Hsp70 reduces α-synuclein aggregation and toxicity

J. Klucken¹, Y. Shin¹, E. Masliah², B.T. Hyman¹ and P.J. McLean¹*

¹ Alzheimer’s Disease Research Laboratory, Harvard Medical School, Massachusetts General Hospital, 114 16th Street, Charlestown, MA 02129, USA

² Department of Neurosciences, University of California San Diego, School of Medicine, La Jolla, CA, USA

* Corresponding author: Pamela J. McLean, Ph.D., phone: +1 617 726 1263, fax: +1 617 724 1480, e-mail: pmclean@partners.org

Running title: Hsp70 reduces α-synuclein aggregation and toxicity
Summary

Aggregation and cytotoxicity of misfolded alpha-synuclein is postulated to be crucial in the disease process of neurodegenerative disorders such as Parkinson's disease and dementia with Lewy bodies. In this study we detected misfolded and aggregated alpha-synuclein in a triton X-100 insoluble fraction as well as a high molecular weight product by gel electrophoresis of temporal neocortex from DLB patients but not from controls. We also found similar triton X-100 insoluble forms of alpha-synuclein in an alpha-synuclein transgenic mouse model, and in an in vitro model of alpha-synuclein aggregation. Introducing the molecular chaperone Hsp70 into the in vivo model by breeding alpha-synuclein transgenic mice with Hsp70 overexpressing mice led to a significant reduction in both the high molecular weight and detergent insoluble alpha-synuclein species. Concomitantly, we found that Hsp70 overexpression in vitro similarly reduced detergent insoluble alpha-synuclein species and protected cells from alpha-synuclein induced cellular toxicity. Taken together, these data demonstrate that the molecular chaperone Hsp70 can reduce the amount of misfolded, aggregated alpha-synuclein species in vivo and in vitro, and protect from alpha-synuclein dependent toxicity.
Acknowledgment and support:

Supported by NIH NS38372, NIH AG18440, DFG KL 1395/2-1. We thank the Harvard Brain Tissue Resource Center (1R24MH68855), and The Massachusetts Alzheimer Disease Research Center (NIH AG05134-20), and the MGH-MIT Udall Center Brain Bank (NIH NS38372A-06) for neuropathological specimens and M. Ingelsson for helpful discussions and tissue preparation. We thank Dr. W.H. Dillman (UCSD) for the kind gift of the Hsp70 transgenic mice.
Introduction

α-Synuclein is a natively unfolded molecule that can self-aggregate to form oligomers and fibrillar intermediates (1-5) that accumulate to form Lewy bodies (LBs) and Lewy neurites (LNs) in neurons at risk for degeneration in Parkinson's disease (PD) and dementia with Lewy bodies (DLB) (6-14). For the most part, these α-synuclein aggregates are densely compact and can be immunostained for multiple additional components including ubiquitin, synphilin-1, and heat shock proteins (HSPs), which suggests that protein misfolding or degradation is altered in cells that develop LBs. Mouse models of α-synuclein aggregation exist that mimic the findings in human brains and show intracellular α-synuclein aggregates (15-17). Aggregated α-synuclein molecules are less detergent soluble and these detergent insoluble species of α-synuclein can be detected in human brain, transgenic mouse models and in vitro models (18-21). Although it is known that the conformation of α-synuclein in Lewy bodies is significantly different than in the neuropil (22), it is unclear which conformation of α-synuclein contributes to inclusion formation. In addition to the formation of intracellular aggregates, α-synuclein is also cytotoxic. It has been postulated that α-synuclein oligomers found in PD tissue by Western blot analysis represent the toxic species (5).

Heat shock proteins (HSPs) belong to the family of chaperone proteins and are important in both refolding misfolded proteins and directing proteins towards proteasomal degradation (23-25). HSPs can be protective in several neurodegeneration models (26-29) and recent data in the fly model suggest that overexpression of the molecular chaperone Hsp70 protects against α-synuclein-induced degeneration (26,30). Hsp70 and its related co-chaperones may be important in α-synuclein misfolding. In fact, several HSPs and co-chaperones are associated with Lewy
Hsp70 reduces \( \alpha \)-synuclein aggregation and toxicity bodies \((26,31)\) and we previously reported that over-expression of Hsp70 and related molecules can prevent \( \alpha \)-synuclein aggregates formation \textit{in vitro} \((31,32)\). In this study we establish biochemical assays to assess aggregated \( \alpha \)-synuclein and investigate the influence of Hsp70 on these \( \alpha \)-synuclein species. We demonstrate that Hsp70 reduces \( \alpha \)-synuclein aggregation and toxicity via its refolding and degradation activities.
**Experimental procedures:**

