Cathepsin D Is the Main Lysosomal Enzyme Involved in the Degradation of α-Synuclein and Generation of Its Carboxy-Terminally Truncated Species †

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Cathepsin D is the main lysosomal enzyme involved in the degradation of α-synuclein and generation of its carboxy-terminally truncated species†

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Abstract

α-Synuclein is likely to play a key role in the development of Parkinson's disease as well as other synucleinopathies. In animal models, overexpression of full-length or carboxy-terminally truncated α-synuclein has been shown to produce pathology. Although the proteosome and lysosome have been proposed to play a role in the degradation of α-synuclein, the enzyme(s) involved in α-synuclein clearance and generation of its carboxy-terminally truncated species have not been identified. In this study, the role of cathepsin D and calpain I in these processes was analyzed. *In vitro* experiments, using either recombinant or endogenous α-synuclein as substrates and purified cathepsin D or lysosomes, demonstrated that cathepsin D degraded α-synuclein very efficiently, and that limited proteolysis resulted in the generation of carboxy-terminally truncated species. Purified calpain I also cleaved α-synuclein, but carboxy-terminally truncated species were not the main cleavage products, and calpain I activity present in cellular lysates was not able to degrade the protein. Knockdown of cathepsin D in cells overexpressing wild-type α-synuclein increased total α-synuclein levels by 28% and lysosomal α-synuclein by 2-fold. In *in vitro* experiments, pepstatin A completely blocked the degradation of α-synuclein in purified lysosomes. Furthermore, lysosomes isolated from cathepsin D knockdown cells showed a marked reduction in α-synuclein degrading activity, indicating that cathepsin D is the main lysosomal enzyme involved in α-synuclein degradation. Our findings suggest that upregulation of cathepsin D could be an additional therapeutic strategy to lessen α-synuclein burden in synucleinopathies.

Parkinson's disease (PD)[1], dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, and multiple system atrophy are among diseases collectively known as synucleinopathies [1-3]. This group of disorders is characterized by the presence of brain inclusions composed mainly of filamentous aggregates of α-synuclein (α-syn).

The role of α-syn in synucleinopathies is supported by: 1) the identification of α-syn as the main component of Lewy bodies and Lewy neurites, two characteristic pathological inclusions found in all sporadic and familial cases of PD, and in other neurodegenerative diseases [1-3]; and 2) the discovery of point mutations in the α-syn gene in familial forms of autosomal PD [4,5]. A direct link between α-syn and PD is further supported by the discovery that multiplications of the wild-type sequence of the α-syn gene cause autosomal-dominant

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[1]**Abbreviations.** PD, Parkinson's disease; α-syn, α-synuclein; α-synΔC, carboxy-terminally truncated α-syn; P-(α-syn), phosphorylated α-synuclein; tet, tetracycline; CKII, casein kinase II; shRNA, small hairpin RNA.

parkinsonism [6-8]. Mutations in the α-syn gene in familial cases of PD lead to α-syn aggregation by increasing either the propensity of the protein to aggregate (*e.g.* A53T and A30P) or its expression (duplications and triplications).

Genetic and experimental data support the notion that high α-syn levels are a risk factor for the development of synucleinopathies; therefore, stimulation of pathways involved in α-syn degradation to reduce high steady-state levels of the protein could be a therapeutic approach. However, incomplete degradation of α-syn resulting in the generation of carboxy-terminally truncated α-syn (α-synΔC) species can be detrimental, since these fragments are known for their high tendency to self-aggregate and to induce the aggregation of full-length α-syn [9, 10]. α-SynΔC species with molecular weights of around 12 kDa and 10 kDa are ubiquitously found in cells and brain tissue, suggesting that these α-synΔC species are generated as part of normal α-syn metabolism [9]. Yet, their presence is increased in cells expressing disease-linked mutations of α-syn and in PD brains [9], indicating that they might be pathologically relevant.

In vitro and *in vivo* data support the involvement of the proteosomal [11,12] and lysosomal pathways in α-syn degradation [13-15]. The 20S subunit of the proteosome has been shown to produce truncated α-syn fragments similar to those found in patients and animal models [11]. In the lysosomal pathway, α-syn has been proposed to be degraded by chaperone-mediated autophagy [15]. Dysfunction of either the proteosomal or lysosomal pathways could, therefore, trigger or exacerbate α-syn aggregation.

Previous studies from our lab suggest that the lysosomal enzyme cathepsin D is involved in α-syn degradation and generation of α-synΔC species [16]. Another candidate enzyme is calpain I, which has been shown in *in vitro* experiments to cleave both the N- and C-terminus, and the non-amyloid component domain of α-syn [17].

In this study, the role of cathepsin D and calpain I in the proteolytic processing of α-syn was analyzed using cells overexpressing wild-type α-syn.

Experimental Procedures

Antibodies

The following antibodies against α-syn were used: syn-1 from BD Bioscience (Rockville, MA), Ab98 from Mayo Clinic Jacksonville, and LB509 from Santa Cruz Biotechnology (Santa Cruz, CA). The other antibodies used in the study were: P-Ser, that recognizes phosphoserine in α-syn, from Wako (Osaka, Japan), cathepsin D from Dako (Carpinteria, CA), calpain I from Santa Cruz Biotechnology (Santa Cruz, CA), H4B4 antibody against Lamp2 from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), spectrin and GAPDH from Chemicon (Temecula, CA), and ß-actin from Sigma-Aldrich (St. Louis, MO).

Brain tissues

Human and mouse brain samples were obtained from the Mayo Clinic Jacksonville Brain Bank and Animal Facility, respectively. The Brain Bank and Animal Facility protocols are approved by institutional ethics committees.

Cell culture

α-Syn transfectant 3D5 cells, derived from the human neuroblastoma BE2-M17D cell line, express human wild-type α-syn upon tetracycline (Tet)OFF induction [16]. 3D5 cells were maintained in DMEM containing 10% fetal bovine serum, and supplemented with geneticin (400 μg/ml), puromycin (1 μg/ml), and tetracycline (2 μg/ml) (TetON medium) at 37°C in 5% CO_2 atmosphere. α-Syn expression in 3D5 cells was induced by changing the medium (the

day after plating) to DMEM containing 10% fetal bovine serum, and supplemented with geneticin (400 μg/ml) and puromycin (1 μg/ml) (TetTOFF medium).

Immunoblotting analysis

3D5 cells were lysed in 20 mM MES pH 6.8 containing 80 mM NaCl, 1 mM $MgCl_2$, 2 mM EGTA, 10 mM NaH_2PO_4, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin (lysis buffer), and the lysates were centrifuged at 500 g for 15 min at 4°C. The protein concentration in the resulting supernatants was determined using the bicinchoninic acid assay (BCA) from Pierce Biotechnology (Rockford, IL). The supernatants were mixed with Tricine-SDS sample buffer, resolved by electrophoresis using 10–20% Tricine gels from Invitrogen (Carlsbad, CA), and transferred to nitrocellulose membranes for analysis.

Expression and purification of recombinant human wild-type α-syn

A detailed protocol describing the preparation and purification of recombinant α-syn is described elsewhere [18]. The purity of α-syn was estimated by Western blotting to be at least 95%.

Phosphorylation of recombinant α-syn

Four μg of recombinant human wild-type α-syn were incubated at 30°C with 5 μl of casein kinase II (CKII) (New England BioLabs) in 2.5 μl of 10x reaction buffer (New England BioLabs), and supplemented with 2.5 μl of 20 mM ATP. CKII was omitted in control samples. The efficiency of the phosphorylation reaction was assessed by Western blotting by taking aliquots after 1h, 3h, and overnight incubations. Two-thirds and 1/3 aliquots of the reaction were run on SDS-gels and transferred to nitrocellulose membranes for analysis. Blots with 2/3 aliquots were probed with P-Ser antibody and blots with 1/3 aliquots with syn-1 antibody. Maximum phosphorylation levels were achieved after a 3-h incubation with CKII.

