α-Synuclein Aggregation and Membrane Association in a Fission Yeast Model: Implications for PD Pathogenesis

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Abstract

Lewy bodies of α-synuclein protein are prominent characteristics in the Parkinson's disease (PD) pathology. The mechanism of Lewy body formation and consequent cytotoxicity was studied by Brandis et al. (2006) in a newly developed model organism of fission yeast. Though, the level of α-synuclein expression studied was either high or low, the wild-type and A53T familial mutant of α-synuclein followed the nucleation polymerization theory in the process of misfolding and aggregating. At high concentration, α-synuclein formed cytoplasmic aggregates in a concentration and time-dependent manner. However, these aggregates appeared to be independent of cytotoxicity. In this current study, the fission yeast model is used again but to evaluate α-synuclein misfolding, aggregation, and non-toxic properties when expression is moderate. The results indicate moderate α-synuclein expression to obey the nucleation polymerization model. In light of this study, α-synuclein aggregation requires a necessary threshold concentration. Moderately expressed α-synuclein forms soluble aggregates, but at a slightly lower expression. So far, studies in fission yeast cells show that various concentrations of α-synuclein, neither target the plasma membrane nor are toxic. Because α-synuclein misfolding and aggregation is linked to Parkinson's disease, absence of its toxicity in fission yeast is paradoxical. We expect that α-synuclein toxicity may require a membrane binding capacity. In an attempt to induce α-synuclein's localization to the plasma membrane, the content of phospholipids in yeast was increased. Membrane localization and cytotoxicity were still lacking. Needless to say, fission yeast shed provocative insight into α-synuclein's role in PD pathogenesis.

Introduction

Parkinson's disease (PD) is the most common movement disorder in humans characterized by slowed movement, resting tremors, rigidity and postural instability. The disabling symptoms of PD result from dying midbrain striatal dopaminergic neurons. This neurodegenerative disease affects more than 1 million Americans over the age of 55 (Collier et al., 2002). Sporadic occurrences constitute 95% of all PD cases, while genetic occurrences constitute the other 5%. There are two well studied familial mutations in the α-synuclein gene in PD: A30P and A53T. In the disease pathology, the reduction of viable neurons correlates with the accumulation of misfolded α-synuclein into cytoplasmic aggregates called Lewy bodies (Giasson, 2001). Numerous model systems in mice (Dauer and Przedborski, 2003), worms (Lasko et al., 2003), flies (Feany and Bender, 2000) and yeast (Outeiro and Muchowski, 2004) have been designed to elucidate α-synuclein's biology and the molecular mechanism of misfolding, aggregation and cytotoxicity.

Fission yeast (*Schizosaccharomyces pombe)* is the model organism that our lab (Brandis et al. 2006) recently developed to study α-synuclein's misfolding, aggregation, and cytotoxic properties *in vivo*. The advantages of using yeast are that they have a fast life cycle and an easy to manipulate genome. Yeast and humans have conserved functions in cellular pathways such as protein folding, protein degradation, and oxidative stress (Wood et al., 2002). When neurological diseases strike human beings, one or more of these pathways malfunctions. Therefore, yeast can serve as an exquisite model for investigating protein misfolding neurological disorders.

The process of Lewy body formation from misfolded α-synuclein was first tested in *in vitro* experiments. Studies suggest that α-synuclein aggregation follows a hypothetical model called nucleation polymerization (Conway et al., 2000). Previously, misfolded protein aggregation in many neurodegenerative diseases, for instance, beta-amyloidsin Alzheimer's Disease, huntingtin in Huntington's Disease, and prion proteins in Transmissible Spongiform Encephalopathies was well predicted by this model (Caughey and Lansbury, 2003; Perutz and Windle, 2001). The nucleation polymerization hypothesis states that protein aggregation begins with the creation of small oligomer (nucleus) seeds. As time and protein concentration increases, the oligomers polymerize into polymers (aggregates). Thus, the formation of protein aggregates is time and concentration dependent.

