, A30P, A53T, and A30P/A53T (A30P/A53T) α-synuclein and GFP cDNAs were previously subcloned into the pYES2.1 TOPO vector with a galactose inducible promoter (see table 1 for construct, vector, and strain information). Each α-synuclein cDNA was tagged with GFP cDNA in the C-terminus. 5μl of each vector were each used in the transformation mix described above. The pellet was re-suspended in the transformation mix, was incubated for 30 min at 30°C, and was then shocked at 42°C for 25 min. Cells were re-pelleted, the transformation mix was removed, and cells were re-suspended in 1ml H_2O. 10μl or 100μl cell suspension was then plated onto SC-URA Glucose selection media, or 100μl was plated onto YEPD media (for positive control). Colonies were screened with whole cell yeast PCR for conformation of transformation. (Forward Primer: Positive colonies were grown in SC-URA Glucose overnight and were stored at -80°C.

Lysates

*Sod*1 and *sod*2 knockout strains transformed with α-synuclein, GFP, or parent plasmid were grown overnight in 10ml SC-URA glucose. Cells were washed twice with H_2O and were re-suspended in 2mL H_2O. Protein expression was induced by inoculating 25ml of SC-URA Galactose with 1 mL of this cell suspension. Cultures were incubated for 48 hours at 30°C, 200rpm. Cell density of each culture was determined by counting, and 7.5 x 10^7 cells were removed from each culture. Cells were washed twice with 1 ml 50 mM Tris, 10 mM NaN_3 and were re-suspended in 300μl electrophoresis sample buffer (ESB: 2% SDS, 80mM Tris (pH 6.8), 10% glycerol, 1.5% DTT, 1 mg/mL bromophenol blue, and various protease inhibitors and solublizing agents (1% Trixton-X 100, lmM phenylmethylsulfonyl fluoride (PMSF), 1mM benzamide, 1mM sodium orthovanadate, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 10μg/ml E64, 2μg/ml aprotinin, and 2μg/ml chymostatin). 0.3 g of .1mm glass beads was added to each sample. Each sample was vortexed for 3 minutes, and was then boiled for 3 minutes.

Western blotting

15μl boiled cell lysates were loaded onto 10-20% Tris-Glycine SDS gels and were electrophoresed in 1x Tris-Glycine SDS Running Buffer at 130 volts. Gels were transferred onto polyvinylidene difluoride (PVDF) membranes in 1x transfer buffer [Tris Base, Glycine, to 500ml DIH_2O, pH 8.3]. PVDF membranes were pre-soaked in methanol, H_2O, and then in1x transfer buffer. Protein was transferred for 2 hours at 30 volts. After transfer, membranes were soaked in 15ml methanol for 5 minutes, and were washed twice with H_20 for 5 minutes. Membranes were then washed with 10 ml Blocking Buffer (2% casein; TWEEN 20 - 0.05%; 1x TBS) for 30 minutes, and then twice with H_20 for 5 minutes. 10 ml Anti–V5 AP 1° Antibody was then added to the membrane for 2-4 hours (5μl of stock Antibody added to 10ml of Blocking Buffer). Antibody was removed and saved, and then membranes were washed four times with Antibody Wash (40ml of 10x TBS Buffer, 200μL of Polyoxyethylene Sorbitan Monolaurate (TWEEN), and 360mL of Sterile Water for a total of 400mL) for 5 minutes each. Membranes were washed with H_20 3 times for 2 minutes, and then color development solution was added (0.7ml of DMF and 30mg of NBT in 0.3 ml of H_20, and 15mg of BCIP in 1mL of DMF, both added to 100mL of Tris Buffer, light sensitive solution). Bands on the membrane were allowed to develop to desired intensity. After two more 5 minute H_20 washes, membranes were dried in soft tissue paper.