Cathepsin D and calpain I cleavage of α-syn

Lysates (56 μg of protein) from 3D5 cells after 7 days in TetOFF medium were incubated at 37°C with 10 mU of cathepsin D in cathepsin D buffer (Sigma-Aldrich) and in the presence or absence of pepstatin A. Seven μl out of the 40 μl reaction volume, were taken at the time points indicated in the Figures and combined with loading buffer. Human and mouse brains were extracted with lysis buffer using a manual homogenizer. Brains extracts containing 140 μg of protein were incubated at 37°C with 10 mU of cathepsin D in cathepsin D buffer.

For cathepsin D cleavage of phosphorylated α-syn (P-α-syn), 320 ng of either α-syn control (incubated in kinase reaction buffer without CKII) or P-(α-syn) were incubated at 37°C with 5 mU of cathepsin D in a total volume of 40 μl. Five μl aliquots were taken at 0, 20 min, 1h, 3h, and after overnight incubation for Western blot analysis.

For calpain I cleavage of endogenous synuclein, lysates from 3D5 cells after 7 days in TetOFF medium were extracted with calpain I extraction buffer from a Biovision (Mountain View, CA) assay kit. Samples containing 100 μg of protein were incubated at 37°C in calpain I reaction buffer (Biovision) with 1 μl calpain I (~ 120 mU), or with calpain I and 1 μl of 62.5 mM calpain inhibitior III (Calbiochem). Control samples were incubated in calpain I buffer without the enzyme. Eight μl aliquots, out of a total reaction volume of 35 μl, were taken after 0, 1h, 4h, and overnight incubations, and mixed with loading buffer to stop the reaction before Western blot analysis.

Cathepsin D and calpain-1 activity assays

Cathepsin D activity was measured using an assay kit from Sigma. The protocol was basically as described by the manufacturer. Ten and 5 μg of cell lysates and lysosomes (see isolation protocol below) were assayed in a 96-well plate using an internally quenched fluorescent substrate. Pepstatin A was added to duplicate samples, and fluorescent values in these samples were used to determine cathepsin D specific fluorescence signals. The plates were incubated at 37°C and readings were performed at 10 min intervals. Values measured within the linear range of the reaction were used for the calculations.

Calpain I activity was measured using a kit from Biovision. The assay is based on fluorescence emittion upon calpain I cleavage of a fluorogenic substrate. Briefly, 3D5 cells were lysed with the extraction buffer supplied with the kit and the lysates were centrifugated at 10,000 g. Calpain I activity was measured in supernatants containing 75-100 μg of protein and data obtained within the linear range of the reaction were used for the calculations. Enzyme-specific signals were calculated by subtraction of signals from inhibitor-treated samples. Fluorescence signals for cathepsin D and calpain I assays were detected with Spectra max M2 plate reader and collected using Soft Max Protein 4.6 software (Molecular Devices).

Lysosomal isolation

Lysosomes from 3D5 cells were isolated using a lysosomal enrichment kit from Pierce Biotechnology. Cells from three 15 cm plates at ~70% confluency were lysed following the manufacturer's instructions. During the entire protocol the samples were maintained at 4°C. The lysates were combined with OptiPrep to a final concentration of 15%, and placed on top of a discontinuous density gradient with the following steps from top to bottom: 17%, 20%, 23%, 27%, and 30%. After centrifugation for 2h at 145,000 g, the top fraction containing the lysosomes was collected. Other membrane fractions present in the gradient were also collected and combined. The lysosomal fraction and the rest of the cellular membranes were diluted at least three times with PBS, and pelleted by centrifugation for 1h at 18,000 g. The membranes were washed once with PBS and recovered by centrifugation at 18,000 g.

Assay of α-syn degrading activities in subcellular fractions

When endogenous α-syn was used as a substrate, lysates and subcellular fractions (50 μg of protein) from 3D5 cells induced to produce α-syn for 7 or 8 days in TetOFF medium were assayed. When recombinant α-syn was used as a substrate, 2 μg of the protein were incubated with 5 μg of fractions from 3D5 cells maintained in TetON medium (to prevent α-syn expression). The reactions were carried out at 37°C in cathepsin D buffer and in a total volume of 45 μl. Overnight reactions were supplemented with 20 μg/ml pepstatin A, or 5 μg/ml leupeptin, or a cocktail of protease inhibitors (Sigma) with broad specificity for the inhibition of serine, cysteine, and metalloproteases. Five μl aliquots were removed at the time points indicated in the Figures, and immediately mixed with loading buffer to stop the reaction.

Cathepsin D and calpain I lentiviral shRNA knockdown

shRNA MISSION™ RNA interference vectors targeting cathepsin D and calpain I were obtained through a partnership agreement between Sigma and the Mayo Clinic RNA Interference Technology Resource. The Virapower lentiviral expression kit (Invitrogen) was used to produce lentiviral stocks in the packaging cell line 293FT according to the manufacturer's protocol.

Transduction of 3D5 cells was performed in 24-well plates as follows. Cells cultured for 4 days in TetOFF medium were plated (30,000 cells per well) using DMEM with 10% FBS and without antibiotics. The following day, the medium was removed and replaced with 250 μl of

virus-containing medium diluted with DMEM containing 10% FBS and supplemented with 6 μg/ml of Polybrene (Sigma). After overnight incubation, the virus-containing medium was replaced with DMEM containing 10% FBS. Three days later the cells were harvested and lysed for analysis.

Statistical analysis

Data is presented as the mean ± SEM from a minimum of three experiments. Statistical comparisons were made by Student's t-test. Statistical significance was inferred at $P < 0.05$.

Results

Limited cathepsin D proteolysis of γ-syn generates γ-synΔC species.

Initial experiments to study the cleavage of α-syn by cathepsin D were performed using commercial cathepsin D (from bovine origin) and lysates from a neuroblastoma cell line (3D5) generated in our lab. 3D5 cells inducibly overexpress wild-type human α-syn upon removal of tetracycline from the medium (TetOFF medium) [16]. Following 7 days of α-syn induction in TetOFF medium, lysates from 3D5 cells were prepared and incubated at 37°C with 10 mU of cathepsin D in the presence or absence of pepstatin A, a cathepsin D inhibitor. Aliquots from the reaction were taken at the time points indicated in Figure 1A and analyzed by Western blot. A time-dependent degradation of full length α-syn with a concomitant generation of lower molecular weight α-syn species, some of which comigrated with endogenous 12 kDa and 10 kDa α-synΔC species (indicated with broken and full arrows, respectively, in Figure 1A), were observed after probing the blot with syn-1 antibody. Lack of reactivity of the cathepsin D-generated species with C-20-R, an antibody against the C-terminal domain of α-syn, confirmed that these species were C-terminally truncated (Figure 1A). Furthermore, cathepsin D-generated fragments showed the same immunoreactivity towards the Ab98 (epitope aa 98-115) and LB509 (epitope aa 115-122) antibodies as did the endogenous 12 kDa and 10 kDa α-synΔC species (Figure 1A).

α-SynΔC species have been reported to be below detectable levels in mouse brain [9]. Since mouse and human α-syn sequences differ in two amino acids in the region that contains the putative cleavage sites to generate these species, we investigated if resistance to cathepsin D cleavage is responsible for the apparent low abundance of these species in mouse brain. Extracts from mouse and human brains were incubated with cathepsin D, and the reactions were analyzed at various time points. α-Syn in mouse and human brain extracts was susceptible to cathepsin D cleavage, and 12 kDa and 10 kDa α-synΔC species were observed in both samples (indicated by broken and full arrows, respectively, in Figure 1B). Therefore, low levels of α-synΔC species in mouse brain are unlikely to be caused by the resistance of mouse α-syn to cathepsin D cleavage.

