α-Synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome

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Received 18 October 2001; revised 24 October 2001; accepted 25 October 2001

First published online 8 November 2001

Edited by Jesus Avila

Abstract **α-Synuclein has been implicated in the pathogenesis of Parkinson's disease based on mutations in familial cases of the disease and its presence in Lewy bodies. Here we show that overexpression of wild-type human α-synuclein is sufficient to induce inclusion formation in SH-SY5Y cells. In this cellular model, proteasome inhibition leads to an increase of α-synuclein accumulation in vivo without ubiquitylation. In accordance, we find that in vitro, unmodified α-synuclein can be directly degraded by the 20S proteasome. These findings suggest an ubiquitin-independent mechanism of proteasomal degradation for α-synuclein and other natively unfolded proteins.** **© 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: α-Synuclein; 20S proteasome; Lewy body

1. Introduction

α-Synuclein is a natively unfolded protein of uncertain function that is abundantly expressed in human brain [1–3]. Genetic studies have identified two mutations in the α-synuclein gene in early-onset autosomal dominant Parkinson's disease (PD) [4,5]. PD is characterized neuropathologically by the presence of filamentous inclusions known as Lewy bodies (LB) and Lewy neurites [6]. α-Synuclein was found to be the main component of LB filaments in PD, as well as in dementia with LB (DLB) and in glial cytoplasmic inclusions of multiple system atrophy, which are referred to as the α-synucleinopathies [7–9]. LB pathology and α-synuclein aggregation in neurons may contribute mechanistically to their dysfunction and degeneration as also suggested by animal models [10–13]. Although the mechanism by which α-synuclein accumulates in neurons is largely unknown, impaired degradation could be involved.

Conjugation of multiple copies of ubiquitin to cellular targets (ubiquitylation) is a central signal for proteolytic degradation by the 26S proteasome complex [14]. The 26S proteasome is composed of two 19S regulatory 'caps' in association with a 20S core complex, the latter of which contains the protease active site. Recent work has suggested a potential in vivo role for the free 20S particle in the ubiquitin-independent degradation of oxidatively damaged proteins [15] or certain small relatively unfolded proteins such as the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ [16].

The great abundance of ubiquitin-immunoreactivity in LB has long been taken as evidence that altered function of the ubiquitin-proteasome pathway is a primary suspect in PD pathogenesis [17]. Consistent with this idea, genetic studies of familial cases of PD and juvenile Parkinsonism have revealed mutations in enzymes involved in this pathway [18,19] and proteasomal function is impaired in sporadic cases of the disease [20]. Moreover, impaired degradation of mutant α-synuclein by the proteasome was shown in SH-SY5Y cells [21]. However, the link between α-synuclein accumulation into LB and proteasome impairment is complicated by the fact that up to 10% of inclusions in α-synucleinopathies were found to be negative for ubiquitin [8,9,22] and that α-synuclein degradation by the proteasome has recently been questioned [23].

To investigate α-synuclein degradation and the role of proteasome impairment in α-synuclein accumulation, we produced stably transfected SH-SY5Y cells over-expressing wild-type (wt) α-synuclein. We found that chronic inhibition of the proteasome in vivo increases accumulation of non-ubiquitylated α-synuclein. Furthermore, we show that α-synuclein can be directly degraded by the 20S proteasome in an ubiquitin-independent manner. These findings suggest that ubiquitin conjugation is not an absolute requirement for proteasome-mediated α-synuclein degradation and that proteasome dysfunction could be related to non-ubiquitylated α-synuclein inclusions that have been reported in α-synucleinopathies [8,9,22].

2. Materials and methods

2.1. Cell culture

SH-SY5Y human dopaminergic neuroblastoma cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (Life Technologies), supplemented with 10% fetal calf serum (Life Technologies) and 1% penicillin/streptomycin. For proteasome inhibition, cells were grown to 60–70% confluency and treated for 24 and 48 h with 10 μM lactacystin (Affinity Research Products). α-Synuclein half-life was investigated in transfected cells by inhibiting protein synthesis with 50 μg/ml cycloheximide (Sigma-Aldrich) for 6, 12 and 19 h.

