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Distinct cleavage patterns of normal and pathologic forms of α-synuclein by calpain I *in vitro*

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Abstract

Parkinson's disease (PD) is characterized by fibrillary neuronal inclusions called Lewy bodies (LBs) consisting largely of alpha-synuclein (α-syn), the protein mutated in some patients with familial PD. The mechanisms of α-syn fibrillization and LB formation are unknown, but may involve aberrant degradation or turnover. We examined the ability of calpain I to cleave α-syn *in vitro*. Calpain I cleaved wild-type α-syn predominantly after amino acid 57 and within the non-amyloid component (NAC) region. In contrast, calpain I cleaved fibrillized α-syn primarily in the region of amino acid 120 to generate fragments like those that increase susceptibility to dopamine toxicity and oxidative stress. Further, while calpain I cleaved wild-type α-syn after amino acid 57, this did not occur in mutant A53T α-syn. This paucity of proteolysis could increase the stability of A53T α-syn, suggesting that calpain I might protect cells from forming LBs by specific cleavages of soluble wild-type α-syn. However, once α-syn has polymerized into fibrils, calpain I may contribute to toxicity of these forms of α-syn by cleaving at aberrant sites within the C-terminal region. Elucidating the role of calpain I in the proteolytic processing of α-syn in normal and diseased brains may clarify mechanisms of neurodegenerative α-synucleinopathies.

Keywords: α-synuclein, calpain I, fibrillization, Lewy bodies, Parkinson's disease.

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Parkinson's disease (PD) is a progressive neurodegenerative disease characterized clinically by resting tremor, rigidity, bradykinesia, and postural instability (Gibb 1989) in association with degeneration of dopaminergic neurons and the accumulation of Lewy bodies (LBs) in the substantia nigra pars compacta (Forno 1996). LBs include many proteins, but the major component of LB filaments is alpha-synuclein (α-syn), a 140 amino acid protein with an N-terminal segment (amino acids 1–60) characterized by four imperfect repeat (KTKEGV) motifs (Clayton and George 1999). The non-amyloid component (NAC) of amyloid plaques comprises the middle section of α-syn (amino acids 61–95). This region harbors two additional repeat motifs, while the C-terminus of α-syn is enriched in aspartate, glutamate, and proline (Uversky and Fink 2002). The secondary structure of native α-syn is unfolded but, in the presence of acidic phospholipids, it can assume an α-helical structure. α-Syn exists as β-pleated sheets in aggregates in diseased brains (Clayton and George 1999). Beta-synuclein (β-syn) and gamma-synuclein (γ-syn) are homologous with α-syn, but they lack the highly amyloidogenic 12 amino acids (71–82) in the NAC region of α-syn. Unlike α-syn, neither β-syn nor γ-syn fibrillize *in vitro*, and neither of these homologues of α-syn are found in the LBs of PD or related disorders known as neurodegenerative α-synucleinopathies (Giasson *et al*. 2001).

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Abbreviations used: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, A-β precursor protein; α-syn, alpha-synuclein; β-syn, beta-synuclein; DTT, dithiothreitol; γ-syn, gamma-synuclein; LBs, Lewy bodies; LC/MS, liquid chromatography/mass spectrometry; MAPs, microtubule-associated proteins; NAC, non-amyloid component; PD, Parkinson's disease; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween-20.

Growing evidence indicates that α-syn plays a crucial role in the pathogenesis of PD and other α-synucleinopathies. α-Syn is the major building block of abnormal filamentous aggregates in the LBs of sporadic PD, and two different α-syn mutations (A53T, A30P) are linked with familial PD. α-Syn has been identified in the LBs of dementia with LBs (DLB), and the LB variant of Alzheimer's disease (AD), in the glial cytoplasmic inclusions of multiple system atrophy, and in the neuronal inclusions and spheroids of neurodegeneration with brain iron accumulation, type I (Galvin *et al*. 2001). Although the function of α-syn is currently unknown, it may play a role in synaptogenesis (Hsu *et al*. 1998), act as a chaperone for other proteins (Souza *et al*. 2000), or regulate membrane stability and turnover (Murphy *et al*. 2000; Narayanan and Scarlata 2001; Sharon *et al*. 2001).