Phosphorylation of Ser 129 in α-syn is an important post-translational modification that might play a role in α-syn pathology. Phosphorylation has been reported to increase the propensity of α-syn to aggregate, and it is apparently the most common α-syn modification found in Lewy bodies [19]. Ser 129 in α-syn is close to the putative cleavage sites for the generation of synΔC species. Therefore, we investigated if phosphorylation of Ser 129 affects cathepsin D cleavage of α-syn. For these experiments, recombinant human wild-type α-syn was phosphorylated with CKII. Unphosphorylated and phosphorylated α-syn were incubated in parallel with 10 mU of cathepsin D, and the reactions were analyzed at the time points indicated in Figure 1C. After a 30-min incubation, most full-length α-syn was no longer detected in either the unphosphorylated or phosphorylated samples (Figure 1C). The cathepsin D-cleavage profile of recombinant α-syn was slightly different to that of endogenous α-syn (compare A and B panels with panel C in Figure 1). The two main species generated by partial cleavage of

recombinant α-syn by cathepsin D were of approximately 12 kDa and 11 kDa molecular weight (indicated with broken and empty arrows, respectively, in Figure 1C), and the 10 kDa species was very faint. This profile is in agreement with a previous mass spectrometry analysis of cathepsin D-cleavage products of recombinant α-syn [20]. Higher levels of α-synΔC species were detected in the phosphorylated than in the unphosphorylated sample after overnight incubation (Figure 1C). The apparent resistance of α-synΔC species to cathepsin D cleavage could not have been caused by phosphorylation of Ser 129, since that residue is absent in α-synΔC species. It is possible that other sites phosphorylated by CKII affected further cathepsin D degradation of α-synΔC species.

Lysosomal fractions are highly enriched in α-syn degrading activity

To determine if cleavage of α-syn by endogenous cathepsin D also generates α-synΔC species, lysosomes were isolated from 3D5 cells after inducing α-syn expression for 7 days. In addition to the lysosomal fraction, the initial cell lysate and two other subcellular fractions were included in the experiments: cytosolic (supernatant of 100,000 g centrifugation) and cellular membranes lacking lysosomes. The purity of the lysosomal fraction was assessed by Western blot using membrane bound (Lamp2) and soluble (cathepsin D) lysosomal markers. More than 80% of these markers were recovered in the lysosomal fraction (for representative profiles of the lysosomal purification see Figures 2A and 4A), confirming the purity of this fraction. Cathepsin D analysis of the lysosomal fractions also provided an estimation of lysosomal integrity in the preparations.

Reactions containing equal amounts of protein from the lysate and each subcellular fraction were incubated in cathepsin D buffer pH 4 at 37°C. Samples were incubated for 4h or overnight (O/N lanes in Figure 2). Overnight incubations were performed in the absence (- lanes in Figure 2) or presence of the following protease inhibitors: pepstatin A (P), leupeptin (L) (an inhibitor of cysteine proteases, frequently used to inhibit cathepsins other than cathepsin D), or a cocktail of protease inhibitors (PI) that also includes leupeptin, but not pepstatin A. After a 4-h incubation without inhibitors, full-length α-syn was undetectable in the lysosomal fraction and was significantly reduced in the lysate and cytosolic fraction (Figure 2A). No changes in full-length α-syn were observed in the membrane fraction that did not contain lysosomes (Figure 2A). In the lysate and cytosolic samples, in addition to a reduction in full-length α-syn, a parallel increase of the 12 kDa α-synΔC species (indicated with a broken arrow in Figure 2A), and to a lesser, extent of the 10 kDa α-synΔC species (indicated with a full arrow) was observed. After overnight incubation in the absence of inhibitors, full-length α-syn was completely degraded in all the fractions. Addition of pepstatin A to the reactions prevented the degradation of α-syn in all fractions, except in the lysosomal fraction (Figure 2A). In the lysate and cytosolic samples containing leupeptin or the cocktail of protease inhibitors, the signals of full-length α-syn and the 12 kDa α-synΔC species were slightly higher than in the samples without inhibitors.

Because the degradation of α-syn was very fast in the lysosomal fraction, it was not possible to observe intermediate products of the cleavage. Therefore, in a following experiment, the aliquots were taken after shorter incubation times. As shown in Figure 2B, only a very weak signal of the α-syn degradation products was observed after 30 min. After a 2-h incubation in the presence of pepstatin A some of the full-length α-syn was preserved. Samples supplemented with leupeptin or the protease inhibitor cocktail showed slightly higher signals of full-length and α-synΔC species when compared to the sample incubated without inhibitors (Figure 2B).

Analysis of α-syn degradation in the lysosomal fraction proved to be challenging due to the relative low α-syn signal (approximately 2% of α-syn present in the lysates was recovered in the lysosomal fraction) and the high levels of proteolytic activity. Furthermore, relatively high levels of truncated α-syn species were present in this fraction (see time point 0 in Figure 2B). Therefore, to compare α-syn degrading activities between the subcellular fractions

independently of their α-syn content, fractions from non-induced (TetON medium) 3D5 cells were prepared and incubated with 2 μg of recombinant human wild-type α-syn. To slow down the reaction in the lysosomal fraction, the amount of protein used in the reactions was reduced by ten-fold. As in the previous experiment, overnight samples were left without protease inhibitors or were supplemented with pepstatin A, leupeptin, or a cocktail of protease inhibitors, and incubated at 37°C. After a 2-h incubation, two low molecular weight α-syn species were observed only in the lysosomal fraction: one of approximately 12 kDa and the other just below it of approximately 11 kDa (indicated by broken and empty arrows, respectively, in Figure 2C). This degradation profile was similar to the one obtained when recombinant α-syn was exposed to purified cathepsin D (Figure 1C). After overnight incubation without protease inhibitors, the 12 kDa and 11 kDa α-syn species were also observed in the lysates, and to a lesser extent in the cytosolic fraction. In the lysosomal fraction, the α-syn cleavage profile was similar in samples without inhibitors and with either leupeptin or protease inhibitors cocktail. In contrast, when pepstatin A was included in the lysosomal sample, the cleavage of recombinant α-syn was completely blocked (Figure 2C). In the lysate the generation of α-syn fragments was significantly reduced not only in samples that contained pepstatin A, but also in samples supplemented with leupeptin or the cocktail of protease inhibitors. In this experiment, little α-syn degradation activity was present in the cytosolic and membrane fractions, probably due to the ten-fold dilution of the fractions.

In summary, α-syn degrading activities in each subcellular fraction was proportional to its cathepsin D content, and therefore, highest in the lysosomes. The main degrading activity in the fractions was pepstatin A-sensitive. However, in the lysate and cytosolic fraction, additional proteolytic activities were likely to play a role in α-syn degradation.

To determine the optimum pH of the α-syn degradation activity present in the lysosomes, the experiment described in Figure 2 was repeated at different pHs, ranging from 4 to 7 in one unit increments. Maximum activity was achieved at pHs 4 and 5, which is consistent with the optimum pH of cathepsin D (data not shown). Thus far, we identified an endogenous activity in 3D5 cells that generated α-synΔC species. This activity is highly enriched in lysosomes, and based on its inhibition by pepstatin A, and optimum pH, is likely to be cathepsin D.

Reduction of cathepsin D activity using shRNAs results in increased α-syn levels.