2.2. Plasmid construction and transfection

Wt human α-synuclein cDNA was subcloned into pcDNA3+ (Invitrogen). For transfection, cells were grown to 60–80% confluency, exposed to 20 μl of Lipofectin Reagent (Life Technologies) and 2 μg of plasmid DNA for 6 h in serum-free medium at 37°C, 5% CO_2. G418 (Life Technologies) at 300 μg/ml was added 48 h following transfection for selection of stable cell lines.

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Abbreviations: LB, Lewy body; PD, Parkinson's disease; wt, wild-type; DLB, dementia with Lewy bodies

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PII: S0014-5793(01)03115-5

2.3. Immunoblotting

SH-SY5Y cells were lysed in RIPA buffer which contained a cocktail of protease inhibitors. Protein amount was determined using a protein assay kit (Bio-Rad). For immunoblotting, 10–20 μg of cell lysates were loaded per lane and separated on 15% SDS–PAGE and transferred onto Immobilon P (Millipore). After blocking with 1% gelatine in TBS, blots were incubated overnight with polyclonal antiserum PER4 (1:1000) directed against the C-terminus of α-synuclein or anti-human α-synuclein antibody LB509 (1:5000; Zymed). Bound antibodies were visualized using the avidin–biotin–peroxidase method and 3,3′-diaminobenzidine as substrate (Vectastain Elite kit, Vector Laboratories). For anti-ubiquitin monoclonal antibody (1:500; Chemicon), anti-ubiquitin polyclonal antiserum (1:500; Dako) and anti-βAPP (1:100; Boehringer Mannheim), membranes were incubated in 5% milk in TBS and developed with peroxidase-conjugated rabbit anti-mouse (1:2000) or goat anti-rabbit (1:5000) antiserum and enhanced chemiluminescence (NEN Life Science).

2.4. Immunocytochemistry

SH-SY5Y cells were fixed in 4% paraformaldehyde and incubated overnight at 4°C with anti-α-synuclein antisera PER4 (1:500), or PER7 (against the first 120 amino acids; 1:1000) and anti-ubiquitin polyclonal antiserum (1:1000; Dako) or monoclonal antibody (1:250; Chemicon). Cells were then incubated for 2 h at room temperature with biotinylated secondary antibody (Vector Laboratories), followed by fluorescein-avidin (Vector Laboratories) for 1 h at room temperature. Cell nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI; 1 ng/μl; Sigma-Aldrich). Fluorescence was analyzed using a Leitz DMRB microscope.

2.5. Purification of inclusions and electron microscopy

The nature of α-synuclein inclusions was investigated by electron microscopy of sarcosyl-extracted preparations as previously reported for dispersed α-synuclein filaments from DLB brain [8]. For immunogold labeling, antiserum PER4 (1:100) was used.

2.6. Proteasome activity assays

Cells treated with 10 μM lactacystin for 12, 24 and 48 h and untreated control cells were lysed in 50 mM Tris buffer, pH 7.4, containing 5 mM ATP, 10 mM $MgCl_2$, 1 mM DTT with repeated cycles of freezing and thawing. Ten micrograms of protein from each lysate were treated with or without lactacystin (10 μM final concentration) and incubated for 2 h at 37°C in 100 μl of 10 mM Tris, pH 7.5, containing 50 μM of the chymotryptic fluorogenic peptide substrate syccinyl-Leu-Leu-Val-Tyr-AMC; (Affinity Research Products). The reaction was terminated by the addition of 2 ml of 80 mM sodium acetate, pH 4.5. Release of free AMC was determined using a Shimadzu RF-5000 spectrofluorophotometer (excitation 360 nm, emission 460 nm).

