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Abstract
Parkinson's disease is a progressive neurodegenerative disease caused by the death of midbrain dopaminergic neurons. The misfolding and aggregation of the protein α-synuclein and accumulation of oxidants is thought to underlie the cell death, but how α-synuclein becomes toxic is unclear. Using fission yeast as a model organism, where α-synuclein expression induces aggregation but not toxicity, we tested the hypothesis that α-synuclein toxicity can result from the combination of protein aggregation and oxidative stress. Surprisingly, we found that both oxidants (hydrogen peroxide and cumene hydroperoxide) tested in our study resulted in α-synuclein-independent toxicity. Nevertheless, this work illustrates the usefulness of yeasts in evaluating genetic factors and environmental factors like oxidative stress to potentially regulate α-synuclein toxicity linked to Parkinson's disease.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder in which patients suffer from resting tremors, postural rigidity, bradykinesia, and poor balance. These symptoms are the result of the specific death of dopaminergic neurons in the substantia nigra *pars compacta* (Chua and Tang 2006). Most cases of PD are idiopathic and occur during late age, with approximately 10% of PD cases are genetically linked (Eriksen *et al*. 2003). PD is fatal and incurable, but over the last thirty years a variety of drugs have been developed to treat the symptoms.

Key findings in PD research point to the hypothesis that the protein alpha-synuclein plays a critical role in pathogenesis. PD is characterized by accumulation of misfolded α-synuclein in cytoplasmic inclusions called Lewy bodies within the affected dying dopaminergic neurons (Spillantini *et al*. 1998). There are three mutations in the α-synuclein gene that cause dominant and familial PD; A53T (Polymeropoulos *et al*. 1997), A30P (Kruger *et al*., 1998), and E46K (Zarranz *et al*. 2004). Transgenic mice (Masliah *et al*. 2000) and flies (Feany and Bender 2000) expressing the human form of α-synuclein are well established models for replicating the PD pathology (Rochet *et al*. 2004).

In addition to α-synuclein misfolding and aggregation, oxidants accumulate in the dying dopaminergic neurons indicating the involvement of oxidative stress in PD pathogenesis (Olanow and Tatton 1999; Dawson and Dawson 2003; Jenner 2003; Maguire-Zeiss *et al*. 2005). Oxidative stress occurs largely due to mitochondrial dysfunction giving rise to reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and the hydroxyl radicals (Abou-Sleiman *et al*. 2006). The inhibition of complex I of the mitochondria is one of the pathological features in human PD brains (Parker *et al*. 1989; Simon *et al*. 2000; Devi *et al*. 2008). This type of inhibition has been shown to damage dopaminergic neurons in culture (Kweon *et al*. 2004). It generates excess cellular ROS (Dauer and Przedborski 2003), which can cause damage to lipid membranes (Giorgio Lenaz 1998), and modulation of important metabolic and other cellular activities by oxidizing critical proteins (Alam *et al*. 1997). Many studies have approached modeling PD by the use of neurotoxins. Among them are methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) and rotenone which abrogate mitochondrial complex I activity, consequently inducing oxidative stress (Fallon *et al*. 1997; Dawson and Dawson 2003).

Whether alpha-synuclein aggregation and oxidative stress are independent inducers of cellular toxicity or they act in concert to achieve a combinatorial outcome, is still an open question. Low concentrations of oxidants (less than 5 mM) do not typically harm yeast cells (Flattery-O'Brien and Dawes 1998; Izawa *et al*. 1995; Jamieson *et al*. 1992). However, our lab recently showed in a budding yeast model that α-synuclein toxicity can be induced when coupled with oxidative stress, which was induced by exposure to low concentrations of hydrogen peroxide in yeast that lack the mitochondrial anti-oxidant enzyme SOD2 (Sharma *et al*. 2006). This finding supports a role for combinatorial toxicity induced by alpha-synuclein aggregation and oxidative stress.