To conclusively establish the role of cathepsin D in α-syn metabolism, its expression was knocked down in 3D5 cells using a lentiviral-mediated RNAi approach. We tested two dilutions (1/2 and 1/10) of viral preparations of a non-target control (NT) and four constructs (named 32, 34, 35, and 36) expressing small hairpin RNA molecules (shRNA) that target cathepsin D RNA. The efficiency of these constructs to reduce cathepsin D was quantitated using an activity assay. The enzyme activity in 3D5 lysates obtained from cells transduced with NT control was considered 100%. When the highest viral concentration (1/2) was used, cathepsin D activity was reduced by approximately 77% in lysates from cells transduced with constructs 32 and 36, by 93% with construct 35, and by 48% with construct 34 (Figure 3A). A reduced effect on the cathepsin D activity (~50% reduction) was observed when a 1/10 dilution of viral preparation of constructs 32 and 36 was used, while with 34 and 35 constructs the activity was similar to that obtained with a 1/2 dilution (Figure 3A). Western blot analysis showed a similar reduction in cathepsin D expression (Figure 3B).

α-Syn levels in 3D5 cells were analyzed by Western blot 3 days after infection with lentiviral shRNAs. α-Syn expression in lysates from cells transduced with constructs 32, 34, 35, and 36 was calculated and expressed as a percentage of α-syn expression in 3D5 lysates obtained from cells transduced with NT control. A significant increase ($P < 0.05$) in α-syn levels was observed with all the constructs: 29% with 32 and 35, 39% with 35, and 22% with 36.

To better assess the extent of the effect of cathepsin D knockdown on α-syn levels, subcellular fractions from 3D5 cells were isolated after lentiviral infection with NT control and constructs 32 and 35. Five μg of lysates and subcellular fractions were analyzed by Western blot. In the three samples (NT, 32, and 35), Lamp2 and cathepsin D were mostly recovered in the lysosomal fractions indicating the high level of purity of these fractions (Figure 4A). Comparison of cathepsin D levels between fractions from cells transduced with NT, and constructs 32 and 35 gave an indication of the knockdown efficiency (Figure 4A).

As expected, the main impact of cathepsin D knockdown on α-syn levels was observed in the lysosomal fraction (Figure 4A). α-Syn was higher in lysosomes isolated from cells transduced with construct 35, the most efficient construct in reducing cathepsin D expression (Figure 4A). Data from three experiments showed that constructs 32 and 35 increased lysosomal α-syn by 70% and 135% ($P < 0.05$), respectively, when compared to NT controls.

To determine if P-(α-syn) is degraded in the lysosomes, lysosomal fractions from the experiment shown in Figure 4A were re-run, and blots were probed with antibodies against α-syn and P-(α-syn) at position 129 (syn-1 and P-Ser, respectively in Figure 4B). P-(α-syn) signal in the samples followed the same trend observed in the blot probed with syn-1 antibody. The amount of P-(α-syn) in the samples was inversely correlated with their cathepsin D content, *i.e.*, highest in sample 35 (lowest cathepsin D expression), followed by sample 32 (intermediated cathepsin D expression), and was not detected in sample NT (highest cathepsin D expression) (Figure 4B).

To establish if knockdown of cathepsin D reduced the capability of lysosomes to degrade α-syn *in vitro*, activity assays similar to the ones previously described in Figure 2 were performed. Lysosomes isolated from cells transduced with NT, 32, or 35 constructs were incubated in cathepsin D buffer without any supplementation. After a 40-min incubation at 37°C, α-syn was reduced by 80 % in the NT lysosomal fraction that contained normal levels of cathepsin D activity. In contrast, even after a 2-h incubation, no reduction of α-syn was observed either in the 32 or 35 lysosomal fractions (Figure 4C). NT and 32 lysosomal samples were also assayed using recombinant α-syn as a substrate. Aliquots of the NT lysosomal sample, analyzed after a 2-h incubation, showed an increase in α-synΔC species. After a 4-h incubation, a marked reduction (more than 50%) of full-length α-syn, and a further increase of α-synΔC species was observed in the NT lysosomal sample. After the same incubation time no degradation of full-length α-syn was observed in the 32 lysosomal fraction (Figure 4D). Overnight incubation caused the complete degradation of full-length α-syn in the NT lysosomal fraction, but some full-length α-syn was still present in the 32 lysosomal fraction. These results further support the role of cathepsin D in α-syn degradation.

Endogenous calpain I activity is not sufficient to cleave α-syn

As mentioned in the Introduction, calpain I was proposed to participate in the metabolism of α-syn. Previous *in vitro* studies with calpain I showed that α-syn is cleaved by the enzyme. Although, data from two different labs disagree as to which are the main cleavage products [17,21], both studies suggest that calpain I cleavage at the C-terminus of α-syn generate fragments similar to endogenous α-synΔC species.

To investigate if endogenous calpain I activity can cleave α-syn, we lysed 3D5 cells using a calpain I extraction buffer from a commercial activity assay kit. Endogenous calpain I activity in the lysates was detected using a fluorogenic substrate supplied with the kit (data not shown). The lysates were incubated in calpain I reaction buffer under three different conditions: without any supplementation, with 120 mU of exogenous calpain I, or with exogenous calpain I plus calpain inhibitor III. Spectrin, an endogenous calpain substrate was used as a positive control. After a 2-h incubation, endogenous calpain I activity was not able to cleave either spectrin or

α-syn (− lanes in Figure 5A). During the same incubation time, spectrin was completely cleaved (the signal in the upper band shifted to the lower band) in samples supplemented with exogenous calpain I (+ lanes in Figure 5A). In contrast, in these samples only partial cleavage of α-syn by exogenous calpain I was observed, and a fragment migrating between full-length syn and the 12 kDa α-syn species was detected. Endogenous 12 kDa and 10 kDa α-synΔC species (indicated by broken and full arrows, respectively, in Figure 5A) were also noticeably reduced by exogenous calpain I. No further cleavage of full-length α-syn was observed after longer incubation times (6h and O/N). Cleavage of spectrin and α-syn was prevented in samples incubated in the presence of calpain inhibitor (+i lanes in Figure 5A).

In an attempt to improve the detection of calpain-cleaved fragments migrating in SDS-gels at the position of endogenous α-synΔC, we repeated the experiment with a different 3D5 lysate and doubled the amount of calpain I added. This time, after a 2-h incubation, full-length α-syn was completely degraded, but only faint signals of a series of fragments migrating below the full-length band were detected (Figure 5B). These experiments indicate that α-syn is not a good calpain I substrate, at least when compared to spectrin, and that α-synΔC species are not the main products after partial cleavage of the protein.

Reduction of calpain I activity in 3D5 cells with shRNAs does not significantly increase α-syn

To determine the role calpain I in maintaining steady-state levels of α-syn, the enzyme was knocked down using shRNAs. In addition to the NT control, four shRNA constructs (named 31, 32, 33, and 34) targeting different regions of calpain I RNA were tested. As was done for cathepsin D, two dilutions of medium (1/2 and 1/10) containing viral particles were used to infect 3D5 cells, and lysates were analyzed 3 days after infection. Reduction in calpain I expression was quantitated by Western blot analysis and calculated as a percentage of its value in NT controls. All four constructs were able to reduce calpain I expression by at least 70%. When a 1/2 dilution of the viral-containing medium was used for infections, the remaining calpain I expression was around 20% in cells infected with constructs 31 and 34, and 30% in cells transduced with constructs 32 and 33 (Figure 6A). Interestingly, infections with constructs 31 and 34 produced neurite outgrowth in 3D5 cells (data not shown). Similar morphological changes were previously reported with calpain inhibitors [22, 23]. The 1/2 dilution of viral preparations of constructs 31, 32, and 33 produced statistical significant increases of about 20% in α-syn levels (P=0.0046) (Figure 6B).

Discussion

In this study, we provide evidence supporting the role of cathepsin D in the metabolism of α-syn. Our data indicate that cathepsin D is the main lysosomal enzyme involved in the degradation of α-syn and generation of synΔC species.