Toxicity analysis

Growth curve: Transformed knockouts and 4741 parent strain were grown in 10ml SC-URA glucose overnight at 30°C, 200 rpm. Cells were harvested at 1500 x g for 5min at 4°C, and were washed twice in 5ml H_20. Cells were re-suspended in 5 ml H_20 and were counted. Flasks with 25ml SC-URA galactose were each inoculated to 2.0x10^6cells/mL density. At 0, 3, 6, 12, 18, 24, and 36 hours, 1ml of cell culture was removed and placed in a cuvet to measure absorbance using Hitachi U-2000 Spectrophotometer. Absorbance reading was plotted against time points to produce a growth curve.

Spotting: Transformed knockouts and 4741 parent strain were grown in 10ml SC-URA glucose vernight at 30°C, 200 rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice in 5ml H_20. Cells were re-suspended in 2ml H_20 and 400 μl of cell suspension was added to 10ml SC-URA raffinose. Cultures were incubated for 3-5 hours at 30°C, 200rpm. Cell density of each culture was then determined by counting, and 2.0x107 cells were removed. Enough media was added to make a final volume of 1 ml cell culture. 100μl of this culture was added to the first lane of a microtiter plate. The next 5 lanes contained 80μl H_20. 20μl of the 100μl of culture was removed from the first lane and added to the second and was mixed. 20μl of this mixture was removed and added to the third lane, and so on, until there were 5 5-fold serial dilutions for each cell culture. These cells were plated either by using a frogger or by pipeting 2μl of each dilution onto SC-URA glucose or SC-URA galactose media plates. Plates were incubated at 30°C until desired cell growth was obtained.

GFP Microscopy

Cells were grown overnight in 10ml SC-URA glucose at 30°C at 200rpm. Protein expression was induced with SC-URA galactose media as previously described. After a desired amount of induction, cells were harvested at 1500 x g at 4°C for 5 minutes and were washed twice with 5 ml H_20. Cells were re-suspended in 100-1000μl SC-URA glucose, and 10μl cell suspension was pipetted onto a slide and visualized under a Nikon fluorescent microscope to determine α-synuclein localization.

Acknowledgements

This study was made possible by Dr. Shubhik DebBurman, and other lab members, Sara Herrera, Jessica Price, Arun Paul, Lavinia Sinitean, and Tasneen Saylawala. This work was supported by a grant from the National Science Foundation and National Institutes of Health.

References

Alim MA, Ma QL, Takeda K, Aizawa T, Matsubara M, Nakamura M, Asada A, Saito T, Kaji H, Yoshii M, Hisanaga S, Ueda K. Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein. J Alzheimers Dis. 2004 Aug;6(4):435-42; discussion 443-9.

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science*. 2003 Jan 10;299(5604):256-9. Epub 2002 Nov 21.

Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron*. 2003 Sep 11;39(6):889-909.

Davidson WS, Jonas A, Clayton DF, George JM.Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *Journal of Biological Chemistry*. 1998 Apr 17;273(16):9443-9.

Ding Q, Keller JN. Proteasome inhibition in oxidative stress neurotoxicity: implications for heat shock proteins. *Journal of Neurochemistry*. 2001 May;77(4):1010-7.

Eliezer David, Esin Kutluay, Robert Bussell, Jr and Gillian Browne. Conformational properties of α-synuclein in its free and lipid-associated states. *Journal of Molecular Biology*. Volume 307, Issue 4 , 6 April 2001, Pages 1061-1073.

Engelender S, Kaminsky Z, Guo X, Sharp AH, Amaravi RK, Kleiderlein JJ, Margolis RL, Troncoso JC, Lanahan AA, Worley PF, Dawson VL, Dawson TM, Ross CA. Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions. *Nature Genetics*. 1999 May;22(1):110-4.

Feany MB, Bender WW. A Drosophila model of Parkinson's disease. *Nature*. 2000 Mar 23;404(6776):394-8.

Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F.A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. *Annals of Neurology*. 2002 Mar;51(3):296-301.

Hattori N. [Etiology and pathogenesis of Parkinson's disease: from mitochondrial dysfunctions to familial Parkinson's disease] *Rinsho Shinkeigaku*. 2004 Apr-May;44(4-5):241-62.