We recently developed a fission yeast model for PD and found these yeasts are resistant to α-synuclein toxicity, despite the extensive □formation of α-synuclein aggregates□ (Brandis *et al*. 2006). To better understand why fission

yeast is protective against α-synuclein toxicity, we reasoned that, just like in budding yeast, a combinatorial insult such as oxidative stress might be needed for α-synuclein to induce cellular toxicity. In this study, we treated α-synuclein expressing fission yeast with two exogenous oxidants (hydrogen peroxide and cumene hydroperoxide) at concentrations that are not toxic to yeasts, and we predicted that α-synuclein-dependent toxicity would be induced in a combinatorial manner with oxidative stress. Finally, we hypothesized that α-synuclein cellular distribution would be altered under such condition. Because oxidative stress has been linked to the initiation and progression of Parkinson's disease, the research aim of this study is to understand an *in vivo* relationship between oxidative stress and the toxic property of α-synuclein aggregation. In light of these two major events in the pathology of the disease, supporting evidence will help uncover of how neurodegeneration occurs.

Materials and Methods

S. Pombe Expression Vectors

Polymerase chain reaction (PCR) was used to amplify C-terminal green fluorescence protein (GFP)-tagged wild-type α-synuclein fusion cDNA from the α-sunclein-GFP containing pYES2/TOPO *S. cerevisiae* vectors constructed by Sharma *et al*. (2006):

forward primer, 5'-GGGGCCAAGCTTGCCATGGATGTATTCATGAAAGGA-3';
reverse primer, 5'-TTTGTAGAGCTCATACATGCCATG-3'.

Similarly, PCR was used to amplify GFP cDNA from GFP-pYES/TOPO *S. cerevisiae* vectors constructed by Sharma *et al*. (2006):

forward primer, 5'CCCGGGACCATGGCCAGCAAAGGAGAAG-3';
reverse primer, 5'-TTTGTAGAGCTCATACATGCCATG-3'.

These PCR products were subcloned, according to the manufacturer's protocol (Invitrogen), into fission yeast pNMT-1 TOPO-TA expression vector. The following two vector constructs were previously created for each experiment: GFP, and wild-type α-synuclein. α-Synuclein was tagged at the C-terminus with GFP. The pNMT-1 vector and GFP in pNMT-1 vector served as expression controls.

Yeast Strains

The TCP1 strain (h-*leu1-32*; Invitrogen) of fission yeast was kindly provided by Judy Potashkin, Rosalind Franklin University of Medicine and Science.

Yeast Transformation

S. Pombe strains were transformed with pNMT TOPO-TA vectors (Alfa *et al*. 1993) using the lithium-acetate transformation method (Burke *et al*. 2000).

S. Pombe Expression

For selection, yeast cells were grown on synthetic-complete media lacking leucine (PDM-Leu). Presence of α-synuclein constructs was confirmed by polymerase chain reaction (PCR). The pREP vector, containing a thiamine repressible promoter, allowed for regulated α-synuclein expression. Yeast cells were first grown overnight in PDM-Leucine (10 μM thiamine) media at 30°C. Cells were washed with water and diluted to log-phase (5×10^6 cells/mL) in Edinburgh minimal medium (EMM) lacking thiamine media to induce expression and grown to the time points desired for various measurements.

Fluorescence Microscopy

Yeast cells were first grown overnight at 30^oC in EMM containing thiamine (10 μM). After 24 hr, cells were pelleted at 1500g for 5 minutes, washed twice in 10 ml dH_2O, resuspended in 10 ml EMM without thiamine, of which 125 mL cells were used to inoculate 25 mL EMM without thiamine (to express α-synuclein). At desired expression time points for microscopy, cells were harvested at 1500g (4^oC) for 5 min and were washed in 5 mL water. Then cells were resuspended in 100-1000 μL EMM+T, of which 10 μL was pipetted onto a slide. Slides were viewed using Nikon TE-2000U fluorescence microscope at 1000X magnification. Images were deconvoluted using MetaMorph software version 4.2. Cells were first viewed under differential interference contrast (DIC) microscopy, and then viewed for GFP fluorescence. For analysis, the number of cells in the field containing α-synuclein aggregates, intracellular structure localization or plasma membrane localization was analyzed. The field was then moved three turns on the field control knob, and the process was repeated in a new field. At least 750 cells were evaluated for each sample. α-Synuclein phenotypes were scored as percent of total cells observed.