Until recently, this hypothesis in PD was unsupported by *in vivo* experiments. However, using live fission yeast, Brandis et al. (2006), showed that α-synuclein aggregates on the basis of the nucleation polymerization theory *in vivo*. In the study, there was evidence of large insoluble clumps of α-synuclein aggregates which reminds us of the Lewy bodies that form in the dying neurons of a Parkinson's disease patient. At high concentration of α-synuclein in fission yeast, the aggregates formed in large numbers, but at low concentration there were no aggregates. In the presence of multiple α-synuclein aggregates, toxicity in fission yeast cells was surprisingly absent. Also, the α-synuclein in fission yeast, in contrast to the other family of yeast called budding yeast, never localized to the plasma membrane (Brandis et al., 2006).

On the other hand, research with budding yeast has provided key insight into α-synuclein misfolding and its ability to disrupt cellular pathways leading to cytotoxicity (Outeiro and Muchowski, 2004; Cooper et al., 2006). In studies of budding yeast, when α-synuclein was expressed at high concentration, it formed aggregates with toxic effects (Outeiro and Linquist, 2003). In other studies, α-synuclein aggregates did not have toxic effects without additional genetic knockouts causing proteasomal dysfunction (Sharma et al., 2006).

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Generally, budding yeast and fission yeast have inequivalent α-synuclein cellular localizations. In budding yeast models, wild-type and A53T mutant α-synuclein associate with the plasma membrane and may form aggregates within the cells. A30P mutant α-synuclein, on the other hand, remains cytoplasmically diffused (Dixon et al., 2005; Outeiro and Lindquist, 2003; Zabrocki et al., 2005; Sharma et al., 2005). In the fission yeast model, we also observe A30P mutant α-synuclein to be cytoplasmically diffused. However, wild-type and A53T mutant α-synuclein do not associate with the plasma membrane and form cytoplasmic aggregates in a time and concentration dependent manner (Brandis et al., 2006).

So far, the nucleation polymerization hypothesis can explain why there are more α-synuclein aggregates when the protein is expressed in high concentration in contrast to when the protein is expressed in low concentration (Brandis et al., 2006). In light of the nucleation polymerization hypothesis, in the first part of this study, we evaluate the expression of moderate concentration of α-synuclein in fission yeast. At moderate levels of α-synuclein, an intermediate number of α-synuclein aggregate formations are expected. According to Brandis et al. (2006), these aggregates should not influence cytotoxicity.

In the second part of this study, we examine the effect of cellular lipid content on toxicity. Cytotoxicity has yet to be observed in fission yeast. In budding yeast, cytotoxicity and -synuclein membrane localization coexist (Sharma et al., 2006). The biochemical property of α-synuclein gives this protein the specificity to bind to lipid membranes, inviting the question of why is there no α-synuclein dependent cytotoxicty in fission yeast (Sharon et al., 2001). It is possible that toxicity is contingent on α-synuclein membrane localization. Here, we use dimethyl sulfoxide (DMSO) to increase the overall lipid content in the fission yeast. In the Murata et al. (2003) study, upon exposure to DMSO, there is an induction of membrane proliferation in budding yeast. The same induction of phospholipid biosynthesis is expected to occur in fission yeast. This change in the physiology of fission yeast may encourage α-synuclein migration towards the cell membranes. According to Volles et al. (2001), membranes are the possible locations in the cell where α-synuclein can form toxic protofibrils (oligomers). These protofibrils can lead to the formation of cytoplasmic aggregates (Rochet et al., 2004) or possibly toxic cytoplasmic aggregates (Lashuel at al., 2002). Therefore, we inquire if the α-synuclein's membrane localization is key to cytotoxicity.

Materials and Methods

S. Pombe Expression Vectors

Human wild-type and A53T mutant α-synuclein cDNAs were a gift from Christopher Ross (Johns Hopkins University). A30P and A30P/A53T mutant α-synuclein were created from wild-type and A53T mutant α-synuclein, respectively, using site-directed mutagenesis (Invitrogen). Polymerase chain reaction (PCR) was used to amplify C-terminal green fluorescence protein (GFP)-tagged α-synuclein (wild-type, A30P, A53T, A30P/A53T) fusion cDNA from the α-sunclein-GFP containing pYES2/TOPO *S. cerevisiae* vectors constructed by Sharma et al.(2006): forward primer, 5'-GGGGCCAAGCTTGCCATGGATGTATTCATGAAA GGA-3'; reverse primer, 5'-TTTGTAGAGCTCATACAT GCCATG-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'-TTTGTAGAGCTCATACATGCCA TG-3'.