The mechanisms governing the turnover of α-syn also are unclear, but this could be a critical aspect of disease mechanisms in α-synucleinopathies, similar to the key role that aberrant processing of the Aβ precursor protein (APP) and Aβ peptides play in AD. Indeed, the α-syn-rich LBs of PD and senile plaques formed by Aβ in AD are filamentous lesions that share common physiochemical properties of amyloid, and it is plausible that the mechanisms underlying the formation of both, including abnormal proteolytic processing, are similar. Although several lines of evidence implicate the proteasome in the metabolism of α-syn, not all reports agree on this point (Bennett *et al*. 1999; Ancolio *et al*. 2000; Gai *et al*. 2000; Chung *et al*. 2001; McLean *et al*. 2001; Shimura *et al*. 2001). For example, Ancolio *et al*. (2000) showed that α-syn is not ubiquinated in HEK293 cells, and proteasomal inhibition did not protect α-syn from degradation nor did it modify the cellular concentration of α-syn. Alternatively, α-syn is predominantly localized to the pre-synaptic terminal, which suggests that it may be a substrate for soluble or membrane-associated proteases such as the calcium-activated neutral protease calpain I. Calpain I is activated by increases in intracellular calcium and cleaves many proteins including the microtubule associate proteins (MAPs) known as MAP1A, MAP2, and tau (Billger *et al*. 1988; Bednarski *et al*. 1995; Mercken *et al*. 1995), and the NMDA receptor subunit 2 (NR2; Guttmann *et al*. 2001). Calpain I also may be involved in the formation and secretion of Aβ in AD (Nixon *et al*. 1994; Yamazaki *et al*. 1997), the accumulation of p25 in AD (Lee *et al*. 2000), abnormal cleavage of the mutant huntingtin protein in Huntington's disease (Gafni and Ellerby 2002), and the accumulation of tau in frontal temporal dementia (Yen *et al*. 1999). Because the role of calpain I in the degradation of α-syn in PD has not been explored, we investigated whether wild-type α-syn, mutated α-syn (A53T, A30P), and fibrillized α-syn are substrates of calpain I, and whether normal or abnormal calpain I cleavage might contribute to the fibrillization and aggregation of α-syn into LBs.

Materials and methods

Materials

Porcine calpain I and calpain inhibitor III (MDL 28170; Z-Val-Phe-CHO) were purchased from Calbiochem (San Diego, CA, USA). The horseradish–peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratory (West Grove, PA, USA). Tris–tricine gels were from Invitrogen (Carlsbad, CA, USA). The bicinchoninic acid protein assay (BCA) and enhanced chemiluminescence reagents were purchased from Pierce Chemical Company (Rockford, IL, USA). Bovine serum albumin (BSA) was purchased from Roche Bioscience (Palo Alto, CA, USA). 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer was purchased from Sigma (St Louis, MO, USA).

Antibodies

Syn 303, Syn 211 and Syn 102 are mouse antiα-syn monoclonal antibodies that recognize epitopes with amino acid residues 2–4, 121–125, and 130–140 in human α-syn, respectively (Giasson *et al*. 2000; Duda *et al*. 2002). Antibodies Syn h119 and Syn h163 are mouse antiα-syn monoclonal antibodies with epitopes that map to amino acid residues 71–82 and 20–43, respectively, of α-syn (unpublished observations). SNL-1 is an affinity-purified rabbit polyclonal antibody raised to a peptide corresponding to amino acids 104–119 in α-syn (Giasson *et al*. 2000).

Calpain I cleavage of α-syn proteins

Calpain I cleavage of purified recombinant human α-syn proteins (including wild-type, fibrillized, and mutant species) was carried out using methods similar to those described in our previous studies (Guttmann *et al*. 2001). Briefly, α-syn protein was incubated with calpain I in a buffer containing 40 mM HEPES (pH 7.2) and 5 mM dithiothreitol (DTT) at 37°C. Reactions were initiated by addition of calcium (1 mM final). To stop the proteolysis, aliquots were removed from the reaction mixture and added to an equal volume of 2× Invitrogen sodium dodecyl sulfate (SDS) stop buffer at various time points, heated in a boiling water bath, and stored at −20°C until use.

Expression and purification of wild-type and mutant species of α-syn

Human wild-type and mutant (A30P, A53T) α-syn were expressed and purified according to Giasson *et al*. (1999). cDNAs for each of the proteins were subcloned into the bacterial expression vector pRK172 and expressed in *Escherichia coli* BL21. The bacterial pellets were resuspended in high-salt lysis buffer including protease inhibitors, heated to 100°C for 10 min, then centrifuged at 70 000 *g* for 30 min. After dialysis against 10 mM Tris (pH 7.5), the supernatants were applied to a Mono Q column and eluted with a 0–0.5 M NaCl gradient. The BCA assay, using BSA as a standard, was used to determine the protein concentration. To create fibrillized forms of α-syn, α-syn proteins were incubated at 37°C in 100 mM sodium acetate (pH 7.0) with continuous shaking for 48 h. Samples were centrifuged at 150 000 *g* for 30 min and HEPES buffer (40 mM, pH 7.2, 5 mM DTT) was added.