Growth Curve
Cells were grown in 5 ml PDM-Leu overnight at 30°C in the incubator which rotates at 200 rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice in 5 ml H_2O. Cells were re-suspended in 5 ml H_2O and were counted. Flasks with 25 ml EMM were each inoculated with 2 x 10^6 cells/ml density. At 0, 6, 12, 18, 24, 36 and 48 hours, and in duplicate measurements, 1 ml of cell was removed and placed in a cuvette to measure absorbance using a Hitachi U-2000 Spectrophotometer. Averaged absorbance readings were plotted against time points to produce a growth curve.

Spotting Analysis
For spotting, cells were grown to mid-log phase in PDM-Leu normalized to equal densities (2 × 10^7 cells/mL), serially diluted (5-fold) into 96-well microwell plates. Cell cultures either containing parent vector, GFP or, wild-type α-synuclein serially diluted 5 times, were spotted onto sets of repressing (+thiamine) and inducing (-thiamine) plates, that contained 0, 0.4 or 2 mM H_2O_2. Photographs of the plates were scanned after 2 days of growth.

Western Analyses
Yeast cells (2.5 × 10^7 cells/mL) were washed in 50 mM Tris (pH 7.5) and 10 mM NaN_3 and solubilized in electrophoresis sample buffer ([ESB] Burke *et al*. 2000) containing 2% sodium dodecyl sulfate (SDS), 80 mM Tris (pH 6.8), 10% glycerol, 1.5% dithiothreitol, 1 mg/mL bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.7 µg/mL pepstatin A, 0.5 µg/mL leupeptin, 10 µg/mL E64, 2 µg/mL aprotinin, and 2 µg/mL chymostatin). Samples were run on precast 10–20% Trisglycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to PVDF membranes, and Western blotting was performed with different monoclonal antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 (Invitrogen) for most blots to probe for α-synuclein, and Anti □-Actin (Abcam) for most expression experiments, as a measure of loading control in duplicate blots.

Statistical Analyses
Statistical significance was established using a T-Test comparing the means of control cells containing no α-synuclein at 18 and 24 hours in treated versus untreated conditions.

H_2O_2 and Cumene Hydroperoxide Treatment
H_2O_2 and Cumene Hydroperoxide (CHP) were purchased from Sigma-Aldrich. Cells were grown in 10 ml PDM-Leu overnight at 30°C. Cell harvesting and calculating cell density for inoculation was performed as described above. H_2O_2 and CHP were added to the selected media at concentrations of 0 mM, 0.4mM and 2 mM. Afterwards, yeast cells were inoculated into the media. Then the cells were observed at the desired time points for the following experiments: Western Analysis, Growth Curve, Spotting Analysis and Fluorescence Microscopy.

Results
H_2O_2 induces α-synuclein-independent toxicity in fission yeast
Our goal was to evaluate if α-synuclein dependent