These PCR products were subcloned, according to the manufacturer's protocol (Invitrogen), into each of these three fission yeast pNMT TOPO-TA expression vectors: pNMT1 (for high expression), pNMT41 (for intermediate expression), and pNMT81 (for low expression) vectors. Note that these vectors added a V5-epitope and a 6X histidine epitope at the C-terminal end of the subcloned α-synuclein-GFP sequence. These vectors were transformed into *Escherichia Coli*, again according to the manufacturer's protocol (Invitrogen). Positive transformants were verified for correctly oriented subcloned cDNA by standard bacteria whole-cell PCR. Plasmid vectors were purified using a Qiagen miniprep kit, and the respective subcloned DNA sequences were confirmed (University of Chicago sequencing facility). The parent pNMT1, pNMT41, and pNMT81 pREP vectors were kindly provided by Judy Potashkin (Rosalind Franklin University of Medicine and Science, North Chicago, IL).

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 vectors using the lithium-acetate transformation method (Alfa et al., 1993).

Fluorescence Microscopy

S. pombe cells were first grown overnight at 30°C in Edinburgh minimal medium (EMM [Invitrogen]) containing thiamine (10μM [to repress α-synuclein expression]). After 24h, cells were pelleted at 1500g for 5 minutes, washed twice in 10ml dH_2O, resuspended in 10 ml EMM without thiamine, of which 125 μL 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°C) for 5 min and were washed in 5 mL water. Then cells were resuspended in 100-1000 uL EMM+T, of which 10 uL was pipetted onto a slide. Slide of cell culture was viewed using Nikon TE-2000U fluorescence microscope at 1000X magnification. Images were deconvoluted using MetaMorph software version 4.2. In order to quantify α-synuclein aggregates, cells were first viewed under differential interference contrast (DIC) microscopy, and total cell count in the field was determined and viewed for GFP fluorescence. The number of cells in the field containing 1, 2, and 3+ aggregates was determined. 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 treatment. Aggregates were scored as percent of total cells in the field that expressed 1, 2, and 3+ aggregates.

Western Analyses

Yeast cells ($2.5x10^7$ cells/ml) were washed in 50 mM Tris (pH 7.5), 10 mM NaN_3 and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 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, 2µg/ml aprotinin, and 2 µg/ml chymostatin). Samples were run on pre-cast 10-20% Tris-Glycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to PVDF membranes and Western blot was performed with anti-V5 AP monoclonal antibody using standard protocols and detected for alkaline phosphatase activity.

Growth Curve

Cells were grown in 10 ml EMM+T 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_20. Cells were re-suspended in 5 ml H_20 and were counted. Flasks with 25 ml EMM were each inoculated with $2.0x10^6$cells/ml density. At 0, 6, 12, 18, 24, and 36 hours, and in duplicate measurements, 1 ml of cell culture was removed and placed in a cuvet to measure absorbance using a Hitachi U-2000 Spectrophotometer. Averaged absorbance readings were plotted against time points to produce a growth curve.

DMSO Treatment

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Cells were grown in 10 ml EMM+T overnight at 30°C. Steps of cell harvesting and calculating cell density for inoculation was done according to the appropriate experiment. The cells had high expressing α-synuclein pNMT-1 vectors. Before inoculation, DMSO was added in the range of 0 to 10% in EMM media. The concentration of DMSO exposure was increased only up to 10%, technically exceeding this percent of DMSO would do harm to the cell (Zabrocki et al., 2005). After the DMSO mixed well in the media, yeast cells were inoculated, and grown. Then the cells were observed at the desired time points for the following examinations: Growth Curves and Fluorescence Microscopy.