Western blotting

A total of 0.5 μg of recombinant α-syn protein cleaved by calpain I were loaded in each well on 16% Invitrogen Tris–tricine gels and

separated by electrophoresis. The gels were either stained with Coomassie blue or transferred electrophoretically to a nitrocellulose membrane. To determine the cleavage pattern, the membrane was blocked in 3% BSA fraction V in Tris-buffered saline with Tween-20 (TBST) for 1 h and incubated overnight at 4°C in antibodies that bind defined epitopes or amino acid sequences in α-syn (Giasson *et al*. 2000). Following rinses in TBST, membranes were incubated in horseradish–peroxidase-conjugated secondary antibody for 1 h then developed with enhanced chemiluminescence reagents. Immunoblots were analyzed using ImageQuant 5.0 (Amersham Biosciences, Piscataway, NJ, USA), to calculate the integrated intensity of the pixels in the region of interest after subtraction of background. Results are reported as integrated intensity.

Purification of calpain I-generated fragments of α-syn

To purify the fragments of calpain I-cleaved α-syn, the reaction was stopped with a final concentration of 2 mM EDTA, concentrated using a Centricon YM-3 centrifugal filter (Millipore, Bedford, MA, USA), and filtered through Millipore Centricon with a 30-kDaA molecular weight cut-off filter. The sample was then analyzed by a Hewlett Packard high-performance liquid chromatography (HPLC) system with a diode array detector using an octadecyl silica gel reverse-phase column (5 μm; 4.6 × 250 mm; Jupiter; Phenomenex Torrance, CA, USA). Solvent A was 0.1% trifluoroacetic acid in ultra-pure water, and solvent B was acetonitrile. Peptides were eluted using an increasing linear gradient of solvent B from 25 to 45% in 60 min then to 60% in 5 min with a flow rate of 1 mL/min. The HPLC detector was set at 210 and 280 nm. The peptide peaks in the chromatogram were collected, dried down in a speed-vacuum system, and resuspended in a small volume of either 20% acetonitrile/0.1% formic acid for mass spectrometry analysis or in water for western blot analysis.

Mass spectrometry studies

Electrospray ionization mass spectrometry was performed on an Agilent 1100 series quadrupole mass spectrometer equipped with an atmospheric pressure ion source and operating in positive ion mode. The collected peptides were directly injected into the ionization needle using acetonitrile : 0.1% formic acid (20 : 80 v/v) as the mobile phase, with a flow rate of 0.1 mL/min. Data were analyzed using the Hewlett Packard Chemstation software. Theoretical masses of peptide fragments were calculated using the peptide tool of Chemstation software. Observed masses were measured within ± 1 Da of theoretical mass.

Identification of the α-syn fragments

To determine the N-terminal sequence of the fragments generated from cleavage of α-syn by calpain I, a sample of the reaction mixture was loaded onto a 16% Tris–tricine gel then transferred to a polyvinylidene difluoride (PVDF) membrane (Pall Corporation Ann Arbor, MI, USA) in CAPS buffer (10 mM, pH 11) in 10% methanol. The membrane was then stained with Coomassie Blue, and the appropriate bands were excised from the membrane and submitted for sequencing to the Mayo Protein Core Facility or the School of Veterinary Medicine at the University of Pennsylvania. To determine the sequence of the fragments purified from the liquid chromatography/mass spectrometry (LC/MS) analysis of the reaction mixture, the samples purified from HPLC were subsequently analyzed by mass spectrometry and western blotting.

Western blot analysis of tissue from transgenic mice

Generation and characterization of transgenic (Tg) mice, as well as sequential biochemical fractionation of the tissue, is previously described in detail in Giasson *et al*. (2002). Pathology in A53T Tg mice resembling inclusions from human brain, was age-dependent and paralleled the onset of the disease. No pathology was found in non-transgenic and wild-type Tg mice. In this study, 10 μL of the high salt fraction from the cortex, cerebellum, and spinal cord of wild-type Tg mice, and A53T Tg mice, were electrophoresed in parallel with calpain I cleaved fibrillized recombinant α-syn on a 16% SDS Tris–tricine gel and subjected to western blot analysis as described above.