2.7. In vitro 20S proteasomal degradation of α-synuclein

Recombinant α-synuclein was expressed and purified as described previously [3]. Purified human 20S proteasomes (0.5 nM; Affiniti Research) were incubated with α-synuclein (1.8 μM) in a final volume of 30 μl of 25 mM Tris–HCl, pH 7.5, at 37°C. At different time points, 5 μl of reaction mixture was removed. Samples were separated on 5–20% SDS–PAGE and transferred to nitrocellulose (Hybond C-Super; Amersham). Membranes were blocked in 5% milk in TBS, and probed with anti-human α-synuclein antibody LB509 (1:5000; Zymed) for 2 h at room temperature. Blots were developed with peroxidase-conjugated rabbit anti-mouse antiserum (1:5000) and enhanced chemiluminescence (NEN Life Science). As a control, human α-lactalbumin (Sigma-Aldrich) was incubated with 20S proteasomes under identical conditions to α-synuclein and blots were processed essentially as above, except membranes were blocked in 0.1% BSA, and probed with anti-human α-lactalbumin antiserum (1:15 000; Sigma-Aldrich).

2.8. Statistical analysis

To ascertain differences among the various treatment groups of SH-SY5Y cells, logistic regression analysis was performed to allow adjustment for inter-experiment variation. All experiments were repeated four times.

3. Results

3.1. Over-expression of wt α-synuclein in SH-SY5Y cells leads to formation of intracellular inclusions

To study the metabolism and aggregation of wt α-synuclein in vivo, we generated SH-SY5Y cell lines stably over-expressing untagged wt human α-synuclein. Immunoblotting analysis confirmed that these cells significantly over-expressed α-synuclein compared to non-transfected cells and cells transfected with empty vector (controls) as shown in Fig. 1e. Using a panel of anti-α-synuclein antibodies, we found that control cells showed a diffuse distribution of α-synuclein in the cell soma (Fig. 1a). This was more intense in transfected cells 10–15% of which showed α-synuclein-positive cytoplasmic inclu-

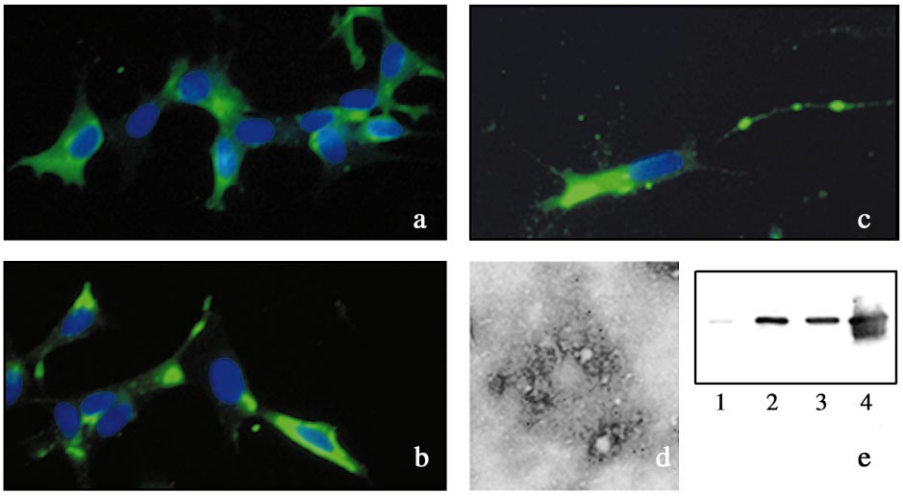

Fig. 1. α-Synuclein expression and accumulation in transfected SH-SY5Y cells. α-Synuclein staining in control (a) and stably transfected SH-SY5Y cells (b,c) using anti-α-synuclein antisera PER7 (a,b) and PER4 (c). α-Synuclein inclusions were present in cell bodies (b) and processes (c). Ultrastructurally, these inclusions were made of amorphous aggregates of α-synuclein as shown by immunogold electron microscopy (d) performed with PER4 antiserum. In (e), an increase of α-synuclein expression in transfected cells (lanes 2 and 3) compared to control cells (lane 1) is shown by immunoblotting using LB509 antibody. Endogenous, over-expressed and recombinant α-synuclein (lane 4) were detected as 19 kDa bands, in accordance with the expected apparent molecular weight of full-length protein.