Our *in vitro* studies using purified cathepsin D showed that limited exposure to the enzyme produced α-syn species with immureactivity and mobility in SDS-gels similar to the 12 kDa and 10 kDa α-synΔC species present in 3D5 lysates and brain extracts (panels A and B in Figure 1). Furthermore, mass spectrometry and amino acid analyses characterization of 12 kDa α-syn species from human brain [9] and of the species generated by cathepsin D cleavage [20] identified Glu123-Ala124 as one the cleavage sites.

In addition to cleave monomeric α-syn, cathepsin D cleaved insoluble α-syn generating a α-synΔC species of ~10 kDa (data not shown), suggesting that cathepsin D has access only to the exposed C-terminus of α-syn, but not to other regions of the molecule in the insoluble aggregate. The fact that cathepsin D did not cleave α-syn beyond the accessible C-terminus should not diminish its potential usefulness in novel therapeutic approaches, since soluble

oligomeric α-syn species and not insoluble α-syn aggregates are believed to be the toxic species [24,25]. These soluble oligomeric α-syn species have been reported to be degraded primarily in the lysosomes [26]. We also observed that in 3D5 samples high molecular weight α-syn species, likely to include soluble oligomeric species, were cleaved by cathepsin D (data not shown).

Since phosphorylation of α-syn at Ser-129 has been linked to pathology [19,27], it is important to establish if this modification alters the susceptibility of α-syn to degradation. Cuervo and colleagues recently showed that α-syn with a mutation that mimics phosphorylation was not taken into the lysosomes for degradation *via* chaperone-mediated autophagy [28]. In *in vitro* studies, we showed that cathepsin D cleaved both phosphorylated and unphosphorylated α-syn with the same efficiency (Figure 1C). Furthermore, we detected P-(α-syn) in lysosomal fractions, and, as was the case for unphosphorylated α-syn, the levels of P-(α-syn) were inversely correlated with the amount of cathepsin D present in the lysosomes (Figure 4B). Taken together, these results indicate that phosphorylation of α-syn does not prevent cathepsin D degradation of the protein in lysosomes.

Lysosomal fractions isolated from 3D5 cells were able to generate truncated α-syn species similar in molecular weight to those obtained with purified cathepsin D (Figure 2C). Degradation of α-syn and generation of α-synΔC species in the lysosomal fractions appeared to be sequentially linked. Cleavage of α-syn C-terminus by cathepsin D is likely to be the first and/or fastest step in the proteolytic processing of the protein.

The cleavage profiles obtained with lysosomes in the presence of protease inhibitors were very informative. The proteolytic activity that produced α-synΔC species in the lysosomal fraction was completely inhibited with pepstatin A, an aspartic protease inhibitor (Figure 2C). Since cathepsin D is the most abundant lysosomal aspartic protease, this experiment supports its role in the proteolytic processing of α-syn. Other subcellular fractions with lower levels of cathepsin D than lysosomes also showed α-syn degradation activity. The proteolytic activity in these fractions was also blocked by pepstatin A, and was proportional to their cathepsin D content. Since the conditions of the assay, buffer and acidic pH, were optimal for lysosomal enzymes, we cannot rule out the presence of other α-syn degrading activities in the cytosolic fraction.

The role of cathepsin D in the proteolytic processing of α-syn was further supported by cathepsin D knockdown experiments. Lenti-viral shRNA constructs targeting different regions in the cathepsin D RNA reduced the enzyme activity to 25% or less of that in controls, and increased α-syn by 28% ($p < 0.05$) in 3D5 cells. This increase in the steady-state levels of α-syn in 3D5 cells after 3 days of reduced cathepsin D activity may seem modest, but it is significant considering that the half life of α-syn in these cells is close to 48h (data not shown). Similar increases in α-syn levels were reported when proteosomal and lysosomal activities were inhibited in another cellular model of synucleinopathies [14].

Isolation of lysosomes after RNAi knockdown of cathepsin D revealed that in this subcellular compartment α-syn was increased by 2-fold when compared to NT controls. The inverse correlation between α-syn levels and cathepsin D activity was also demonstrated in *in vitro* experiments using lysosomes with normal and reduced cathepsin D activity (Figure 4B). These data, together with the complete inhibition of α-syn degradation by pepstatin A in lysosomal fractions (Figure 2C), indicate that cathepsin D is the main lysosomal protease involved in α-syn degradation.

Calpain I, an enzyme that it is not directly associated with either the lysosomal or proteosomal pathways, has been proposed to play a role in α-syn cleavage. Its role in α-syn aggregation remains controversial, since previous reports differ in identifying the main α-syn fragments that are generated as a result of calpain I cleavage. This is an important issue because it could

help determine if calpain I primarily prevents α-syn aggregation by cutting the protein preferentially in the non-amyloid component domain [17] or fosters aggregation by generating amyloidogenic α-synΔC species [21].

We confirmed previous reports that calpain I can cleave α-syn. However, in agreement with one of these studies [17], α-syn does not seem to be very susceptible to the enzyme, and cleavage of α-syn by calpain I generates only small amounts of α-synΔC. Calpain I knockdown experiments, aimed to determine its role in α-syn degradation, showed a similar increase in cellular levels of α-syn to that observed in cathepsin D knockdown experiments (compare Figures 3C and 6B). However, since calpains are proposed to be involved in a variety of cellular events, including autophagosome formation, that could impact α-syn metabolism [29], we cannot rule out the possibility that the observed effect of calpain I knockdown on α-syn is indirect and not due its role in α-syn degradation.

Our data suggest that impaired cathepsin D activity would result in increased α-syn levels leading to its aggregation. Although, a significant reduction in cathepsin D activity (more than 50%) was needed to observe an effect on α-syn levels in our cell model, it is possible that a chronic moderate enzymatic deficiency in an animal model would result in higher levels of α-syn.

Total cathepsin D deficiency in knockout animals gives a phenotype resembling the human disease neuronal ceroid lipofuscinosis [30]. Cathepsin D-deficient mice are born normally but die in about 4 weeks due to massive intestinal necrosis, thromboembolia, and lymphopenia [30]. Because of the short life of cathepsin D knockout animals, significant accumulation of α-syn in their brains is unlikely to occur. Heterozygous animals, on the other hand, have a life span close to normal; therefore, analysis of α-syn in the brain of these animals could be very informative.

With the identification of cathepsin D as the main lysosomal enzyme involved in α-syn degradation, upregulation of cathepsin D activity in animal models of synucleinopathies should be considered as one of the therapeutic strategies for the treatment of these diseases.