Results

Calpain I cleavage of α-syn

Recombinant α-syn was a substrate of calpain I. Breakdown products of α-syn were visible with Coomassie blue staining in a time-dependent manner (Fig. 1a). Four major cleavage products of the full-length α-syn protein (apparent molecular mass 16 kDa) were identified, including one prominent fragment at approximately 8.5 kDa (fragment 1), two fragments running as a doublet at approximately 6 kDa (fragments 2 and 3), and a single fragment at approximately 5 kDa (fragment 4). Occasionally, a band migrating just below fragment 1 (indicated by the asterisk) was detected by Coomassie blue stain. Cleavage of α-syn by calpain I was concentration-dependent, as increasing concentrations of calpain I resulted in faster proteolysis of full-length α-syn and the generation of low molecular mass forms (data not

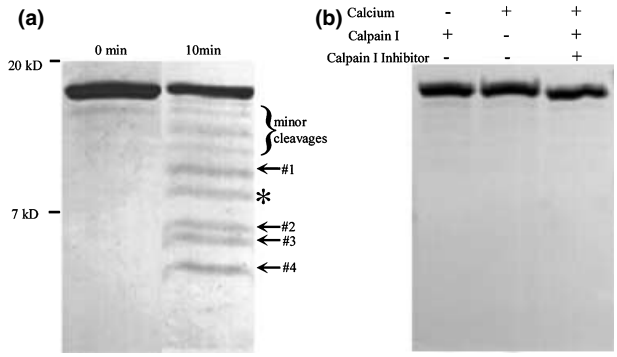

Fig. 1 Calpain I cleavage of α-syn. Coomassie blue stain of SDS–polyacrylamide gels of α-syn incubated with (a) 2.0 U/mL calpain I and 1 mM calcium (b) no calcium with calpain I (lane 1), no calpain I with calcium (lane 2), or calpain I and calcium in the presence of calpain inhibitor III (10 μM) (lane 3). Calpain I cleavage resulted in degradation of full-length α-syn and the appearance of four major cleavage fragments indicated by arrows 1–4 and minor fragments just less than full length. Occasionally, a less prominent fragment just below fragment 1 was present, indicated by the asterisk. No cleavage was observed in the absence of calpain I or calcium or in the presence of calpain inhibitor III. Lines indicate molecular weight markers.

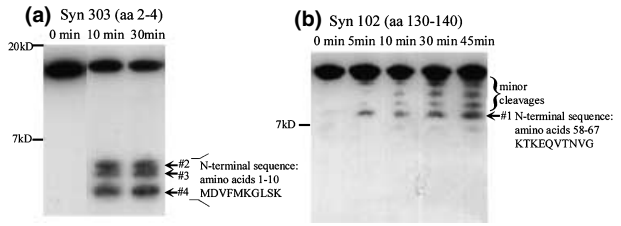

Fig. 2 Calpain I cleavage of α-syn detected by immunostaining. (a) Immunostaining of α-syn with antibody Syn 303 recognizing amino acids 2–4 of α-syn. Full length was recognized at ~16 kDa and calpain I-generated fragments were found at ~6 kDa (doublet) (fragments 2 and 3) and 5 kDa (fragment 4). (b) Overexposure of immunostaining with antibody Syn 102 recognizing amino acids 130–140. A minor amount of N-terminal cleavage occurred with the identification of one major fragment at ~8.5 kDa (#1). Fragments 1, 2, 3, and 4 were N-terminally sequenced. Fragments 2–4 began with amino acid 1, and fragment 1 began with amino acid 58. The four minor fragments indicated by the bracket may correlate with cleavage at each of the four repeat motifs in the N-terminal regions of α-syn. These fragments were stable *in vitro* as they remain present for up to 45 min. Arrows indicate fragments, and lines indicate molecular weight markers.

shown). No cleavage was noted when the reaction was carried out in either the absence of calpain I or in the absence of calcium or in the presence of calpain inhibitor III (Fig. 1b).

We then sought to identify the location of cleavages in α-syn by calpain I using antibodies to specific epitopes of α-syn in conjunction with western blotting. Antibody Syn 303 recognized amino acids 2–4 of the N-terminal region, and antibody Syn 102 recognized amino acids 130–140 of the C-terminal region of α-syn (Fig. 2). Following 10-min incubations, breakdown products were visible with both antibodies. The N-terminal antibody, Syn 303, identified two fragments with approximate molecular masses of 6 kDa (fragments 2 and 3) and a fragment around 5 kDa (fragment 4), all of which comigrated with the fragments of the same size visible by Coomassie blue stain (Fig. 1a). In contrast, the C-terminal antibody, Syn 102, identified one prominent fragment at approximately 8.5 kDa (fragment 1) which comigrated with the Coomassie blue-stained fragment at that molecular mass. This antibody also recognized small amounts of several fragments that ran only slightly faster than full-length α-syn. This overexposed immunoblot (Fig. 2b) emphasizes these evenly spaced minor fragments. These data indicate that the major cleavages of α-syn by calpain I occur in the middle of the protein based on molecular mass of the products (fragments 1–4), with no cleavage in the C-terminal region, and very limited cleavage in the N-terminal end. The N-terminal cleavage appeared to be evenly spaced, suggesting cleavage at the four repeat motifs in this segment of α-syn (Fig. 2b). The presence of three N-terminally labeled fragments and a single C-terminally labeled fragment suggests that some fragments may be further degraded, or that their structure may change in a manner which masks the epitopes of the C-terminal antibody.