**Human and mouse tissue**

α-Synuclein transgenic animals (line D) have been described (16), Hsp70 overexpressing mice were a kind gift of W.H. Dillmann (33) (UCSD, California, USA). Fresh-frozen brain tissue (temporal cortex) from twelve subjects (10 men and 2 women) with a pathological diagnosis of dementia with Lewy body disease (DLB) and eight subjects (4 men and 4 women) without a neurodegenerative disease that served as control were obtained from the Harvard Brain Tissue Resource Center, The Massachusetts Alzheimer Disease Research Center, and the MGH-MIT Udall center Brain Bank. The mean age of death for the DLB cases was 80.5 years (±6.4 SD), and 73 years (±12.1 SD) for controls. The mean post-mortem interval was 14.3 hours (±9.9 SD) for DLB and 13.2 hours (±1.6 SD) for control cases. Brain tissue was homogenized in 10 vol (w/v) of cold lysis buffer (Tris/HCl 50 mM pH 7.4, NaCl 175 mM, EDTA 5mM pH 8.0, protease inhibitor cocktail (Roche, Basel, CH)) and sonicated for 10 sec. (total cell lysates).

**Plasmid construction**

The constructs for human wild type untagged α-synuclein and its C-terminal tagged version (93 amino acid long tag referred to as Syn-T), and synphilin-1 have been described previously (31,32). The C-terminal tagged α-synuclein is a truncated α-synuclein-eGFP (enhanced green fluorescent protein) fusion protein that has 93 amino acids of eGFP fused to the carboxy-terminus of α-synuclein. This fusion protein is no longer fluorescent but has a propensity to aggregate when over-expressed in H4 cells. Co-transfection with synphilin 1 further enhances this aggregation (32) and thus co-transfection of SynT plus synphilin 1
functions as our α-synuclein inclusion model. Briefly, cDNA encoding the genes were cloned into pcDNA3.1 or pSI (Promega, Madison, WI, USA) expression vectors. Human Hsp70 cDNA was kindly provided by J.-C. Plumier, Massachusetts General Hospital, and subcloned into pcDNA3.1 (Clontech, Palo Alto, CA, USA).

**Cell culture and transfection**

Human H4 neuroglioma cells (HTB-148 - ATCC, Manassas, VA, USA) were maintained in OPTI-MEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. H4 cells were passaged 24 h prior to transfection and plated in four-well chamber slides for immunocytochemistry (Labtek, Nalgen-Nunc, Naperville, IL, USA) or 100 mm cell culture dishes for analysis of cell lysates (Corning inc., Corning, NY, USA). Cells were transfected with equimolar ratios of plasmids using Superfect (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Co-transfection with an empty pcDNA3.1 vector was used as control. After 48 hours cells were washed with cold PBS, harvested by scraping in cold lysis buffer without detergents (Tris/HCl 50 mM pH 7.4, NaCl 175 mM, EDTA 5mM pH 8.0, protease inhibitor cocktail (Roche, Basel, CH)) and sheared 1x through a 30½ G needle followed by sonication for 10 sec. (total cell lysates).

**Detergent solubility fractionation and gel electrophoresis**

Detergent solubility was performed by adding Triton X-100 to total cell lysates (final concentration 1%) and incubating for 30 min on ice followed by centrifugation (15,000 x g, 60 min, 4°C). The supernatant was designated as *triton X-100 soluble fraction* and the pellet was redissolved in 2% SDS containing lysis buffer and sonicated for 10 seconds (*triton X-100 insoluble fraction*). Additional washing of the triton X-100 insoluble pellet was found to not alter...
the α-synuclein expression in this fraction (data not shown) and was omitted from the experiments. Protein concentration was determined using a Lowry protein assay. 20-40 ug of each cell lysate was loaded onto 4–20% or 10-20% Tris–Glycine gels (Invitrogen, Carlsbad, CA, USA) for Western blot analysis. SDS-PAGE was performed with SDS-containing running and sample loading buffer, whereas native-PAGE (not containing any detergent) was performed with native (SDS-free) running and sample loading buffer (Invitrogen, Carlsbad, CA, USA). Protein was transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA) and blocked in blocking buffer (Lycor, Lincoln, NE, USA) for 1 h prior to addition of primary antibody (anti-α-synuclein - Syn-1 1:1000 – BD Transduction Lab, San Jose, CA, USA; or anti ubiquitin – SPA200 1:1000 – Stressgen, Victoria, BC, Canada) at room temperature for 1–2 h or overnight at 4°C. Following three TBS-T washes, infrared fluorescent labeled secondary antibodies (IRDye 800 anti-rabbit or anti-mouse, Rockland Immunochemicals, Gilbertsville, PA, USA 1:3000 or Alexa-680 anti-rabbit or anti-mouse, Molecular Probes, Eugene, OR, USA 1:3000) were incubated at room temperature for 1 h and immunoblots were processed and quantified using the Odyssey infrared imaging system (Lycor, Lincoln, NE, USA). Blots were also probed for actin (anti-actin, AC40, Sigma-Aldrich, St. Louis, MO, USA), or proteins on the gels were stained with coomassie blue (quantified using Odyssey infrared imaging system as loading controls). A mock/untransfected cell lysate was included in all cell culture experiments to control for non-specific and endogenous signal.