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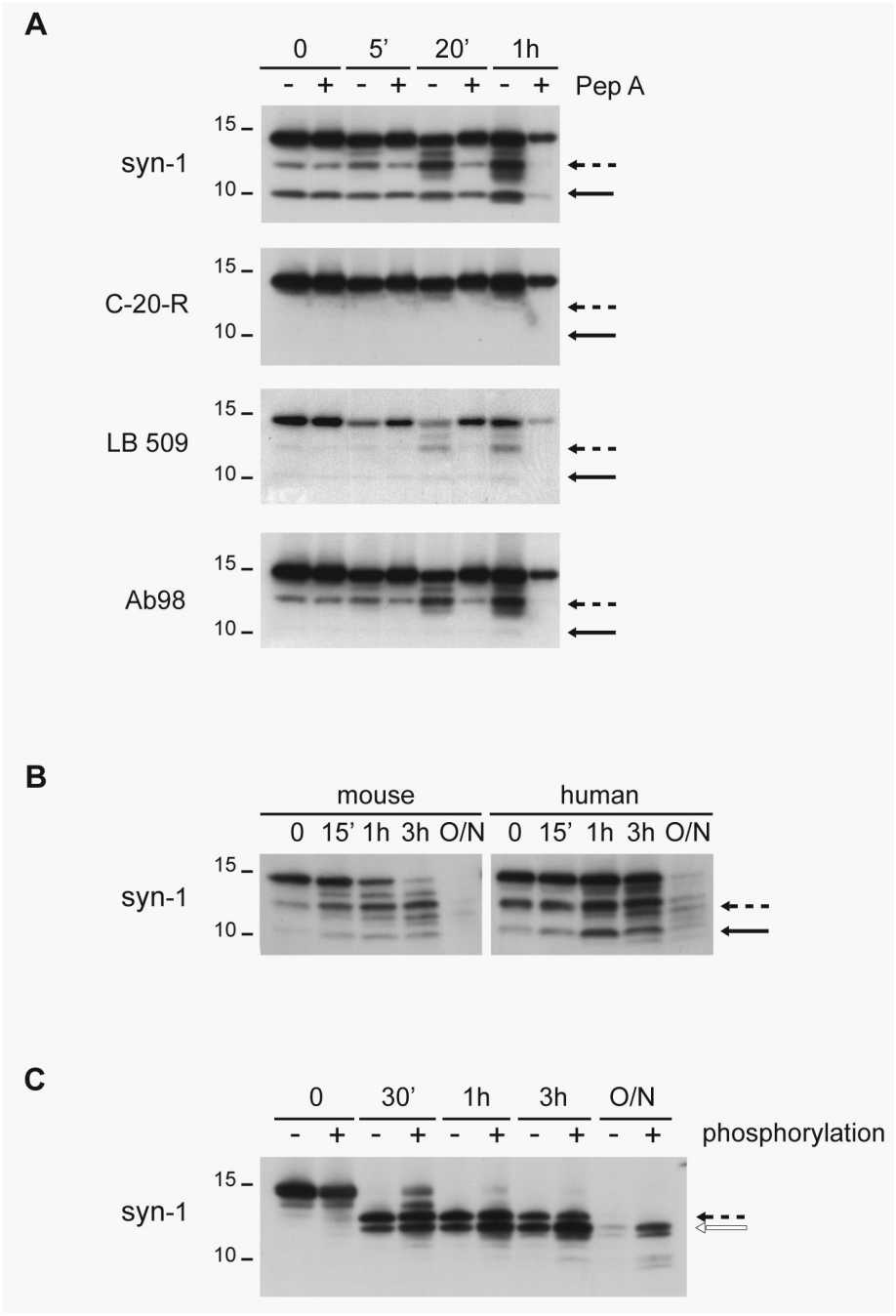

Figure 1. α-SynΔC species are generated by cathepsin D cleavage of α-syn

Lysates from 3D5 cells expressing α-syn (*A*), mouse and human brain extracts (*B*), and unphoshporylated (−) and phosphorylated (+) recombinant α-syn (*C*), were incubated with cathepsin D in pH 4 buffer at 37°C. Aliquots from the reactions were taken at the indicated time points and analyzed by Western blot. The top blot in panel *A*, and blots shown in panels *B* and *C* were probed with syn-1 antibody (epitope aa 91-99). The other blots in panel A were probed with C-20-R antibody against the C-terminus of α-syn, LB 509 (epitope aa 115-122), and Ab98 (epitope aa 98-115). The positions of 12 kDa and 10 kDa α-synΔC species are indicated with broken and full arrows, respectively. The empty arrow in panel C indicates the position of 11 kDa α-synΔC species. Representative blots of three experiments are shown.

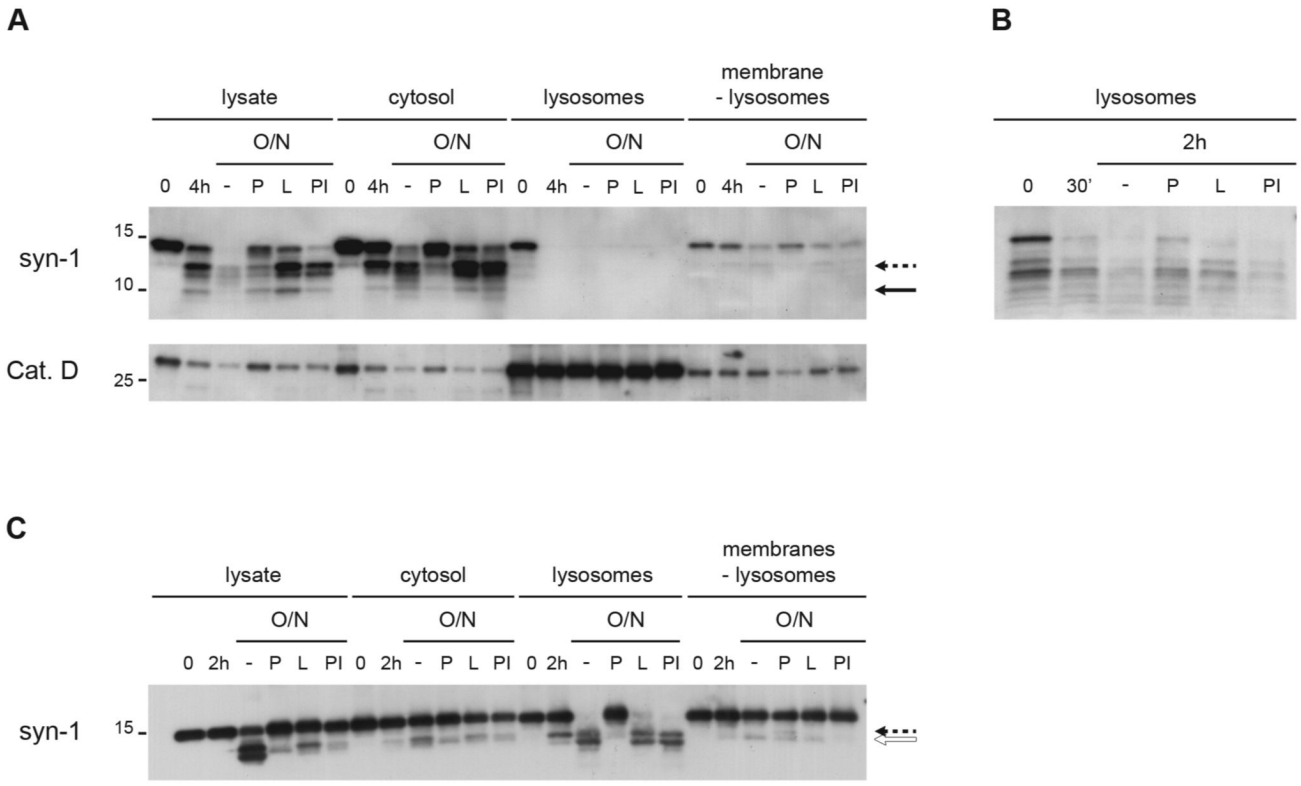

Figure 2. α-Syn degrading activities in subcellular fractions are proportional to their cathepsin D content

A. Subcellular fractions (56 μg of protein) from 3D5 cells isolated after 7 days in TetOFF medium were incubated in cathepsin D buffer pH 4 at 37°C. *B.* Lysosomal fractions from the same preparation described in *A* were analyzed at shorter incubation times. *C.* Subcellular fractions (5 μg of protein) from 3D5 cells cultured in TetON medium (background α-syn expression in these cells was not detectable during the time of exposure shown in the Figure) were incubated with 2 μg of recombinant human wild-type α-syn in cathepsin D buffer. Overnight (O/N) samples shown in Panels *A* and *C*, and 2h samples in *B* were incubated without inhibitors (−), with pepstatin A (P), leupeptin (L), or a cocktail of protease inhibitors (PI). Aliquots at the time points indicated in the Figure were analyzed by Western blot using syn-1 antibody. The blot shown in panel *A* was also probed with a cathepsin D antibody. The positions of 12 kDa and 10 kDa α-synΔC species are indicated with broken and full arrows, respectively. The empty arrow in panel *C* indicates the position of 11 kDa α-synΔC species. Representative blots from four experiments are shown.