sions (Fig. 1b, c). Control experiments where the primary antibody was omitted did not show any immunoreactivity (data not shown). In order to investigate the ultrastructural characteristics of these inclusions, we performed electron microscopy and immunogold labeling in cell extracts, which showed immunogold-labeled amorphous rather than filamentous material (Fig. 1d). No obvious toxicity was detected in cells with inclusions using DAPI staining or MTT assays (data not shown).

3.2. *Proteasome inhibition in vivo leads to accumulation of non-ubiquitylated α-synuclein*

Fluorogenic assays showed significant proteasome inhibition in transfected cells even 48 h following treatment with 10 μM lactacystin (Fig. 2a), without significant toxicity as assessed by MTT assays (data not shown). Using 10 μM lactacystin for 48 h for chronic proteasome inhibition, we found increased α-synuclein immunoreactivity on immunoblots of lysates from treated cells over-expressing α-synuclein (Fig. 2b). Semi-quantitative analysis using densitometry (Scion Image 1.62c Software, Scion Corp) showed a two-fold increase in α-synuclein immunoreactivity after 48 h of proteasome inhibition. No changes were observed after 24 h treatment. This is consistent with the finding that α-synuclein was remarkably stable in our transfected cells for over 19 h, as shown by de novo inhibition of protein synthesis with 50 μg/ml of cycloheximide. Under similar conditions, the short-lived βAPP protein used as control disappeared within the first 6 h (Fig. 2c). Furthermore, complete proteasome inhibition led to accumulation of poly-ubiquitylated substrates and disappearance of free ubiquitin (Fig. 2d). However, in the same preparations, no ubiquitylated α-synuclein was found either directly or following immunoprecipitation with anti-α-synuclein antibodies (data not shown).