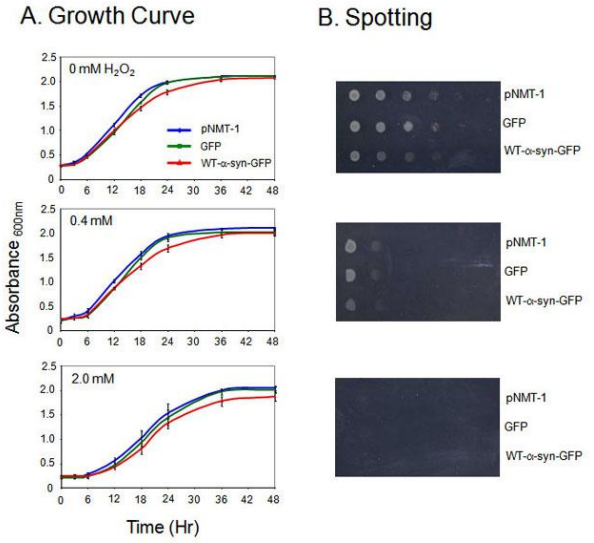

Figure 1. Fission yeast cells treated with H_2O_2. A. Growth Curve: Fission yeast were grown in EMM-T for 48 hours expressing WT (wild-type) □-synuclein along with pNMT-1 parent plasmid and GFP as controls. Over this time course, the cells were treated with 0.4mM or 2mM H_2O_2 and compared to the cells that were not treated with H_2O_2. Each of the points on the curves represents the mean from three trials on measurement of absorbance. In addition, the standard error of the mean for a 95% confidence interval is represented by the y-bar on each of the points on the growth curves. Significant growth difference exists between pNMT-1 in 0mM H_2O_2 (n=3, p_{18hr} = 0.0199 and p_{24hr}= 0.0762) and pNMT-1 in 2mM H_2O_2. B. Spotting: Serially diluted cells expressing parent pYES2 plasmid, GFP, or WT-a-synuclein were spotted onto EMM-T (inducing) plates. This experiment was repeated twice.

toxicity could be induced with oxidative stress in fission yeast, an organism in which α-synuclein expression has minimal toxic effect on its own (Brandis *et al*., 2006). We reasoned that H_2O_2 exposure would function as an additional insult on the existing stress of α-synuclein expression (McCormacka *et al*., 2002; Abou-Sleiman *et al*., 2006). As we exposed fission yeast cells to various concentrations of H_2O_2, the growth curve shifted to the right, particularly at 2mM, indicating a toxic effect (Figure 1A). Surprisingly, this slow growth was not α-synuclein dependent, since cells that expressed GFP alone or contained only the parent pNMT1 vector were also growth impaired (Figure 1A). To confirm this effect, we also evaluated fission yeast colony survival on plates by a serial dilution assay. Similar to growth curve analysis, we observed a more striking dose-dependent toxicity with H_2O_2 that was not dependent on the presence of α-synuclein (Figure 1B). With a 2 mM H_2O_2 exposure, no fission yeast growth was detected on the agar plates with or without α-synuclein (Figure 1B).

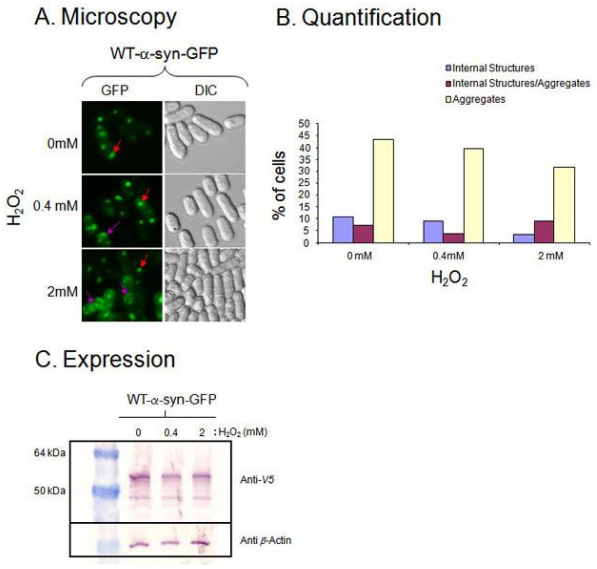

Figure 2. GFP Microscopy and α-synuclein expression in fission yeast cells treated with H_2O_2. A. Microscopy: Images were captured at 18 hour after induction in EMM-T. Aggregates (red arrow) were noticeable with H_2O_2 treatment, along with small number of cells showing α-synuclein localized to intracellular structures (purple arrow). α-Synuclein aggregates and intracellular structure localization did not increase with higher dose of H_2O_2. B. Quantification: Cells expressing WT-a-synuclein were counted and characterized by the following phenotypes: intracellular structure localization (blue bar), intracellular structure localization and aggregates (red bar), or aggregates (yellow bar). Bars represent the percent of 600-800 total cells which display those various a-synuclein phenotypes at different concentration of H_2O_2. C. Expression: For all the samples, α-synuclein expression was induced for 24 hr in EMM-T media and detected using anti-V5. Anti α-Actin antibody showed equal loading of protein.