**Immunocytochemistry**

Cells were plated into 4-well chamber slides (Nunc, Naperville, IL, USA) and transfected as described above. 48 hours later the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at RT. After washing with PBS cells were permeabilized in TBS
Hsp70 reduces α-synuclein aggregation and toxicity containing 0.1% Triton X100 for 20 min at RT. After blocking in 1.5% normal goat serum containing TBS for 1 hour cells were incubated with primary antibody in blocking solution for 2 hours at RT or overnight at 4°C followed by washing with PBS and secondary antibody incubation for 1 hour. As a control for antibody specificity 1 µl of antibody solution was preabsorbed with 20 µg of recombinant protein (α-synuclein – Alpha-Diagnostics inc., San Antonio, TX, USA; Hsp70 – Stressgen, Vancouver, BC, USA) in PBS for 2 hours at 37°C followed by 16 hours at 4°C. After centrifugation at 4°C (15,000 rpm) for 15 minutes the immune complex free supernatant was diluted in blocking solution and applied to the cells 2 hours followed by washing with PBS and secondary antibody incubation for 1 hour. After a final wash, slides were cover slipped with aqueous mounting solution (GVA, Zymed, San Francisco, CA, USA) and subjected to fluorescence microscopy using a Nikon Eclipse TE300 inverted microscope.

**Toxicity assay**

Toxicology was analyzed 24 hours after transfection by measuring the release of adenylate kinase (AK) from damaged cells into the culture media using the ToxiLight™ (Cambrex, Walkersville, Maryland, USA) according to the manufacture’s protocol.

**Quantification of cells containing inclusions**

The number of cells containing α-synuclein immunopositive inclusions were assessed by immunocytochemistry using a Nikon Eclipse TE300 inverted microscope with a 20x objective as follows: Cells were assessed by an observer blind to the transfection conditions (i.e. the co-transfected plasmid). Approximately 300 to 400 cells, from two wells, were assessed for each experiment. A total of four experiments were performed with each condition. A positively
transfected cell was scored on the presence of significant \( \alpha \)-synuclein immunostaining compared to background (which in all cases was negligible). A transfected cell containing inclusions was scored on the presence of a detectable aggregate of \( \alpha \)-synuclein immunostaining. A cell was considered positive for inclusions independent of the size or number of inclusions. The percentage of cells containing inclusions compared to the total number of transfected cells was recorded.

**Statistical analysis**

Statistical analysis for comparison of groups was performed by ANOVA, with Fisher's probability of least significant differences (PLSD) post hoc test for significance.
Results

*Triton X-100 insoluble monomeric and HMW species of α-synuclein in DLB*

The current understanding of the pathophysiology of α-synuclein is that α-synuclein can form oligomers that then further aggregate into protofibrils and fibrils and result in tightly packed α-synuclein in Lewy bodies. To assess this process in human brains we performed biochemical assays using protein extracts from temporal neocortex of DLB cases and control brains. This brain area has only modest numbers of LBs and LNs in DLB (34). Detergent solubility testing revealed a triton X-100 insoluble fraction containing α-synuclein species that formed a high molecular weight (HMW) product ranging from 60 kD to 250 kD on SDS-PAGE (figure 1A). In addition to HMW aggregated α-synuclein, we also detected SDS soluble α-synuclein species in the triton X-100 insoluble fraction that ran at its expected monomeric weight on SDS-PAGE (~16 kD – figure 1B). Interestingly, quantification of these species from 12 DLB cases and 8 control brains on SDS-PAGE showed a significant increase in both the HMW and the monomeric triton X-100 insoluble fraction of α-synuclein in DLB brain compared to control brain. The HMW species and the monomeric α-synuclein found in the triton X-100 insoluble fraction was ~2.5 fold or ~2.0 fold higher in DLB, respectively (figure 1C). Analysis of α-synuclein levels in total protein fractions on SDS-PAGE showed no significant difference in total (SDS soluble) or triton X-100 soluble monomeric α-synuclein between DLB and control brain (figure 1C). Thus, DLB differs from control brain due to the presence of increased α-synuclein in triton X-100 insoluble fractions, rather than a change in the total amount of α-synuclein.
Hsp70 reduces α-synuclein aggregation and toxicity