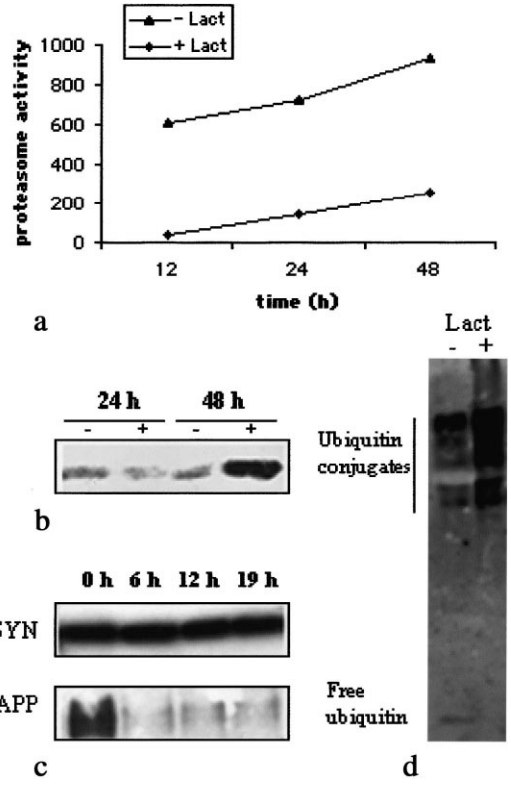

Fig. 2. Lactacystin treatment leads to accumulation of non-ubiquitylated α-synuclein. a: Graph indicating chymotryptic proteasomal activity (arbitrary units) in cells treated with (+Lact) or without (−Lact) lactacystin after 12, 24 and 48 h. b: Cells over-expressing α-synuclein were treated (+) or not (−) with 10 μM lactacystin for 24 and 48 h and immunoblotted using antiserum PER4. Semi-quantitative analyses using densitometry revealed a two-fold increase in the accumulation of α-synuclein only after 48 h. c: α-Synuclein and βAPP expressions were analyzed by immunoblotting cell extracts treated with cyclohexamide and tested at the indicated time points. While α-synuclein is stable at all time points, βAPP is almost completely degraded within the first 6 h. d: Total protein lysates were analyzed by immunoblotting with anti-ubiquitin antibodies before (−) and after (+) lactacystin treatment. Note the accumulation of poly-ubiquitin conjugates and the disappearance of free ubiquitin following inhibition of the proteasome pathway.

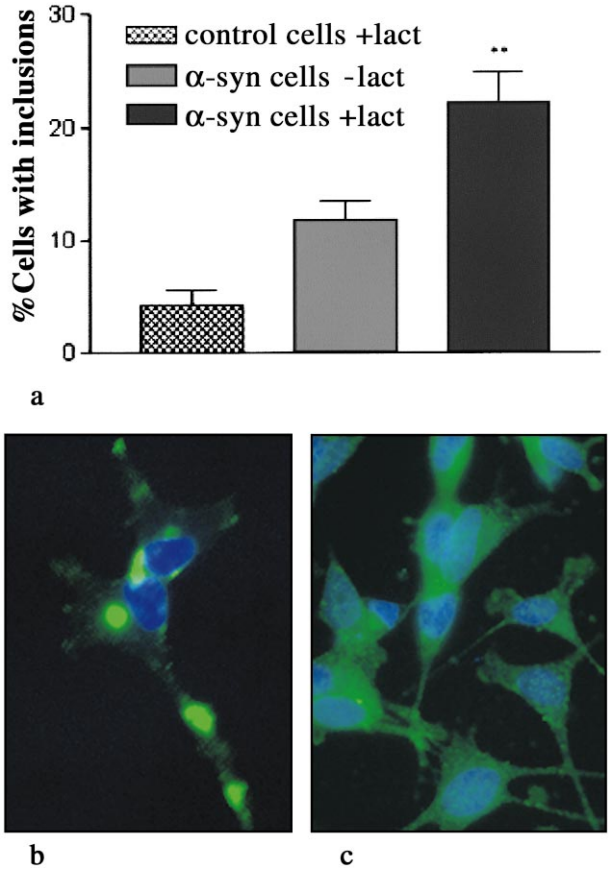

Fig. 3. Proteasome inhibition increases the formation of ubiquitin-negative α-synuclein-positive inclusions. a: A significant increase in the percentage of cells with α-synuclein-positive inclusions was found ($P < 0.006$, $n = 4$) in treated (α-syn cells +lact) compared to untreated cells (α-syn cells −lact) over-expressing α-synuclein. A small number of cells with α-synuclein-positive inclusions were also observed in treated control cells (control cells +lact). The increase in inclusion number after lactacystin treatment was clearly demonstrated with both PER7 (b) and PER4 (not shown) antisera. No inclusions were stained using either anti-ubiquitin polyclonal antiserum (c) or monoclonal antibody (not shown) before or after lactacystin treatment.

3.3. *Proteasome inhibition increases the formation of ubiquitin-negative α-synuclein inclusions*

To investigate whether α-synuclein accumulation following lactacystin treatment was linked to aggregation, we quantified cells with α-synuclein-positive inclusions after inhibition of the proteasome in vivo for 48 h. We found a two-fold increase