Results

Moderately Expressed α-Synuclein Aggregates in Live Cells

α-synuclein localization in fission yeast is screened as the expression of the protein, promoted by pNMT-41 vector, is in moderate concentration. Fluorescence microscopy indicates A30P, A53T, and A30P/A53T mutant α-synuclein localizations. The protein expression level is slightly higher than the expression by pNMT-81 vector (refer to Figure 5C: Western Analysis in Brandis et al., 2006). A53T α-synuclein forms cytoplasmic aggregates. Meanwhile, A30P and A30P/A53T α-synuclein remain cytoplasmically diffused. Even in this moderate concentration, α–synuclein never localizes to the plasma membrane. These expression characteristics from all isoforms of α-synuclein in live fission yeast match previous findings (Brandis et. al, 2006) (Figure 1).

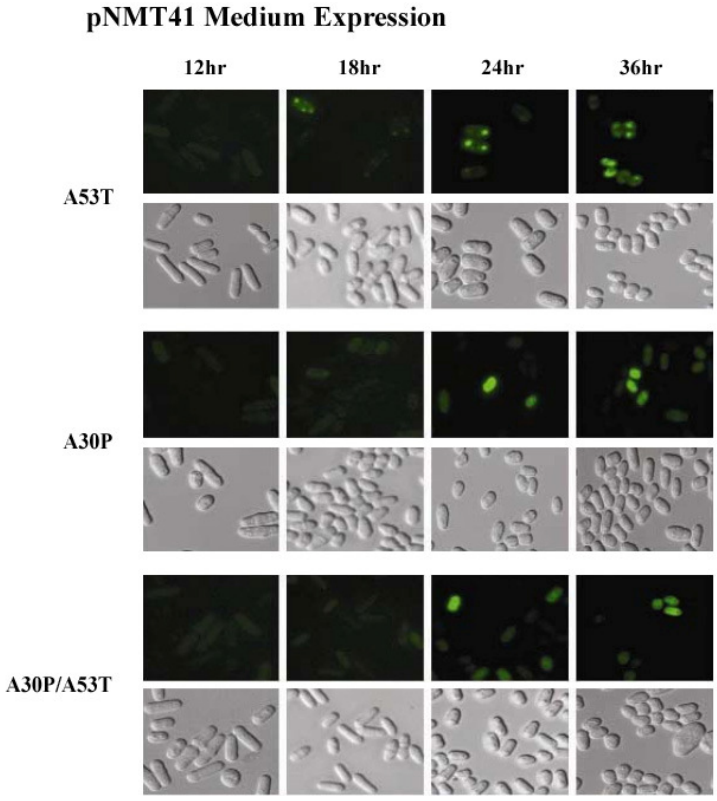

Figure 1. Fluorescence Microscopy of pNMT-41 Medium Expression: α-Synuclein was expressed in moderate concentration by pNMT41 promoter vector. These yeast were grown in EMM without thiamine. Images were captured at the indicated times over 36 hours. A53T α-synuclein began to form aggregates at the 18th hour time point. A30P and A30P/A53T exhibited diffuse cytoplasmic fluorescence throughout the time course.

Quantification of Moderately Expressed α-Synuclein Aggregates

Quantifying the aggregates for A53T mutant α-synuclein is necessary to make qualitative assessment on how these aggregates form. At moderate concentrations of α-synuclein, nucleation polymerization hypothesis predicts a delayed formation of an intermediate number of aggregates. The data showed that formation of aggregates was time-dependent, as they started appearing at the 18th hour (Figure 2A). Additionally, when compared to Brandis et al. (2006), the data supports the predictions made by