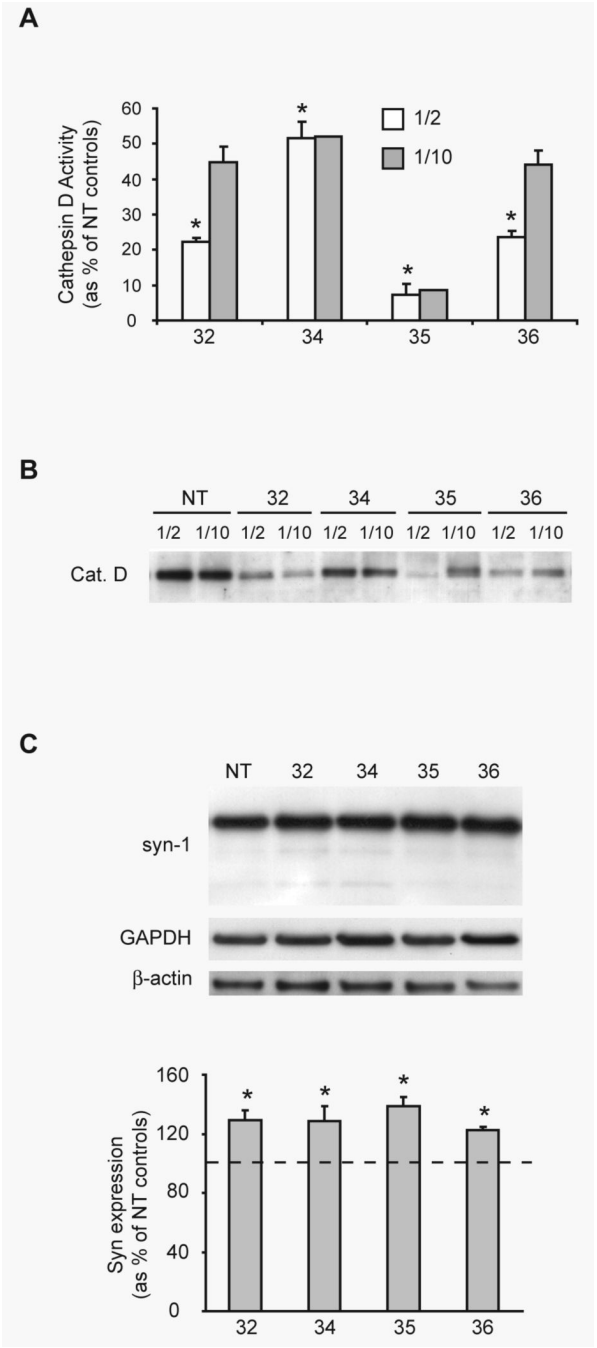

Figure 3. Reduction of cathepsin D activity in 3D5 cells causes an increase in α-syn levels

3D5 cells maintained for 4 days in TetOFF medium were transduced with 1/2 and 1/10 dilutions of control (NT) or of four constructs targeting cathepsin D RNA. Three days after transduction, cathepsin D activity and expression in the cell lysates were evaluated. *A.* Values in the linear range were plotted for each construct dilution, and expressed as a percentage of the values obtained with NT samples. The reduction in cathepsin D activity with a 1/2 dilution of the constructs was: 78% with 32 (n=7), 48% with 34 (n=3), 93% with 35 (=3), and 76% with 36 (n= 4). *=P<0.05 compared to NT control. *B.* Western blot analysis of cathepsin D in the 3D5 lysates after transduction with 1/2 and 1/10 dilutions of NT, 32, 34, 35, and 36 constructs. *C.* Western blot analysis of α-syn from 3D5 lysates after transduction with a 1/2 dilution of NT,

32, 34, 35, and 36 constructs. The blot was probed with antibodies against α-syn (syn-1), GAPDH, and ß-actin. Quantitation of α-syn levels was done by scanning the X-ray films and using ImageQuant software. The values were normalized to GAPDH, and expressed as a percentage of the NT value. The percentages were as follows: construct 32, 29% (n=7); 34, 29% (n=4); 35, 39% (n=5); and 36, 22% (n=4). Similar values were obtained when ß-actin was used for the calculations. The broken line in the plot indicates 100%. *=P<0.05 compared to NT control.

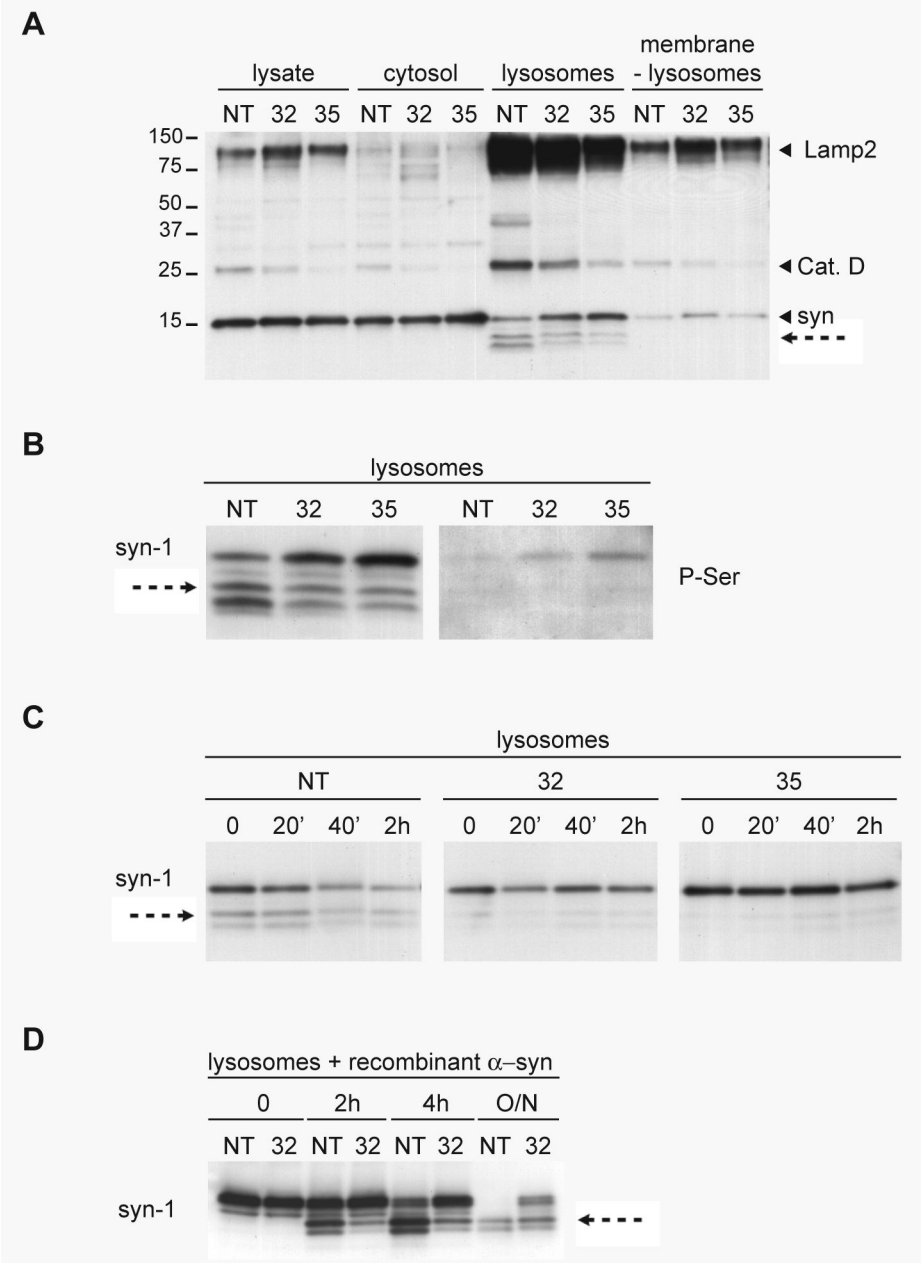

Figure 4. α-Syn is resistant to degradation by lysosomes with reduced cathepsin D activity