**Triton X-100 insoluble and HMW α-synuclein species in α-synuclein transgenic animals**

We next asked if similar α-synuclein species were present in a mouse model of α-synuclein pathology. As reported by Masliah et al., α-synuclein transgenic mice (line D) have a substantial number of α-synuclein inclusions in the cortex that resemble some of the features of LBs found in human brain (16). To determine if different aggregation states of α-synuclein led to the same biochemical features we observed in human DLB cortex, we examined total cell lysates from the cortex of 6 month old α-synuclein transgenic and non-transgenic animals. Cell lysates were subjected to native PAGE and immunoblotted for α-synuclein. Interestingly, the HMW α-synuclein species running between 90 and 150 kD were detected in α-synuclein transgenic animals to a greater extent than in control animals (figure 2A). We next examined whether α-synuclein in the transgenic animals also displayed abnormal detergent insolubility. Indeed, ~2 fold more triton X-100 insoluble α-synuclein species were present on SDS-PAGE in α-synuclein transgenic animals than in control animals (figure 2B). Under SDS denaturing conditions, the triton X-100 insoluble α-synuclein resolved into only monomeric 16 kD species, suggesting that aggregated α-synuclein species are not as tightly packed and detergent resistant in transgenic animals as in DLB cases.

**Prevention of HMW and Triton X-100 insoluble α-synuclein species by Hsp70 in mice**

Recent data in the fly model suggest that overexpression of the molecular chaperone Hsp70 protects against α-synuclein-induced neuronal degeneration (26,30). In addition, data from our laboratory demonstrate that over-expression of Hsp70 and related molecules can
Hsp70 reduces α-synuclein aggregation and toxicity

prevent α-synuclein aggregates from forming in vitro (31) and that LBs immunostain robustly for heat shock proteins (26,31). Here we tested the hypothesis that overexpression of Hsp70 can reduce abnormal triton X-100 insoluble α-synuclein species observed in the α-synuclein transgenic mice. We bred α-synuclein transgenic mice with mice overexpressing rat Hsp70 (33). Performing the same biochemical assays from 5 Hsp70 x α-synuclein mice and 3 α-synuclein only expressing mice (5 month old) we found a significant ~5 fold (5.27 ±1.51) reduction in the amount of HMW species of α-synuclein (figure 3A). In addition, the amount of α-synuclein in the triton X-100 insoluble fraction was also decreased in the Hsp70 x α-synuclein animals by ~2 fold (35.74% ±3.40% compared to 17.40% ±6.36% - figure 3C). The total amount of SDS soluble monomeric α-synuclein did not differ between these mice (figure 3B), suggesting that Hsp70 specifically reduced abnormal conformations without changing total amount of α-synuclein.

Effect of Hsp70 on α-synuclein aggregation in vitro

We previously reported that overexpression of C-terminally modified α-synuclein (SynT), as well as the naturally occurring rat syn-2 isoform (which has a different carboxyl terminus) led to the formation of intracellular α-synuclein positive aggregates in H4 cells and that this phenomenon was increased by proteasome inhibition (31,32). By contrast, transient expression of untagged wild type (WT) α-synuclein did not produce intracellular inclusions. Co-expression of SynT with synphilin-1 facilitates the formation of intracellular aggregates (32) but synphilin-1 fails to induce aggregation when co-transfected with wild type α-synuclein. We used co-expression of SynT and synphilin-1 as an in vitro α-synuclein inclusion model (figure 4) to study the protective function of Hsp70 on α-synuclein aggregation.
Interestingly, we observed α-synuclein species that were similar to the species detected in DLB cases and transgenic mice models in protein lysates of H4 cells transfected with Syn-T and synphilin-1. Native PAGE of total protein lysates revealed an α-synuclein positive HMW product between 80 and 200 kD (figure 5A, lane 1) that was not detected in cells transfected with the untagged (non aggregating) wild type α-synuclein (lane 3) or MOCK transfected cells (lane 5). In addition we detected an approximately 55 kD α-synuclein positive band under native PAGE condition in Syn-T and WT-α-synuclein transfected cells, but no monomeric (16kD) form of α-synuclein (figure 5A). Triton X-100 solubility testing revealed triton X-100 insoluble α-synuclein only if cells were transfected with both Syn-T and synphilin-1 (Figure 5 C). Wild type α-synuclein was found in triton X-100 soluble fractions exclusively (Figure 5 C). Co-transfection of synphilin-1 did not alter this pattern compared to omitting synphilin-1 (data not shown).