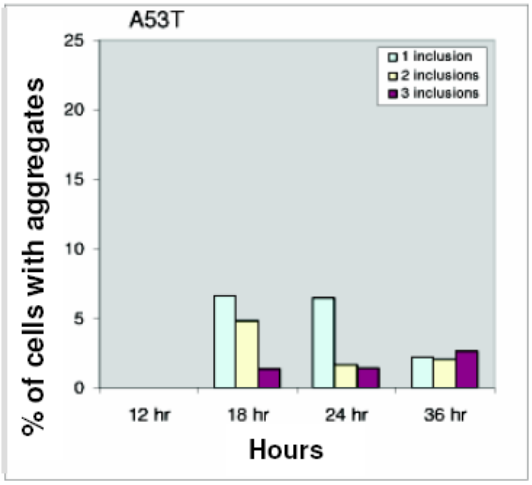

Figure 2A. Time course of quantified A53T α-synuclein aggregation. Cells, cultured in EMM media without thiamine, expressed α-synuclein in moderate concentrations. The number of aggregates formed was quantized in cells expressing A53T α-synuclein over a 36-hr time course. Cells were scored in terms of containing 1 aggregate (blue bar), 2 aggregates (yellow bar), or 3 or more aggregates (red bar). Bars represent percentage of total cells counted in each sample that exhibited the designated number of aggregates per cell.

the nucleation polymerization theory. First, the time of formation of aggregates at moderate concentration was six hours later when compared to the time of aggregate formation at high concentration. Second, throughout the 36 hour time course, the percent of cells forming aggregates were lower for moderate concentration compared to high concentration (Figure 2B).

Toxicity Levels of Moderately Expressed α-Synuclein Aggregates

Brandis et al. (2006) suggest that even at high concentrations of α-synuclein leading to formation of many aggregates, the cytotoxicity levels were minimal. An optical density assay performed in this study showed no toxicity in the cells expressing α-synuclein in moderate concentration either (Figure 3). The control in this growth analysis was a culture of parent vector (pNMT-41) cells that did not express α-synuclein. None of the isoforms of α-synuclein was toxic to fission yeast (Figure 3). Next, we investigated whether membrane localization was critical for toxicity. The lipid content of fission yeast cells was induced to promote α-synuclein to localize to the membrane.

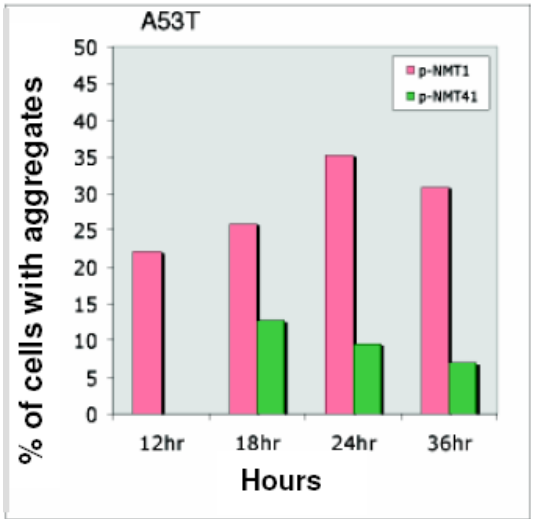

Figure 2B. Comparison of moderate to high expression of quantified A53T α-synuclein aggregation. Comparison of quantified data on A53T α-synuclein aggregates with high expression (red bar) (Quantification data provided by Brandis et al. 2006) compared to moderate expression (green bar) over the 36-hr period. Bars represent percentage of total cells that had 1 or more aggregates per cell.

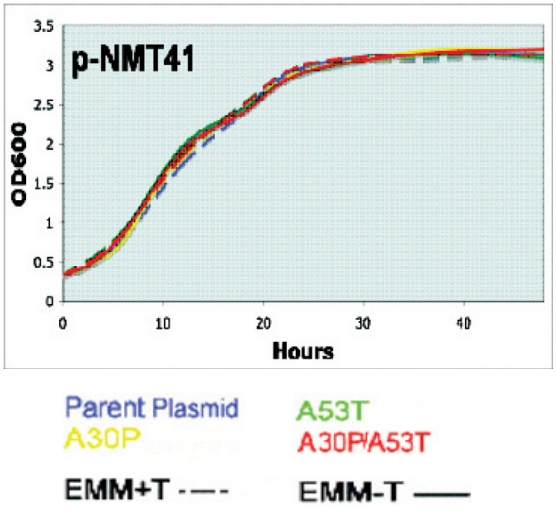

Figure 3. Growth Curve of Cells Moderately Expressing α-Synuclein. OD600 measurements were obtained over 48 hours for cells containing A30P, A53T, and A30/A53T forms of α-synuclein that were tagged with GFP in pNMT41 vector. Cells grown in thiamine (dashed lines) served as control. Concentration-dependent α-synuclein toxicity to fission yeast was not observed.