H_2O_2 induces slight alteration of α-synuclein localization without affecting expression

Next, we examined the cellular distribution of α-synuclein in fission yeast to determine whether its normal localization (cytoplasmic aggregates) changed due to oxidant exposure. Live cell GFP fluorescence images for cells expressing α-synuclein were recorded at 18 hour post-induction. As the concentration of H_2O_2 was increased to 2mM, the predominant α-synuclein localization pattern was still aggregates (A). A less common finding was the localization of α-synuclein into as-yet-unidentified internal cellular structures (Figure 2A). In fact, some H_2O_2-exposed cells had both of these α-synuclein localization patterns (Figure 2A). Quantification of cellular distribution indicated that there was no statistical significance in the number of α-synuclein aggregates between H_2O_2-exposed cells versus untreated cells (Figure 2B). Meanwhile, the expression of α-synuclein remained the same with or without H_2O_2 treatment, as determined by Western blot (Figure 2C).

CHP induces α-synuclein independent toxicity in fission yeast

Lastly, we examined whether the H_2O_2 toxicity on yeast cells had a broader effect representative of more than one oxidant. As such, we induced oxidative stress in fission yeast by exposing them to cumene hydroperoxide. Though the growth curve analysis was conducted only once, the cellular toxicity under CHP treatment was more dramatic than under H_2O_2 treatment (Figure 3). The toxicity was not α-synuclein dependent, since cells that expressed GFP alone or contained the parent vector were also growth impaired (Figure 3).

Discussion

A connection between α-synuclein misfolding, aggregation, and oxidative stress is not fully understood. As examined initially by Brandis *et al*. (2006), S. *pombe* does not exhibit toxicity when α-synuclein is overexpressed. We inquired whether an additional stress (e.g. oxidant exposure) on fission yeast is critical for toxicity to occur. Our