To further confirm this digestion pattern, antibodies to other regions of α-syn were utilized. Fragments 2, 3, and 4 (identified by antibody Syn 303) were also recognized by antibodies directed to the N-terminal portions through amino acid 43 (Table 1). Antibodies directed at C-terminal components after amino acid 71 did not recognize fragments 3 and 4. However, these antibodies did recognize fragment 1, which was labeled by C-terminal antibodies with epitopes from amino acid 71–140. Antibody Syn h119 also recognized fragment 2, suggesting that this fragment may include amino acids recognized by this epitope (amino acids 71–82).

To identify the exact amino acid cleavage sites in α-syn, the major fragments (1–4) identified by Coomassie blue staining were N-terminally sequenced (Fig. 2). As expected, based on the mapping of the cleavage sites using antibodies, sequencing of the major fragments identified by Coomassie blue stain demonstrated N-terminal sequence in fragments 2–4 (MDVFMKGLSK). Fragment 1 began with the sequence KTKEQVTNVG, matching the sequence of α-syn beginning at amino acid 58. This indicates one specific calpain I cleavage

Table 1 Antibody mapping of calpain I cleavage of wild type a-syn

Antibody sequence	Syn 303 2–4	Syn h163 20–43	Syn h119 71–82	SNL-1 104–119	Syn 211 121–125	Syn 102 130–140
Fragment 1	–	–	+	+	+	+
Fragment 2	+	+	+	–	–	–
Fragment 3	+	+	–	–	–	–
Fragment 4	+	+	–	–	–	–

Four major calpain I cleavage fragments were labeled by immunostaining using a panel of antibodies capable of recognizing various epitopes throughout α-syn. Fragment 1 was labeled only by antibodies to the C-terminal region that recognize epitopes between amino acids 71–140. In contrast, fragments 3 and 4 were labeled by antibodies that recognize only the N-terminus but are not reactive to antibodies recognizing beyond amino acid 43. Syn h119 recognized fragment 2 but not fragments 3 and 4.