Is α-Synuclein Membrane Localization Gained With Exposure to DMSO?

Contrary to the prediction, upon DMSO exposure to fission yeast cells, no α-synuclein lipid binding property was observed. α-Synuclein aggregates did not decrease also. The aggregation inducing property of Wt (Figure 4) and A53T and as well as the cytoplasmically

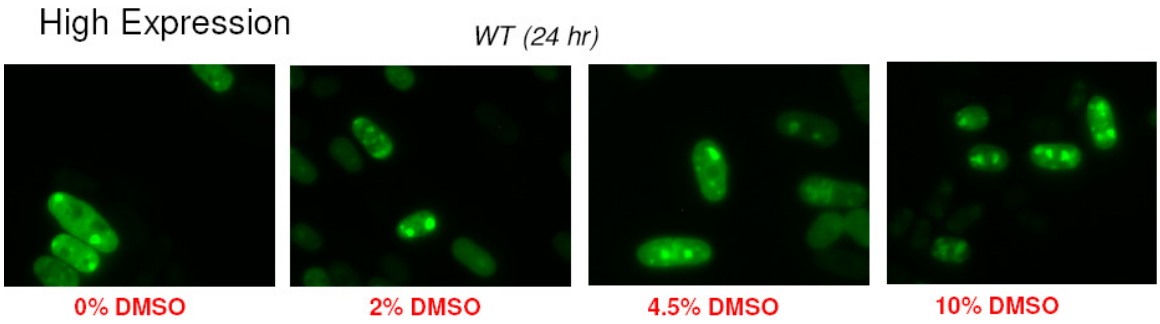

Figure 4. Fluorescence Microscopy of pNMT-1 High expression Cells Exposed to DMSO: Cells were grown in EMM media without thiamine, expressing α-synuclein in high concentration promoted by pNMT-1 vector. Florescence microscopy cell images were captured at the 24th hour. Wt α-synuclein did not gain membrane localization and the cytoplasmic aggregates still remain in fission yeast with exposure to varying concentrations of DMSO.