Jakes R, Spillantini MG, Goedert M.Identification of two distinct synucleins from human brain. *FEBS Letters*. 1994 May 23;345(1):27-32.

Jenco JM, Rawlingson A, Daniels B, Morris AJ.Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins. *Biochemistry*. 1998 Apr 7;37(14):4901-9.

Jo E, McLaurin J, Yip CM, St George-Hyslop P, Fraser PE. alpha-Synuclein membrane interactions and lipid specificity. *Journal of Biological Chemistry*. 2000 Nov 3;275(44):34328-34.

Johnson, BE. Expression and characterization studies of α-synuclein to model Parkinson's Disease in yeast. Lake Forest College Senior Thesis Project. Lake Forest College. 2 Apr 2003.

Junn E, Mouradian MM.Human alpha-synuclein over-expression increases intracellular reactive oxygen species levels and susceptibility to dopamine. *Neuroscience Letters*. 2002 Mar 8;320(3):146-50.

Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, Mandel RJ, Bjorklund A. Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *Journal of Neuroscience*. 2002 Apr 1;22(7):2780-91.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N.Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*. 1998 Apr 9;392(6676):605-8.

Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nature Genetics*. 1998 Feb;18(2):106-8.

Kweon GR, Marks JD, Krencik R, Leung EH, Schumacker PT, Hyland K, Kang UJ. Distinct mechanisms of neurodegeneration induced by chronic complex I inhibition in dopaminergic and non-dopaminergic cells. *Journal of Biological Chemistry*. 2004 Oct 6 [Epub ahead of print].

Lakso M, Vartiainen S, Moilanen AM, Sirvio J, Thomas JH, Nass R, Blakely RD, Wong G.Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human alpha-synuclein. *Journal of Neurochemistry*. 2003 Jul;86(1):165-72.

Mizuno Y, Ohta S, Tanaka M, Takamiya S, Suzuki K, Sato T, Oya H, Ozawa T, Kagawa Y. Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. *Biochemistry Biophysics Research Communications*. 1989 Sep 29;163(3):1450-5.

Ostrerova Natalie, Leonard Petrucelli1, Matthew Farrer, Nitinkumar Mehta, Peter Choi1, John Hardy, and Benjamin Wolozin. α-Synuclein Shares Physical and Functional Homology with 14-3-3 Proteins. *The Journal of Neuroscience*, July 15, 1999, 19(14):5782-5791.

Outeiro TF, Lindquist S. Yeast cells provide insight into alpha-synuclein biology and pathobiology. *Science*. 2003 Dec 5;302(5651):1772-5.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. 1997 Jun 27;276(5321):2045-7.

Sharma, N. Baker's yeast promotes understanding of α-synuclein's functional and pathogenic roles. Lake Forest College Senior Thesis Project. Lake Forest College. 30 Apr 2004.

Slot JW, Geuze HJ, Freeman BA, Crapo JD. Intracellular localization of the copper-zinc and manganese superoxide dismutases in rat liver parenchymal cells. *Laboratory investigation*. 1986 Sep;55(3):363-71.

Tanaka M, Kim YM, Lee G, Junn E, Iwatsubo T, Mouradian MM.Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. *Journal of Biological Chemistry*. 2004 Feb 6;279(6):4625-31. Epub 2003 Nov 19.

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW.Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science*. 2004 May 21;304(5674):1158-60. Epub 2004 Apr 15.

Willingham S, Outeiro TF, DeVit MJ, Lindquist SL, Muchowski PJ.Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. *Science*. 2003 Dec 5;302(5651): 1769-72.

Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG.The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Annals of Neurology*. 2004 Feb;55(2):164-73.

Zhou Y, Gu G, Goodlett DR, Zhang T, Pan C, Montine TJ, Montine KS, Aebersold RH, Zhang J. Analysis of alpha-synuclein-associated proteins by quantitative proteomics. *Journal of Biological Chemistry*. 2004 Sep 10;279(37):39155-64. Epub 2004 Jul 01.