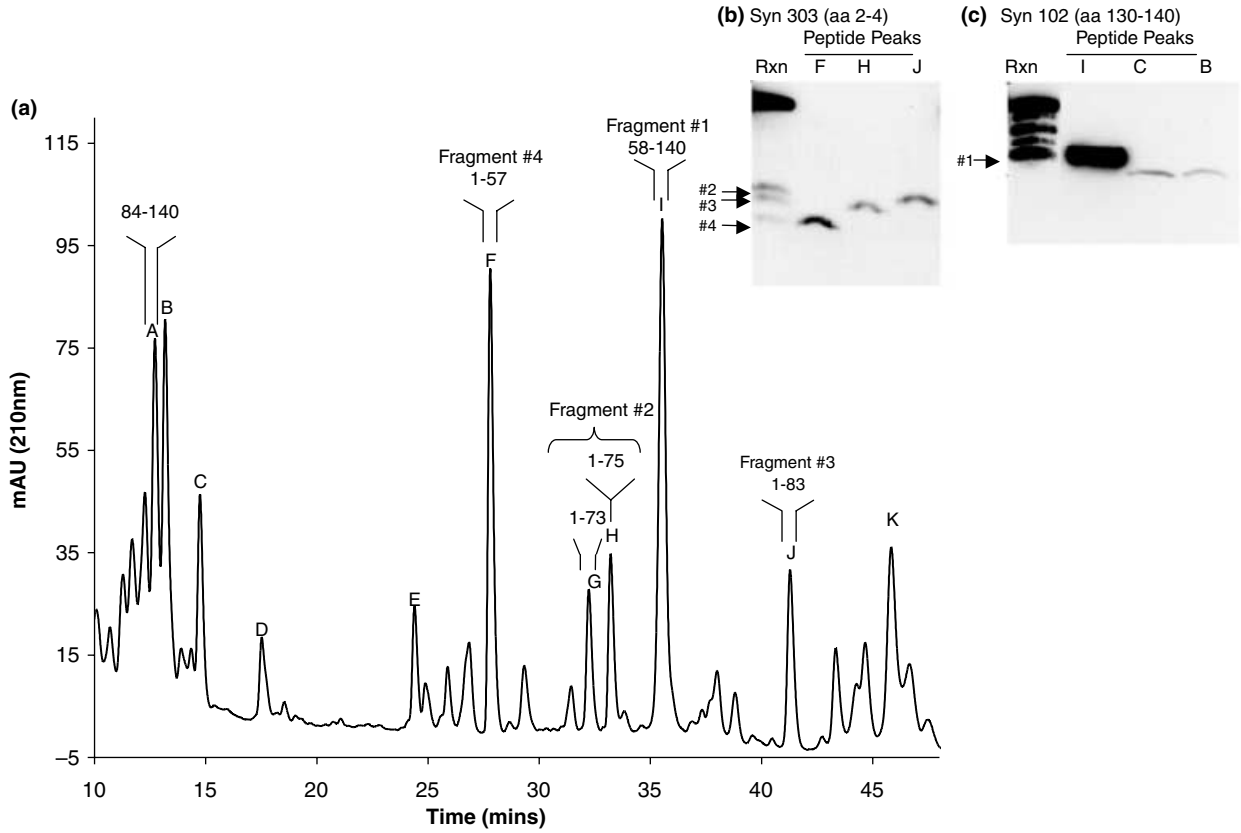

Fig. 3 HPLC chromatograms of calpain I cleaved α-syn and western blot analysis of purified fragments. Full-length α-syn was digested with 2.0 U/mL calpain I as described under Methods. The peptides were eluted from a reverse-phase HPLC column. (a) The peptides generated by calpain I cleavage of α-syn eluted between 12 and 46 min. All major peaks were labeled and further analyzed by mass spectrometry. (b) Immunostaining using the antibody Syn 303 identified peptides containing amino acids (aa) 2–4 and (c) Syn 102 recognized fragments containing amino acids 130–140 from purified peaks collected from HPLC analysis of the reaction mixture. (b) Syn 303 recognized fragments corresponding to amino acids 1–57 (lane 2), 1–75 (lane 3) and 1–83 (lane 4). A sample of the reaction mixture (Rxn) in lane 1 shows the migration of fragments 2–4, indicated by arrows. (c) Syn 102 recognized peptides corresponding to amino acids 58–140 (lane 2), 74–140 (lane 3), and 76–140 (lane 4). Lane 1 shows a sample of the reaction mixture (Rxn) to demonstrate the migration of fragment 1 labeled with the arrow.

site to be between amino acids 57 and 58 (at the fifth repeat motif).

To identify other fragments that may not be detected by western blot analysis as described above, the reaction mixture was analyzed by reverse-phase chromatography. The HPLC elution profiles of the fragments generated by calpain I cleavage of α-syn are shown in Fig. 3(a). The chromatographic peaks were collected, and the peptide masses were determined by mass spectrometry (Table 2). Aliquots of each peak were also tested for immunoreactivity against Syn 303 and Syn 102 antibodies (Figs 3b and c).

Peptides in peaks F, G, H, and J were assigned to peptide fragments corresponding to residues 1–57, 1–73, 1–75, and 1–83, respectively (Table 2) based on their determined molecular masses and their immunoreactivity with antibody Syn 303 (Fig. 3b, data for peak G not shown). Moreover, the peptides in peaks F, H, and J comigrated with fragments 4, 3, and 2 (from Figs 1 and 2), respectively (Fig. 3b), all of which were sequenced as beginning with the first 10 N-terminal amino acids (Fig. 2). Although the chromatogram showed the presence of four N-terminal peptides while the western blot showed only three such fragments, peaks G and H showed the same electrophoretic mobility (not shown) and therefore likely comigrated in the reaction mixture electrophoresed in a SDS gel.

The LC/MS analysis of the reaction mixture also identified the corresponding C-terminal fragments of the four N-terminal peptides described above (Table 2). The peptide in sample I co-migrated with fragment 1 (Fig. 3b, lane 2), having an N-terminal sequence of KTKEQVTNVG (residues 58–67; Fig. 2a). In addition, the observed mass of this peptide corresponded to the peptides 58–140 (Table 2). This confirmed the major cleavage of α-syn by calpain I after amino acid 57. The peptide in peak A (observed mass of

Table 2 Molecular masses of the peptides purified by HPLC

Peaks	Retention time	Observed molecular mass (Da)	Theoretical molecular mass (Da)	Possible sequence matches	Recognition by antibody	Confirmation by N-terminal sequencing
A	13.1	6088.92	6088.72	**84–140**		
B	13.6	6915.75	6916.17	26–96; 30–99; 37–105; 74–138; **76–140**	Syn 102 (aa 130–140)	
C	15.24	7116.0	7116.29	3–37; 4–74; 28–99; 69–138; **74–140**	Syn 102 (aa 130–140)	
D	18.2	3122.1	3121.63	1–31; 104–131		
E	25.2	4041.1	4041.1	1–39; 16–56; 36–76; 73–113		
F	28.7	5791.1	5791.1	**1–57**	Syn 303 (aa 2–4)	+
G	33.1	7360.76	7360.94	55–126; 67–137; **1–73**	Syn 303 (aa 2–4)	+
H	34.1	7560.98	7561.06	49–123; 67–137; **1–75**	Syn 303 (aa 2–4)	+
I	36.4	8685.7	8686.14	20–106; 56–136; **58–140**	Syn 102 (aa 130–140)	+
J	42.2	8388.9	8388.51	57–136; 58–137; **1–83**	Syn 102 (aa 130–140)	+
K	46.7	11356.6	11354.6	29–138; 32–140		