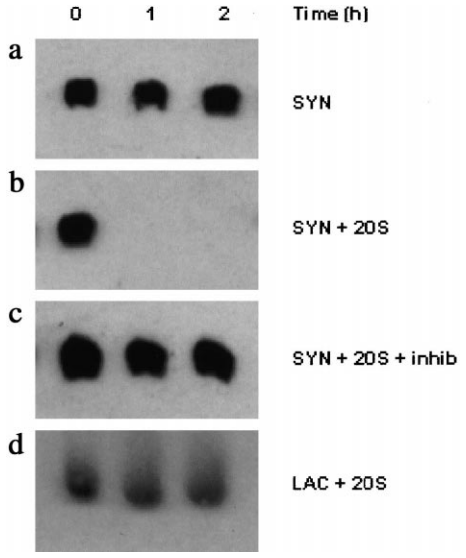

Fig. 4. α-Synuclein is degraded by purified 20S proteasomes in an ubiquitin-independent manner. Immunoblots were developed with anti-α-synuclein antibodies (LB509; panels a–c) or anti-α-lactalbumin antibodies (panel d). a: Purified recombinant α-synuclein alone (1.8 μM) is stable under the conditions of the degradation assay. b: Addition of purified 20S proteasomes (0.5 nM) catalyzes rapid degradation of recombinant α-synuclein, as assessed by loss of immunoreactivity. c: 20S proteasome-catalyzed degradation is completely inhibited by the addition of lactacystin (10 μM). d: Under identical conditions to a–c, purified human α-lactalbumin was completely resistant to 20S proteasome degradation.

($P < 0.006$, β-coefficient = 40.9, 95% confidence interval 11.9–70.02) in the percentage of cells with inclusions (Fig. 3a). Interestingly, although cells over-expressing α-synuclein showed significant increase in α-synuclein-positive inclusion formation after lactacystin treatment (Fig. 3b), two different well-characterized anti-ubiquitin antibodies failed to stain α-synuclein inclusions (Fig. 3c), demonstrating that the aggregated α-synuclein is not ubiquitylated.

3.4. α-Synuclein is degraded by purified 20S proteasomes

The accumulation of α-synuclein and the absence of poly-ubiquitylated forms of the protein suggested that α-synuclein could be degraded by the proteasome in vivo in an ubiquitin-independent manner. Since α-synuclein is natively unfolded, we hypothesized that the 'unfoldase' activity of the 19S regulator may not be necessary for its proteasomal degradation. We therefore performed an in vitro assay to determine whether α-synuclein can be directly degraded by the 20S proteasome. We incubated purified recombinant α-synuclein with purified human 20S proteasomes and found that the 20S proteasome was able to efficiently degrade α-synuclein in an ubiquitin-independent manner (Fig. 4a,b). This reaction was completely inhibited by the addition of 10 μM lactacystin (Fig. 4c). Under similar conditions, human α-lactalbumin, a tightly folded protein of similar molecular size to α-synuclein, used as control, was completely resistant to 20S proteasome degradation (Fig. 4d).

4. Discussion

Our data show that over-expression of wt α-synuclein in SH-SY5Y cells is sufficient to induce its accumulation into cellular inclusions. Furthermore, we find that inhibition of the proteasome with lactacystin leads to an increase in soluble and aggregated α-synuclein. However, neither α-synuclein nor α-synuclein-positive inclusions were ubiquitylated before and after proteasome inhibition, suggesting that ubiquitylation is not an absolute requirement for proteasome-mediated α-synuclein degradation. In agreement with these data, we find that in vitro α-synuclein can be degraded by the 20S proteasome in an ubiquitin-independent manner.