We next asked if Hsp70 can reduce the HMW and the triton X-100 insoluble α-synuclein species in this model. Co-transfection of Hsp70 with Syn-T/synphilin-1 significantly reduced the HMW α-synuclein species (figure 5A lane 2) consistent with the effect of crossing α-synuclein transgenic mice with Hsp70 overexpressing animals (figure 3). It also reduced the 55 kD α-synuclein positive band in Syn-T and WT-α-synuclein transfected cells (figure 5A lane 2, 4). Furthermore, quantification of the 16 kD monomeric α-synuclein on SDS-PAGE in 7 different experiments demonstrated that total α-synuclein expression levels were reduced by 45% by Hsp70 (figure 5B).

Co-transfection with Hsp70 not only decreased total α-synuclein levels, it also reduced the triton X-100 insoluble α-synuclein species in Syn-T transfected H4 cells (Figure 5 C). In order to assess the refolding activities of Hsp70 on misfolded α-synuclein we determined the ratio of triton X-100 insoluble α-synuclein compared to the soluble fraction in six independent...
Hsp70 reduces α-synuclein aggregation and toxicity

experiments. This enabled us to measure changes in the triton X-100 insoluble fraction independent of the total protein levels. A comparison of the triton X-100 insoluble fraction in Hsp70 versus empty vector (control) co-transfected cells revealed that Hsp70 significantly reduced the percentage of α-synuclein in the triton X-100 insoluble fraction by ~50% (Figure 5D). Thus, Hsp70 leads to a decrease in the total amount of (misfolded) Syn-T as well as to a specific decrease in the percentage of Syn-T found in detergent insoluble fraction, suggesting either chaperone-related “refolding” and/or specific degradation of the misfolded detergent insoluble fraction.

**Influence of Hsp70 on cellular toxicity of α-synuclein overexpression**

Because Hsp70 has been shown to be protective in α-synuclein dependent toxicity in *drosophila*, we examined the effect of Hsp70 on the toxicity of Syn-T, which forms macroscopic aggregates, and wild type (WT) α-synuclein, which does not form aggregates, in our cell culture model. Transfection with wild type α-synuclein alone increased the cellular toxicity by ~2 fold (1.75 ±0.16) compared to MOCK transfected cells. Transfection with an irrelevant protein had no effect on toxicity (e.g. GFP, data not shown). Similarly, a ~1.5 fold (1.56 fold ±0.13) increase in toxicity was observed by transfection with Syn-T. Interestingly, co-transfection with Hsp70 significantly reduced the toxicity of both wild type α-synuclein and Syn-T by about 20% (figure 6). Thus α-synuclein appears to be cytotoxic regardless of whether it might form aggregates; Hsp70 can protect in either case.
Discussion

Our data suggest two major conclusions: First, we describe abnormal detergent insoluble forms of α-synuclein in human DLB cortex, α-synuclein transgenic mice, and tissue culture models of α-synuclein aggregation. Although the exact biochemical characteristics vary among these preparations, it seems clear that triton X-100 insolubility highlights a form of α-synuclein that is abnormal in the disease state. Second, we detected amelioration of this biochemical abnormality by over-expression of the molecular chaperone Hsp70 in two experimental systems. Hsp70 may have a role both in refolding and in degradation of misfolded α-synuclein molecules. Intriguingly, Hsp70 is also protective against α-synuclein toxicity. Thus molecular chaperones may be involved in regulating α-synuclein’s biochemical characteristics and toxicity, supporting the idea that molecular chaperones play a role in protecting against α-synuclein mediated neurotoxicity (26).