A. Subcelluar fractions from 3D5 cells overexpressing α-syn and transduced with a 1/2 dilution of shRNA control (NT), and 32 and 35 constructs targeting cathepsin D were analyzed by Western blot. The blot (5 μg of protein per lane) was probed with antibodies against two lysosomal markers: Lamp2 and capthesin D, and with syn-1 antibody. *B*. Blots of NT, 32, and 35 lysosomal fractions containing 2.5 μg and 5 μg of protein per fraction were probed with syn-1 and P-Ser antibodies, respectively. *C*. NT, 32, and 35 lysosomal fractions (50 μg of protein) were incubated in cathepsin D buffer. Aliquots of the reactions were taken at the time points indicated in the Figure and analyzed by Western blot. *D*. Lysosomal fractions (5 μg of protein) from NT and 32 were incubated in cathepsin D buffer with 380 ng of recombinant α-syn. The reactions were monitored by Western blot at different time points. The position of 12

kDa α-synΔC species is indicated with broken arrow. Representative blots of at least three experiments are shown.

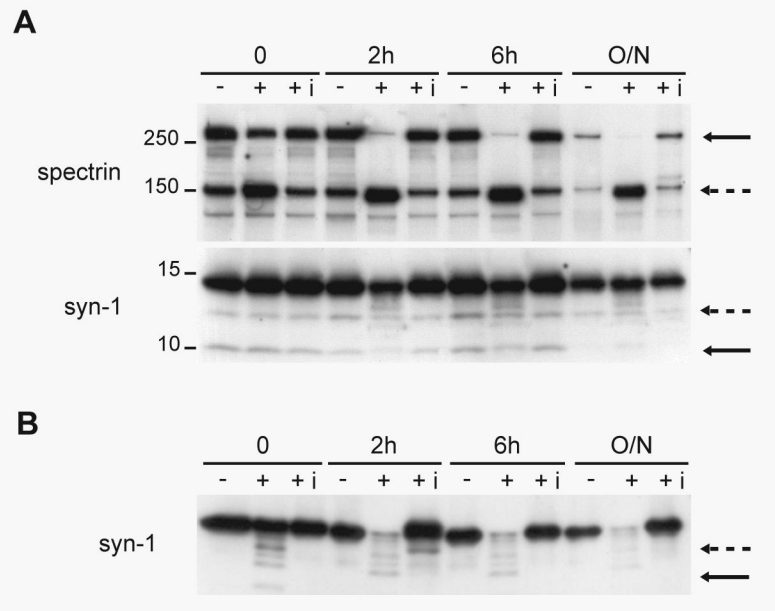

Figure 5. Calpain I degradation of α-syn does not generate significant amounts of α-synΔC species

A. 3D5 cells overexpressing α-syn were lysed with calpain I extraction buffer, and the lysates were incubated in calpain I reaction buffer without supplementation (−), with addition of 120 mU of exogenous calpain I (+), or with 120 mU of exogenous calpain I and calpain III inhibitor (+i). Aliquots at the time points indicated in the Figure were analyzed by Western blot. The blots were probed with antibodies against spectrin and α-syn (syn-1). In the top blot the full and broken arrows indicate the position of uncleaved and calpain-cleaved spectrin, respectively. In the bottom blot the broken and full arrows indicate the position of the 12 kDa and 10 kDa α-synΔC species, respectively. *B.* Lysates from 3D5 were obtained and incubated as described above. In this experiment the amount of calpain I was doubled (240 mU calpain). Representative blots from three experiments are shown.

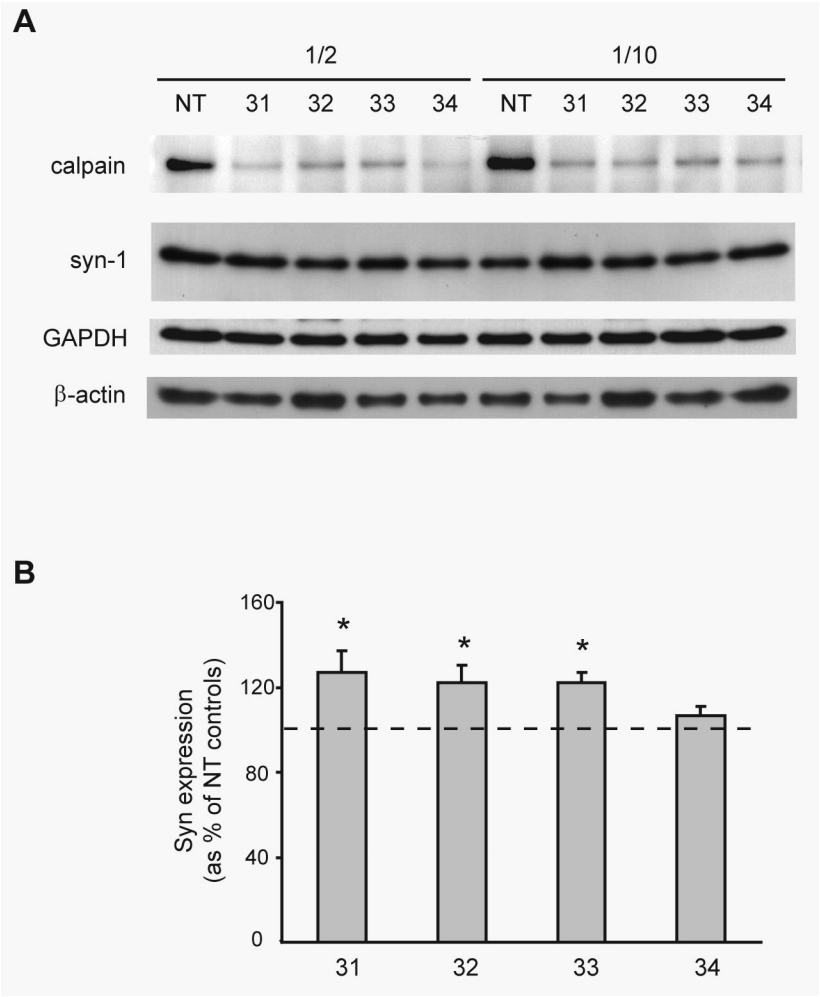

Figure 6. Effect of calpain I reduction on α-syn levels in 3D5 cells

A. 3D5 cells were infected with 1/2 and 1/10 dilutions of viral-medium containing a non-target control (NT) or constructs 31, 31, 33, and 34 targeting calpain I RNA. Three days after infection, cell lysates were prepared and 10 μg from each sample were analyzed by Western blot. The blot was probed with antibodies against calpain I, α-syn (syn-1), GAPDH, and ß-actin. *B.* Quantitation of calpain I and α-syn in 3D5 lysates expressing constructs targeting calpain I was done by scanning the X-ray films and using ImageQuant software. The values were normalized to GAPDH, and expressed as a percentage of the NT value. The percentages were as follows: construct 31, 26% (n=5); 32, 22% (n=7); 33, 22% (n=7); and 34, 7% (n=8). Similar values were obtained when ß-actin was used for the calculations. The broken line in the plot indicates 100%. *=P<0.05 compared to NT control.