The possible sequence matches are listed for each molecular mass. Chemstation software determined the theoretical mass of the fragments based upon the protein sequence. The observed mass was measured within ± 1 Da of the theoretical mass. The final assignment of the proteolytic fragments, determined using antibody immunoreactivity and the determination of the molecular mass by MS, are highlighted in bold.

6088.72 Da) could only be assigned to sequence 84–140, confirming another cleavage site after amino acid 83. The peptides in peaks B and C were assigned, based on their determined molecular masses and immunoreactivity with antibody Syn 102, to correspond to amino acid sequences 76–140 and 74–140, respectively. These data suggest two minor cleavages after amino acids 73 and 75. These fragments are not readily apparent by western blot analysis of the reaction mixture but are noted only after reverse-phase purification and concentration. We cannot fully exclude the possibility that the peptides in peptides B and C are 74–138 and 69–138, respectively. However, this would not agree with the data relating to the N-terminal fragments and would not change the presence of a cleavage site after amino acid 73. Other minor peaks identified by HPLC, such as peak E (Fig. 3a and Table 2), may correspond to further degradation products. Peaks D and K (Fig. 3a and Table 2) suggest a minor cleavage site between amino acids 31 and 32, which corresponded to the third repeat motif in the N-terminal segment of the protein. Collectively, these data, summarized in Table 2, identified the N-terminal and C-terminal fragments of calpain I cleavage of α-syn after amino acids 57, 73, 75 and 83.

Fibrillized α-syn cleavage by calpain I

Incubation of fibrillized α-syn with calpain I under the same conditions as wild-type α-syn revealed the presence of two major fragments (Fig. 4a). This fragment pattern was different than wild-type α-syn (Figs 1 and 2) and was further confirmed by western blotting (Figs 4b and c). The immunoblot of fibrillized α-syn with the N-terminal antibody Syn 303 revealed two prominent fragments (5 and 6) slightly

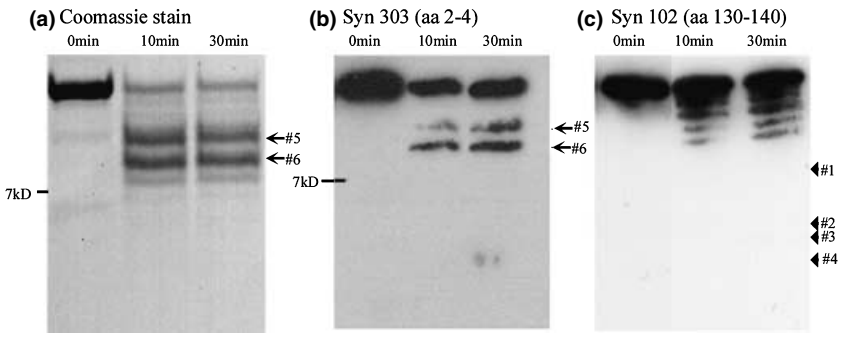

Fig. 4 Calpain I cleavage of fibrillized α-syn. A strikingly different fragment pattern was generated by calpain I-mediated proteolysis of fibrillized α-syn, in which fragments only slightly smaller than full-length synuclein were identified. (a) Coomassie blue stain of SDS–polyacrylamide gels identified fragments 5 and 6. (b) Immunolabeling with antibody Syn 303 identified fragments 5 and 6 as N-terminal segments. (c) Immunolabeling with antibody Syn 102 showed minor cleavages in the N-terminus. Lines indicate molecular weight markers. For comparison, the approximate positions of fragments 1–4 are marked with arrowheads.