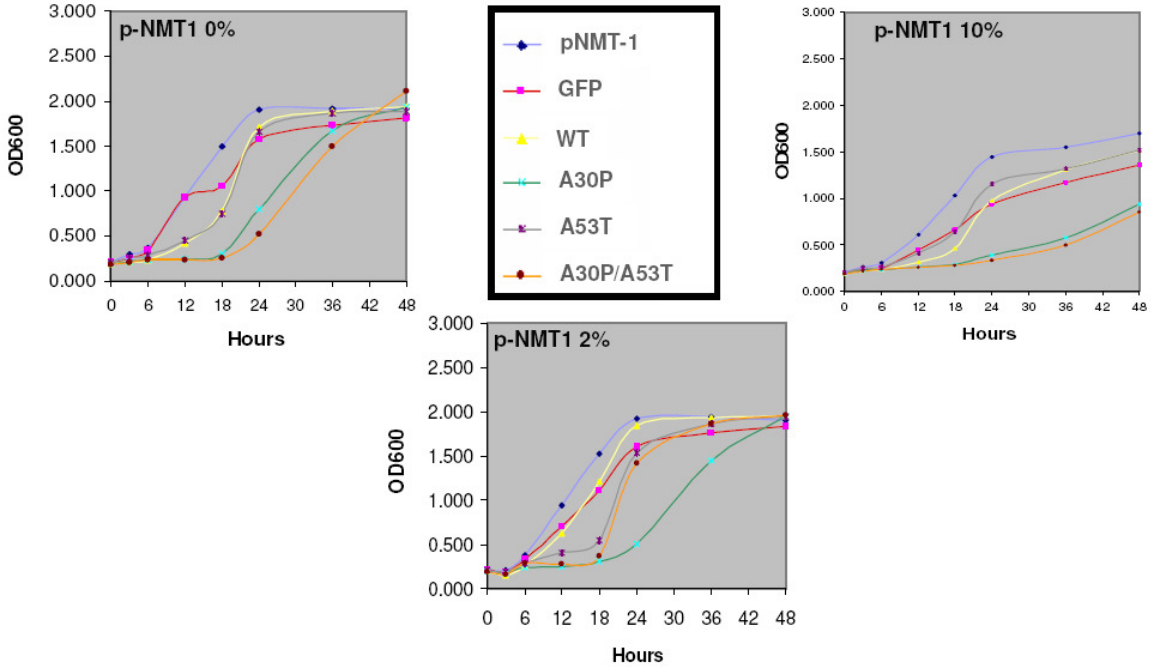

Figure 5. Growth Curve Analysis on α-Synuclein Expressing cells Exposed to DMSO (*1st Trial*): OD600 measurements over 48 hours were obtained for cells that express α-synuclein in high concentration. Control cells had simply GFP or parent-plasmid. Cells were grown in EMM media without thiamine. The growth was measured in exposure to different amounts (0%, 2%, and 10%) of DMSO. The data showed slight inhibition in growth.

diffused property of A30P and A30P/A53T were unchanged in exposure to DMSO (data not shown). These observations were made under fluorescence microscopy.

Are Toxicity Levels Enhanced in Fission Yeast with Exposure to DMSO?

All fission yeast in varying concentrations of DMSO showed a slight inhibition in their growth. We considered this result anomalous because 0% DMSO particularly should not be toxic. It contradicts the findings by Brandis et al. (2006). In turn, we tried to clarify the inconsistencies by rerunning the DMSO treatment experiment (Figure 5).

In the second trial, cells were treated with 0 %, and 4.5 % DMSO. This time, the control group with no exposure to DMSO had normal growth. As the DMSO exposure climbs to 4.5%, toxicity was not observed. Under toxic conditions, there would have been a lag in the growth curves (Figure 6).

Yet, these two trials' results were contradictory. In exposure to DMSO, the first trial

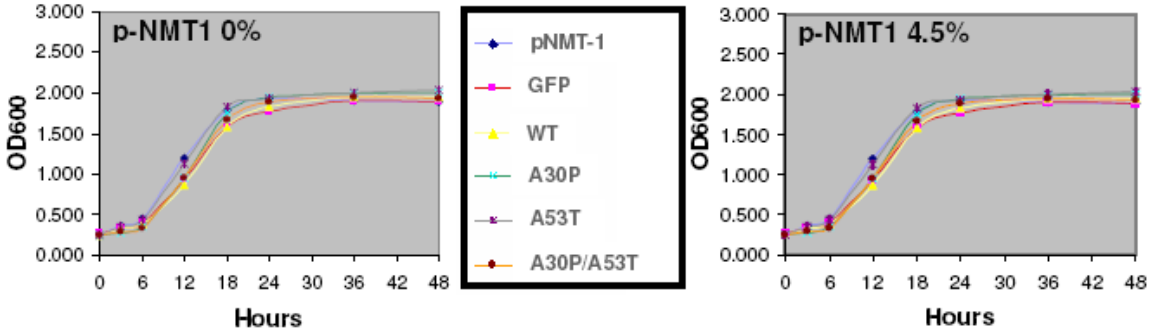

Figure 6. Growth Curve Analysis on α-Synuclein Expressing cells Exposed to DMSO (*2nd Trial*): OD600 measurements over 48 hours were obtained for cells that express α-synuclein in high concentration. Control cells had simply GFP or parent-plasmid. Cells were grown in EMM media without thiamine. The growth was measured in exposure to different amounts (0%, 4.5%) of DMSO. The control growth curves with no DMSO exposure had normal s-shaped curves. DMSO exposure of 4.5% did not cause toxic effects.

showed slight inhibition of cellular growth, while the second trial showed normal growth. In the future, several more trials of DMSO treatment on α-synuclein expressing fission yeast are necessary.

Discussion

From the past study by Brandis et al. (2006) and this current study, we have successfully developed fission yeast as a model organism to study the misfolding, aggregation, and cytotoxic properties of α-synuclein linked to Parkinson's disease. Specifically, work with fission yeast sheds provocative insight into the ideas that concentration of α-synuclein is important for the protein's aggregation and α-synuclein membrane localization might be critical for the protein dependent toxicity.