Whether the proteasome pathway is involved in α-synuclein metabolism has been a matter of debate. An early study by Bennett et al. [21] showed that α-synuclein transiently transfected in SH-SY5Y cells is degraded by the proteasome. However, in their study, Bennett et al. [21] used very high concentrations (40 μM) of a lactacystin catabolite (β-lactone), which could interfere with proteasome-unrelated activities as pointed out by Ancolio et al. [23] who instead found that lower doses of lactacystin (5 μM) did not affect the metabolism of either wt or mutant α-synuclein in HEK cells or murine neurons over-expressing α-synuclein. Although it is possible that proteasome-mediated α-synuclein degradation varies between different cell types, here we show that at low concentrations of lactacystin, non-toxic for SH-SY5Y cells, chronic exposure rather than shorter treatment enhances α-synuclein accumulation. Accordingly, we observed an increase in the accumulation of α-synuclein after 48 h rather than 24 h of proteasome inhibition. This is consistent with the finding that α-synuclein in our cells was stable over 19 h after de novo inhibition of protein synthesis. In agreement with this remarkable stability of cellular α-synuclein, a previous study reported a very long half-life for α-synuclein in stably transfected cells [24].

Interestingly, following lactacystin treatment, we only observed increased non-ubiquitylated α-synuclein. A similar observation was previously reported by Bennett et al. [21] who suggested that this could be due to concurrent de-ubiquitylation of α-synuclein by isopeptidases. However, this is less likely, since in our study we readily detected accumulation of poly-ubiquitylated substrates after lactacystin treatment. Instead, we show for the first time that α-synuclein can be directly degraded by the 20S core of the proteasome in an ubiquitin-independent manner.

α-Synuclein is a natively unfolded protein which only adopts a helical conformation upon binding to lipid membranes [25]. Because of its lack of secondary structure, it is therefore possible that in vivo, unfolded α-synuclein could bypass the need for ubiquitylation and unfolding by the 19S regulator, and enter directly the 20S proteasome where it is degraded. Consistent with this notion, recent reports have shown that other unfolded proteins can be degraded by the 20S proteasome in an ubiquitin-independent manner [16,26].

Here we also show that unmodified full-length α-synuclein over-expression in SH-SY5Y cells leads to accumulation into ubiquitin-negative cytoplasmic inclusions and that this process is further enhanced by inhibition of the proteasome pathway. Several groups, using different cellular models, reported the formation of α-synuclein-positive inclusions, which were variably stained for ubiquitin [27,28]. More recently, Rideout et al. [29] showed that proteasomal inhibition in non-transfected PC12 cells leads to formation of ubiquitin-positive inclusions, some of which contain α-synuclein which is not ubiquitylated. Our observations suggest that ubiquitylation could be a late

event in α-synuclein accumulation. It is possible that α-synuclein is degraded in an ubiquitin-independent manner and ubiquitylation occurs only when the protein becomes post-translationally modified, for example glycosylated [30], or abnormally folded, e.g. aggregated into filaments. However, the 20S proteasome can directly degrade oxidatively damaged proteins [15] and α-synuclein, which is nitrated in PD [31], although post-translationally modified, in this case, could be degraded by the 20S proteasome.

In α-synucleinopathies, α-synuclein degradation could be defective or the proteasome could not cope with the increased amount of protein leading to its accumulation. α-Synuclein could accumulate initially in a non-ubiquitylated form and subsequently becomes ubiquitylated, although there is yet no direct biochemical proof that α-synuclein is ubiquitylated in LB. A number of observations support the hypothesis of an ubiquitin-independent degradation of α-synuclein in PD. First, 20S function is impaired in PD [20] and 20S proteasome is found in LB [32]. Second, up to 10% of all α-synuclein inclusions in α-synucleinopathies are ubiquitin-negative. Furthermore, the evolution of α-synuclein deposits in PD starts with ubiquitin-negative cloud-like staining found in morphologically normal neurons, progressing to more compact classic LB usually immunoreactive for both α-synuclein and ubiquitin [22].

Further studies are needed to clarify whether 20S proteasome impairment in vivo is associated with accumulation of α-synuclein in α-synucleinopathies.

Acknowledgements: We would like to thank Drs R.A. Crowther for electron microscopy and M. Goedert for recombinant α-synuclein. This research was supported by an ORS Award, Trinity College IGS, and the James Baird fund (G.K.T.), the Wellcome Trust (R.L.), and the UK PD Society (M.G.S.).

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