In Parkinson's disease and dementia with Lewy bodies α-synuclein accumulates in aggregates that are densely compact, and can be immunostained for multiple additional components, including the α-synuclein interacting protein, synphilin-1 (35,36). The incremental aggregation process of α-synuclein involves several modifications, misfolding, dimer- and oligomer formation, self-aggregation, and is linked to PD-associated mutations (5,22,37-50). In the current study we detected abnormal α-synuclein species based on their detergent solubility and electrophoretic mobility characteristics in three different systems; human DLB cortex, transgenic mice overexpressing wild type human α-synuclein, and a transiently transfected cell culture system. We found triton X-100 insoluble high molecular weight (HMW) and monomeric α-synuclein species in tissue extracted from the temporal cortex of patients with DLB product.
Both species were significantly increased in DLB patients compared to control brains. Total SDS
and triton X-100 soluble levels of α-synuclein were similar for DLB and control brains, suggesting that HMW and monomeric triton X-100 insoluble/SDS soluble species of α-synuclein specifically represent a portion of total α-synuclein that is abnormal in DLB. Since LBs and LNs are sparse in temporal neocortex, we speculate that the triton X-100 insoluble fraction may represent a biochemical lesion that is independent of the morphological alteration seen in DLB.

We detected a similar increase in triton X-100 insoluble and HMW α-synuclein species in the brains of α-synuclein transgenic mice compared to background mice. These mice develop intracellular α-synuclein inclusions that are associated with decrements of dopaminergic terminals in the striatum and result in motor and dopaminergic deficits (15,16). However, in the triton X-100 insoluble fraction we detected only increased monomeric α-synuclein species, and not the HMW α-synuclein species observed in DLB. This implies that the intracellular α-synuclein inclusions in 6 month old α-synuclein transgenic mice (line D) recapitulate some features of LBs, but lack the most highly aggregated α-synuclein species. Thus, the triton X-100 insoluble HMW species in DLB brain could embody the typical fibrillar components of aggregated α-synuclein in LBs that are not present in α-synuclein transgenic mice (16). These data are in accord with other findings that show detergent insoluble α-synuclein species in cases of DLB and PD and transgenic mice (5,21,51). Different protein processing techniques have been used by these groups which makes it likely that the detergent insoluble fraction of α-synuclein consists of a mixture of different α-synuclein species.

We also found that overexpression of Hsp70 in the α-synuclein transgenic mice protects against the development of abnormal α-synuclein aggregation. When we crossed α-synuclein transgenic mice with Hsp70 overexpressing mice (33), we observed a significant reduction in
Hsp70 reduces α-synuclein aggregation and toxicity

HMW and Triton X-100 insoluble α-synuclein species. This observation is the first direct evidence for an influence of Hsp70 on the aggregation process of α-synuclein in a mammalian in vivo model. Several reports support the idea that heat shock proteins - in particular Hsp70 - are protective in in vivo models of neurodegenerative diseases. Overexpression of Hsp70 leads to reduced toxicity in a model of SCA1 neurodegeneration (52), ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse (53), protects mice from malonate and 3-nitropropionic acid induced toxicity (54), and has some protective effect against cerebral ischemia (55-57). Modest protective effects were seen in a mouse model of Huntington’s disease (58), whereas Hsp70 clearly suppressed polyglutamine-mediated neurodegeneration in drosophila (59). Recent data in a fly model of α-synuclein pathology (60) suggest that overexpression of Hsp70 also protects against α-synuclein-induced degeneration in drosophila (26,30). Since our results demonstrate that the α-synuclein protein levels in α-synuclein transgenic mice and Hsp70 overexpressing x α-synuclein transgenic mice did not differ, we conclude that Hsp70 specifically effects the detergent insoluble species of α-synuclein.

We then evaluated the influence of Hsp70 on α-synuclein aggregate formation in a cell culture model. In in vitro models, overexpression of HSPs provides protection against aggregation or toxicity of polyglutamine aggregates (24,27,29,61-66), polyalanine aggregates (67), and diminishes aggregation and toxicity of mutant SOD in cultured neurons (68). Moreover, we previously reported that endogenous Hsp70 stains α-synuclein aggregates in our in vitro model of α-synuclein aggregation, and that overexpression of Hsp70 or its co-chaperones, prevents aggregates from forming (31). In the current study we illustrate that the biochemical characteristics of this α-synuclein aggregation model are comparable with the findings in DLB brains and in α-synuclein transgenic mice, in that HMW α-synuclein positive products and triton
Hsp70 reduces α-synuclein aggregation and toxicity

X-100 insoluble, SDS soluble monomeric α-synuclein species are formed. Similar to the transgenic mouse model, over-expression of Hsp70 reduced these triton X-100 insoluble species.