α-Synuclein aggregation: concentration is key

The protein's polymerization activity is concentration dependent. At high concentrations, there are many α-synuclein aggregates, while at low concentrations there are no α-synuclein aggregates (Brandis et al, 2006). In the current study, at moderate concentration, α-synuclein forms an intermediate number of aggregates. According to data by Brandis et al. (2006), the protein expression of moderate concentration was only slightly higher than the protein expression of the low concentration. Conversely, the low concentration of α-synuclein was not enough to lead to the formation of insoluble aggregates. Therefore, the evidence indicates that slightly higher (moderate) concentration of α-synuclein is required to form aggregates. We consider the moderate concentration or a concentration near to it as the threshold point of α-synuclein protein concentration that must be present to turn the soluble protein into insoluble aggregates. As protein concentration increases, the oligomers polymerize to form greater numbers of aggregates. Moreover, α-synuclein aggregation activity is time-dependent. Overall, Brandis et al. (2006) and this study display both concentration and time-dependent α-synuclein aggregation properties which support the grounds for the nucleation polymerization model *in vivo*.

Toxicity: is membrane localization key?

To this date, in a fission yeast model, membrane localization has not been observed with cytotoxicity. In the budding yeast model, there is evidence of membrane localization with toxicity (Sharma et al., 2006). This leads to the hypothesis that membrane localization might be key to cytotoxicity. We did not observe α-synuclein dependent toxicity in fission yeast, but we still suspect that toxicity requires α-synuclein membrane localization. In a previous study by Rochet et al. (2004), membrane localization of α-synuclein was shown to be essential in creating toxic protofibrils. Under a different study, the destruction of vesicular membranes by protofibrillar α-synuclein was directly observed by atomic force microscopy (Volles et al., 2001). In our lab, the toxicity of budding yeast was observed when α-synuclein localized at the cell periphery. In budding yeast, the localization of α-synuclein to the plasma membrane happened prior to the formation of α-synuclein aggregates. With the exception of A30P, wild-type and A53T α-synuclein localized to the plasma membrane before forming inclusions (Sharma et al., 2006). Conversely, in our fission yeast model, neither toxicity nor membrane association was observed. Overall, a connection between α-synuclein-dependent toxicity and α-synuclein's association with the plasma membrane is strongly implicated.

Future Proposal

To more conclusively elucidate α-synuclein's membrane association and yeast toxicity, the approaches of treating fission yeast with DMSO must be refined and repeated. Further research is crucial because several yeast models already suggest that membrane localization of α-synuclein is critical to pathogenesis (Dixon et al., 2005; Outeiro and Lindquist, 2003; Zabrocki et al., 2005).

Additionally, this future investigation can be done in budding yeast by reducing the lipid concentration of the membrane and then measuring changes in α-synuclein toxicity. Cho1 and Cho2 are knockouts in budding yeast strain W303 that code for enzymes critical to making major cell membrane phospholipids. Cho 1 encodes for phosphatidylserine synthase enzyme that coverts CDP-DG, a precursor to two of the major phospholipids in the membranes namely PI and PS. Cho 2 encodes a PL methyl transferase to produce PC from PE phospholipids. Using knockouts of these genes, budding yeast can be manipulated to give lipid deficient cells (Carman and Zeimetz, 1996). α-Synuclein must have specificity for binding to one of the major phospholipids. The

knockout of one or more of the major membrane phospholipids may advance the loss of α-synuclein membrane localization. If this loss occurs, we predict the reduction in toxicity levels.

We observed the formation of α-synuclein aggregates in fission yeast that did not acquire the initial membrane localization. This step may have been essential to creating toxic protofibrils and/or toxic cellular aggregates. We predict that the budding and fission yeast models will facilitate the establishment of α-synuclein association with the membrane phospholipids as a necessary characteristic to increase cytotoxicity levels. α-Synuclein is an abundant and broadly expressed protein in the human brain, where it interacts with membranes and vesicular structures (Outeiro and Lindquist, 2003). The α-synuclein property of associating with membranes and its link to pathogenic consequences makes it critical to future research.

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