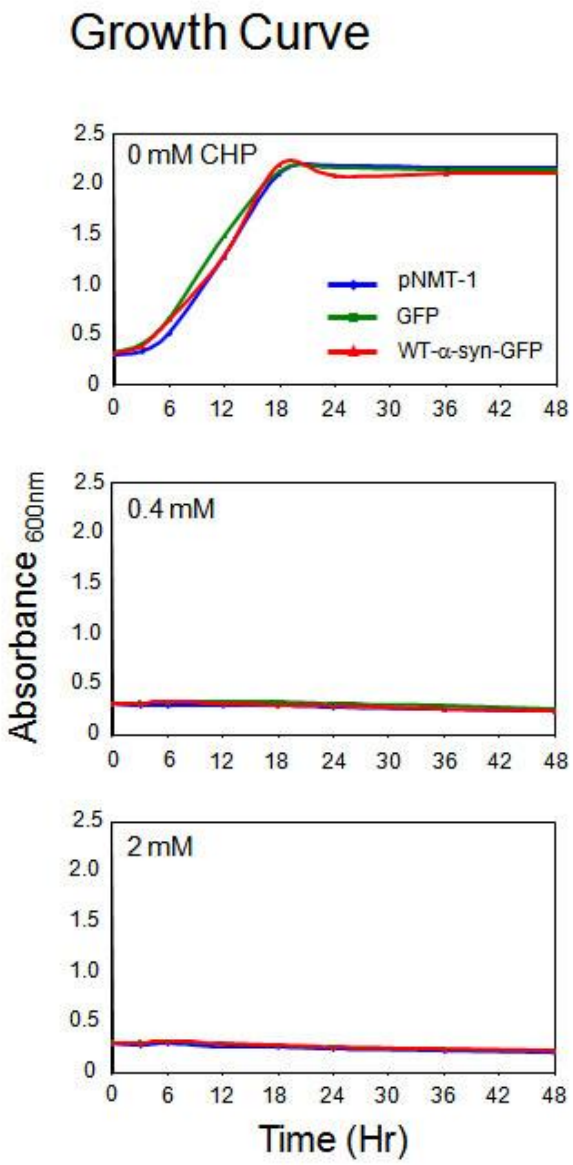

Figure 3. Fission yeast cells treated with CHP. Growth Curve: Fission yeast was grown in EMM-T for 48 hours expressing WT (wild-type) α-synuclein along with pNMT-1 parent plasmid and GFP as controls. Over this time course, the cells were treated with 0.4mM or 2mM CHP and compared to the cells that were not treated with CHP. Each of the points on the curves represents measurement of absorbance from one trial.

major finding is that oxidants (H_2O_2 and CHP) exerted unexpected toxicity on fission yeast at low concentrations, independent of α-synuclein expression and also altered α-synuclein's cellular localization.

Toxicity of oxidants in fission yeast

The slow growth of fission yeast in media containing 2mM H_2O_2 was a surprising finding. At this same concentration, H_2O_2 does not impair growth of budding yeast (Sharma *et al*., 2006). More surprisingly, CHP exerted stronger toxicity on fission yeast than H_2O_2 at low of a concentration as 0.4mM. According to Mutoh *et al*. (2005), fission yeast has close to 100% survival rate at such a low concentration, but they are certainly growth impaired. In their study, they show 10-fold reduction in yeast colony forming ability within four hours after 0.1mM CHP treatment. One possibility for this conundrum is that budding yeast and fission yeast might have varying sensitivity to oxidants. Though fission yeast produce antioxidants and enzymes to break down reactive oxygen species generated by H_2O_2 and CHP, these might not be adequate to maintain cellular redox homeostasis as oxidant concentrations rise (Chen *et al*., 2003). One study points out that a 2mM H_2O_2 concentration inhibits the fission yeast cell growth by 58% (Pekmez *et al*., 2008). Also, the increased H_2O_2 concentrations lower the glutathione (GSH) levels; 2.0 mM H_2O_2 treatment showed significant decrease in GSH level in fission yeast (Pekmez *et al*., 2008). GSH is an abundant thiol that has been shown to protect against the deleterious effects of reactive oxygen species in budding yeast cells (Grant *et al*., 1998; Izawa *et al*., 1995). GSH expression in budding yeast and fission yeast (or within various fission yeast strains) may perhaps be different hence leading to difference in tolerance for H_2O_2 and perhaps also CHP. Along with GSH, there might be group of other regulatory genes in response to oxidative stress that may be responsible for why fission yeast is sensitive to the oxidants.

No enhancement of α-synuclein toxicity with H_2O_2 and CHP treatment

Contrary to our hypothesis, α-synuclein-dependent toxicity was not induced in a combinatorial manner with oxidative stress. Both the α-synuclein expressing cells and those that did not express the protein experienced the same magnitude of growth defect along the time course with exposure to the two oxidants. We did not observe any changes in α-synuclein expression level in treatment with H_2O_2, but contrast that to a study done in neuronal cells, acute exposure to H_2O_2 increases the level of α-synuclein expression where the protein protected neurons from apoptosis (Quilty *et al.,* 2006). Whether α-synuclein is cytotoxic or cytoprotective along with oxidative stress remains a contentious subject.