Our data support the possibility that enhanced chaperone protein function can alter α-synuclein misfolding \textit{in vivo} and toxicity \textit{in vitro}. Hsp70 has activity both for refolding misfolded proteins and, in collaboration with co-chaperones such as CHIP and parkin associated ubiquitinylation pathways (69), promote proteasomal degradation. Further studies will be necessary to distinguish these possibilities; however, the observation that (in \textit{drosophila}) Hsp70 protection from α-synuclein toxicity can be dissociated from prevention of α-synuclein aggregation suggests that Hsp70 acts specifically upon a toxic abnormal species of α-synuclein.
Hsp70 reduces α-synuclein aggregation and toxicity

References

Hsp70 reduces α-synuclein aggregation and toxicity


Hsp70 reduces α-synuclein aggregation and toxicity


Hsp70 reduces α-synuclein aggregation and toxicity


Figure legends

**Figure 1:**

**Triton X-100 insoluble and HMW α-synuclein species in DLB patients:** DLB patients have increased triton X-100 (Tx) insoluble HMW (A) and monomeric 16kd α-synuclein species (B). Quantification of the expression level of different α-synuclein species in brain tissue of temporal cortex of 12 DLB patients and 8 controls are shown in C. Relative expression of HMW species are normalized to control (1.00). Arbitrary expression intensities of total SDS soluble monomeric form of α-synuclein, as well as triton X-100 soluble and insoluble fraction show only a significant increase of triton X-100 insoluble monomeric α-synuclein level and portions.

**Figure 2:**

**Triton X-100 insoluble and HMW α-synuclein species in α-synuclein transgenic mice:** Native HMW and triton X-100 insoluble monomeric species of α-synuclein are increased in the brain of α-synuclein transgenic mice (Masliah line D). Total protein from the cortices of two 6 month old transgenic (Masliah line D and background) and two background mice were analyzed by native PAGE (A). Triton X-100 solubility testing was performed and fractions separated by SDS-PAGE (B).

**Figure 3:**

**Reduced Triton X-100 insoluble and HMW α-synuclein species in Hsp70 overexpressing α-synuclein transgenic mice:** Breeding of α-synuclein transgenic mice with mice overexpressing Hsp70 results in a reduction of HMW (A) and triton X-100 insoluble α-
Hsp70 reduces α-synuclein aggregation and toxicity

synuclein species (C). Levels of total monomeric α-synuclein (SDS soluble) were not changed (B) suggesting a specific reduction in higher aggregated and detergent insoluble forms of α-synuclein.

Figure 4:

In vitro α-synuclein aggregation model: A: Transient transfection of H4 neuroglioma cells with C-terminal tagged α-synuclein (Syn-T) and synphilin-1 results in α-synuclein immunopositive intracellular inclusions that also stain for endogenous Hsp70 (32). B and C: Preabsorption of anti α-synuclein antibody with recombinant α-synuclein protein (B) or anti Hsp70 antibody with recombinant Hsp70 protein (C) served as negative controls for antibody specificity. Reflection images demonstrate the presence of cells in the absence of antibody staining. Syn-T is a truncation of α-synuclein-eGFP with 93 amino acids of GFP fused to the C-terminus of α-synuclein. The fusion protein is not fluorescent but has a propensity to form intracellular aggregates when co-transfected with synphilin-1 (32). White bars indicate 10 μm.

Figure 5:

Hsp70 reduces triton X-100 insoluble and HMW α-synuclein species in vitro: A: HMW α-synuclein species ranging from 80-200 kD could be found by native PAGE (lane1). Hsp70 reduced both HMW α-synuclein in the Syn-T inclusion model (SynT lane 2) and the 55 kD species of untagged wild type (WT) α-synuclein that appears with native PAGE (lane 2,4). Endogenous α-synuclein in MOCK transfected cells also runs at 55 kD (enhanced intensity due to low expression levels –lane 5). B: Quantification of total monomeric Syn-T (34 kD on SDS-PAGE) was also reduced by Hsp70 suggesting enhanced degradation of α-synuclein. C: α-Synuclein was found in the triton X-100 insoluble fraction (“Tx insol”) in the Syn-T inclusion
Hsp70 reduces α-synuclein aggregation and toxicity model whereas WT α-synuclein only appeared in the triton X-100 sol fraction (“Tx sol”). Hsp70 reduced the amount of triton X-100 insoluble α-synuclein in the inclusion model (Syn-T). D: Changes in the proportion of triton X-100 insoluble Syn-T compared to soluble Syn-T was normalized to control (100%) and demonstrated a significant reduction in triton X-100 insoluble fraction of α-synuclein by co-transfection with Hsp70.