H_2O_2 and CHP are powerful oxidizing agents, but their interaction with α-synuclein inside the cell may not have been enough to cause α-synuclein dependent cell death, partly because α-synuclein rapidly forms aggregates in this organism and may prevent such interactions (Brandis *et al*. 2006). Secondly, because the oxidants produced significant toxicity on their own in fission yeast, this may mask any additional toxic contribution that would have come out of a combined effect of both oxidant and α-synuclein expression. Lastly, additional cellular dysfunction maybe required before α-synuclein dependent toxicity can be elevated. We presume that protective measures exist in fission yeast to suppress both α-synuclein toxicity and oxidative stress. For example, the anti-oxidant gene *sod2* was shown to protect budding yeast cells from α-synuclein toxicity (Willingham *et al*., 2003). When the *sod2* gene is deleted, α-synuclein toxicity was exhibited and further enhanced with H_2O_2 treatment (Sharma *et al*., 2006). An important future study should be to repeat our current experiments in fission yeasts knocked down for key anti-oxidant genes. In *S. Pombe*, oxidative stress activates transcription factors that induce the expression of ROS defensive genes. Transcription factors like Atf1, Pap1 and Prr1 (Wilkinson *et al*., 1996; Vivancos *et al*., 2004; Ohmiya *et al*., 1999) which are known to respond to oxidative stress in fission yeast can be targeted for deletion.

In conclusion, it is not clear how oxidative stress is a critical factor to the etiology of Parkinson's disease. On the other hand, the abnormal aggregation of alpha-synuclein into Lewy bodies has acquired much of the focus in the research field. Both of these events could be connected because several *in vitro* studies suggest that increased cellular oxidative stress increases the propensity for alpha-synuclein to aggregate within cells (Hashimoto *et al.*, 1999; Souza *et al.*, 2000). Our current *in vivo* study in fission yeast, where α-synuclein aggregation is prominent but it exhibits no cytotoxicity, was an attempt to dissect how oxidative stress could potentially induce α-synuclein dependent toxicity. Ongoing research that examines α-synucleinopathy with oxidative stress will be helpful for looking at potential therapeutic targets in the oxidative stress pathway to combat this debilitating brain disease.

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References

Abou-Sleiman, PM *et al*. (2006) Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nature Reviews Neuroscience 7: 207–219.

Alam, ZI *et al*. (1997) A Generalised Increase in Protein Carbonyls in the Brain in Parkinson's but Not Incidental Lewy Body Disease. Journal of Neurochemistry 69(3): 1326-1329.

Alfa, C *et al*. eds. (1993) Experiments with Fission Yeast, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Brandis, KA *et al.* (2006) α-Synuclein fission yeast model: concentration dependent aggregation without plasma membrane localization or toxicity, Journal of Molecular Neuroscience 28: 179-191.

Burke, D *et al.* (2000) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, New York.

Chua, CEL and Tang, BL (2006) alpha-Synuclein and Parkinson's disease: the first roadblock. Journal of Cellular and Molecular Medicine 10(4): 1-10.

Chen, D *et al*. (2003) Global Transcriptional Responses of Fission Yeast to Environmental Stress. Molecular Biology of the Cell 14, 214–229.

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

Devi, L et al. (2008) Mitochondrial Import and Accumulation of α-Synuclein Impair Complex I in Human Dopaminergic Neuronal Cultures and Parkinson Disease Brain. Journal of Biological Chemistry 283(14): 9089-9100.

Dawson, TM and Dawson, VL (2003) Molecular pathways of neurodegeneration in Parkinson's disease. Science 302: 819–822.

Eriksen, JL *et al*. (2003) Caught in the Act: alpha-Synuclein Is the Culprit in Parkinson's Disease. Neuron 40: 453–456.

Fallon, J *et al*. (1997) MPP+ produces progressive neuronal degeneration which is mediated by oxidative stress. Experimental Neurology 144(1): 193-8.

Feany, M and Bender, W (2000) A Drosophila model of Parkinson's disease. Nature 23: 294–298.

Flattery-O'Brien, JA and Dawes, IW (1998) Hydrogen Peroxide Causes RAD9-dependent Cell Cycle Arrest in G2 in Saccharomyces cerevisiae whereas Menadione Causes G1 Arrest Independent of RAD9 Function. Journal of Biological Chemistry 273(15): 8564–8571.
Izawa, S *et al*. (1995) Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in Saccharomyces eerevisiae. FEBS Letters 368: 73-76.

Grant, CM *et al*. (1998) Glutathione and Catalase Provide Overlapping Defenses for Protection against Hydrogen Peroxide in the Yeast *Saccharomyces cerevisiae*. Biochemical and Biophysical Research Communications 253(3): 893-898.

Hashimoto, M *et al.* (1999) Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro. NeuroReport 10(4): 717–721.

Jamieson, DJ (1992) *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. Journal of Bacteriology 174(20): 6678-6681.

Kruger, R *et al*. (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. Nature Genetics 18: 106-108.

Kweon, GR *et al*. (2004) Distinct mechanisms of neurodegeneration induced by chronic complex I inhibition in dopaminergic and non-dopaminergic cells. Journal of Biological Chemistry 279: 51783 – 51792.

Lenaz, G (1998) Role of mitochondria in oxidative stress and ageing. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1366(1-2): 53-67.

McEwen, JG *et al*. (1984) Toxic effect of products of oxidative metabolism on the yeast form of Paracoccidioides brasiliensis. The Journal of Medical Microbiology 18(3): 423-428.

McCormacka, AL *et al*. (2002) Environmental Risk Factors and Parkinson's Disease: Selective Degeneration of Nigral Dopaminergic Neurons Caused by the Herbicide Paraquat. Neurobiology of Disease 10(2): 119-127

Masliah, E *et al*. (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 287: 1265-1269.

Mutoh, N *et al*. (2005) Effects of Four Oxidants, Menadione, 1-Chloro-2,4-Dinitrobenzene, Hydrogen Peroxide and Cumene Hydroperoxide on Fission Yeast Schizosaccharmoyces pombe. The Journal of Biochemistry 138: 797–804.

Ohmiya, R *et al.* (1999) A fission yeast gene (prr1+) that encodes a response regulator implicated in oxidative stress response. The Journal of Biochemistry 125: 1061–1066.

Olanow, CW and Tatton, WG (1999) Etiology and pathogenesis of Parkinson's disease. Annual Review of Neuroscience 22: 123–144.

Parker, WD *et al.* (1989) Abnormalities of the electron transport chain in idiopathic Parkinson's disease. Annals of neurology 26(6): 719–723.

Pekmez, M *et al*. (2008) Hydrogen peroxide-induced oxidative damages in *Schizosaccharomyces pombe*. Biologia 63(2): 151-155.

Polymeropoulos, MH *et al*. (1997) Mutation in the α-Synuclein Gene Identified in Families with Parkinson's Disease, Science 276: 2045-2047.

Quilty, MC *et al.* (2006) Alpha-synuclein is upregulated in neurones in response to chronic oxidative stress and is associated with neuroprotection. Experimental Neurology 199(2): 249-256.

Rochet, JC *et al*. (2004) Interactions among alpha-synuclein, dopamine, and biomembranes: some clues for understanding neurodegeneration in Parkinson's disease. Journal of Molecular Neuroscience 23: 23–34.

Sharma, N *et al.* (2006) α-Synuclein budding yeast model: toxicity enhanced by impaired proteasome and oxidative stress. Journal of Molecular Neuroscience 28(2): 161–178.

Simon, DK *et al*. (2000) Mitochondrial DNA mutations in complex I and tRNA genes in Parkinson's disease. Neurology 54: 703-709.

Spillantini, M *et al.* (1998) α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease. Proceedings of the National Academy of Sciences 95: 6469–6473.

Souza, JM *et al.* (2000) Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. Journal of Biological Chemistry 275 (24): 18344–18349.

Vivancos, AP *et al.* (2004) Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. Molecular Microbiology 52: 1427–1435.

Wilkinson, MG *et al.* (1996) The Atf1 transcription factor is a target for the Sty1 stressactivated MAP kinase pathway in fission yeast. Genes and Development 10: 2289–2301.

Willingham, S *et al.* (2003) Yeast genes that enhance the toxicity of a mutant huntingtin or α-synuclein. Science 302: 1769–1772.

Zarranz, JJ *et al*. (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Annals of Neurology 55(2): 164-173.