Figure 6:

Hsp70 protects from α-synuclein toxicity in vitro: Both α-synuclein and Syn-T significantly increase cellular toxicity by 1.75 (±0.16SEM) or 1.56 (±0.13SEM) fold, respectively (n=9, p<0.01, one sample t-test). Hsp70 significantly reduced toxicity for both WT-α-synuclein (A) and Syn-T (B). Toxicity was measured by estimating the amount adenylate kinase released by dead cells into the growth medium 24 hours after transfection of H4 cells.
A: Image showing protein bands with molecular weight markers.

B: Image showing 16 kD α-synuclein bands.

C: Table comparing DLB and control samples

<table>
<thead>
<tr>
<th></th>
<th>HMW smear (&gt;70 kD)</th>
<th>total SDS soluble (16kD)</th>
<th>Tx-soluble fraction (16kD)</th>
<th>Tx-insol. fraction (16kD)</th>
<th>% in insoluble fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DLB</strong></td>
<td>2.68 (±0.44)</td>
<td>45.25 (±4.88)</td>
<td>40.95 (±4.90)</td>
<td>4.30 (±0.39)</td>
<td>11.16% (±1.62%)</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td>1.00 (±0.10)</td>
<td>35.06 (±8.95)</td>
<td>33.46 (±9.01)</td>
<td>1.60 (±0.35)</td>
<td>5.69% (±1.27%)</td>
</tr>
</tbody>
</table>
A: HMW species 
\( \alpha \)-synuclein

B: total (16 kD) 
\( \alpha \)-synuclein

C: 16 kD \( \alpha \)-synuclein

<table>
<thead>
<tr>
<th></th>
<th>Tx-sol.</th>
<th>Tx-insol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )SYN</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Hsp70</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>
### A:

<table>
<thead>
<tr>
<th></th>
<th>Syn-T EV</th>
<th>Syn-T HSP70</th>
<th>WT-syn EV</th>
<th>WT-syn HSP70</th>
<th>MOCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>148 kDa</td>
<td><img src="image1.webp" alt="Image" /></td>
<td><img src="image2.webp" alt="Image" /></td>
<td><img src="image3.webp" alt="Image" /></td>
<td><img src="image4.webp" alt="Image" /></td>
<td><img src="image5.webp" alt="Image" /></td>
</tr>
<tr>
<td>96 kDa</td>
<td><img src="image6.webp" alt="Image" /></td>
<td><img src="image7.webp" alt="Image" /></td>
<td><img src="image8.webp" alt="Image" /></td>
<td><img src="image9.webp" alt="Image" /></td>
<td><img src="image10.webp" alt="Image" /></td>
</tr>
<tr>
<td>50 kDa</td>
<td><img src="image11.webp" alt="Image" /></td>
<td><img src="image12.webp" alt="Image" /></td>
<td><img src="image13.webp" alt="Image" /></td>
<td><img src="image14.webp" alt="Image" /></td>
<td><img src="image15.webp" alt="Image" /></td>
</tr>
</tbody>
</table>

### B:

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS soluble α-synuclein</td>
<td>100%</td>
<td><strong>54.94%</strong> (±6.75%) *</td>
</tr>
</tbody>
</table>

### C:

<table>
<thead>
<tr>
<th></th>
<th>EV</th>
<th>EV</th>
<th>HSP70</th>
<th>HSP70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx sol</td>
<td><img src="image16.webp" alt="Image" /></td>
<td><img src="image17.webp" alt="Image" /></td>
<td><img src="image18.webp" alt="Image" /></td>
<td><img src="image19.webp" alt="Image" /></td>
</tr>
<tr>
<td>insol</td>
<td><img src="image20.webp" alt="Image" /></td>
<td><img src="image21.webp" alt="Image" /></td>
<td><img src="image22.webp" alt="Image" /></td>
<td><img src="image23.webp" alt="Image" /></td>
</tr>
<tr>
<td>Syn-T (35kD)</td>
<td><img src="image24.webp" alt="Image" /></td>
<td><img src="image25.webp" alt="Image" /></td>
<td><img src="image26.webp" alt="Image" /></td>
<td><img src="image27.webp" alt="Image" /></td>
</tr>
<tr>
<td>WT-syn (16kD)</td>
<td><img src="image28.webp" alt="Image" /></td>
<td><img src="image29.webp" alt="Image" /></td>
<td><img src="image30.webp" alt="Image" /></td>
<td><img src="image31.webp" alt="Image" /></td>
</tr>
</tbody>
</table>

### D:

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100 insoluble fraction</td>
<td>100%</td>
<td><strong>52.54%</strong> (±5.87%) *</td>
</tr>
</tbody>
</table>