Table 3 Antibody mapping of calpain I cleavage of fibrillized α-syn

Antibody sequence	Syn 303 2–4	Syn h119 71–82	SNL-1 104–119	Syn 211 121–125	Syn 102 130–140
Fragment 5	+	+	+	+	–
Fragment 6	+	+	+	–	–

Antibody mapping was used to identify the approximate location of the two major calpain I sites in fibrillized α-syn. The two fragments are labeled by antibodies up to amino acid 119. Fragment 5 extends within the epitope of antibody Syn 211 (i.e. amino acids 121–125), while fragment 6 only extends to the SNL-1 epitope (amino acids 104–119).

shorter than full-length α-syn, contrasting with the three low molecular mass fragments revealed by this antibody from wild-type α-syn. The size of the two fragments suggests that the major cleavages of fibrillized α-syn occur in the C-terminal portion of the molecule. When cleavage of fibrillized α-syn was investigated with the C-terminal antibody, Syn 102, a slight accumulation of a series of fragments slightly shorter than full-length was noted (Fig. 4c), which likely reflects minor cleavages at repeat motifs in the N-terminal region. Notably, no appearance of the major C-terminal fragment (#1) at 8.5 kDa, observed in cleavage of wild-type α-syn, was shown in the cleavage of fibrillized α-syn. The lack of accumulation of products labeled with C-terminal antibodies, in spite of levels of cleavage matching cleavage of soluble α-syn (quantified by Syn 102 immunoreactivity; data not shown), again suggested that the major cleavage of fibrillized α-syn was in the C-terminal region, with a small amount of cleavage in the N-terminal region.

The region in which fibrillized α-syn was cleaved by calpain I was examined by probing with antibodies to other components of α-syn to further define the cleavage sites (Table 3). Antibodies recognizing the regions of α-syn from the amino terminus to amino acid 119 labeled the two major breakdown products of fibrillized α-syn, fragments 5 and 6. An antibody (Syn 211) directed to amino acids 121–125 also recognized fragment 5. These results demonstrate that there are two major cleavage sites of fibrillized α-syn, one of which is likely located near amino acid 119, while the other is likely near amino acid 125.

To identify the exact cleavage sites in fibrillized α-syn, the reaction mixture of calpain I-cleaved fibrillized α-syn was subjected to LC/MS analysis similar to monomeric α-syn. The chromatographic peaks were collected and the peptide masses were determined by mass spectrometry. Four major peptides with molecular masses of 11457.88 Da, 12340.24 Da, 10447.25 Da, and 11331.60 Da were identified. Based on these results, the identity of two of the major cleavage products could be directly assigned because their molecular masses matched with unique peptide sequences in α-syn: 1–114 (observed molecular mass 11457.88 Da) and 10–114 (observed molecular mass 10447.25 Da). It is likely that fragment 6 corresponds to 1–114 as antibody mapping of this fragment demonstrates that it reacts with antibodies that span from the extreme N-terminus to the region 104–119, but not 121–125 (Table 3). The peptide 10–114 likely represents the proteolytic fragments that are detected by Coomassie staining below fragment 6 in Fig. 4(a), but which are not recognized by Syn 303 (Fig. 4b). Although the peptide with a molecular mass of 12340.24 Da had more than one possible sequence match within α-syn, it was assigned to 1–122 since this fragment contains the extreme N-terminus based on its immunoreactivity with the antibody Syn 303 (Fig. 4b). This prediction is consistent with antibody mapping of fragment 5, demonstrating that it reacts with antibodies that recognize epitopes from the N-terminus through amino acid 125 (Table 3). Secondary cleavage of peptide 1–122 after amino acid 9 (similar to the cleavage resulting into the peptide 10–114) would produce the peptide 10–122, which corresponds to the molecular mass of the fourth peptide detected by LC/MS analysis: 11331.60. Taken together these results indicate that fibrillized α-syn can be cleaved by calpain I at two major sites in the C-terminal region (i.e. between amino acids 114–115 and 122–123), and these resulting fragments can be further processed by cleavage between amino acids 9 and 10. This cleavage between amino acids 9 and 10 is just before the first the repeat sequence in α-syn analogous to the cleavage site identified unequivocally at the fifth repeat motif (i.e. between residues 57 and 58), and a possible cleavage site at the third repeat motif, i.e. between residues 31 and 32 (see Table 2). Collectively, these data indicated that the minor fragments labeled with the C-terminal antibody Syn 102 (see Figs 2 and 5) are most likely fragments cleaved at the N-terminal repeat motifs.

Calpain I cleavage of mutant forms of α-syn

The mutant forms of α-syn, A30P and A53T, were substrates of calpain I with breakdown products shown by Coomassie blue staining appearing after 10 min of digestion (Fig. 5a and b). A30P α-syn showed a similar pattern of digestion to that of wild-type α-syn, with three fragments less than 7 kDa (fragments 2–4), and a major fragment matching the C-terminal fragment (#1), indicating that A30P was also cleaved predominantly in the middle of the protein (Fig. 5a). In contrast, cleavage of A53T α-syn produced only two major fragments (2 and 3; Fig. 5b). The smallest N-terminal

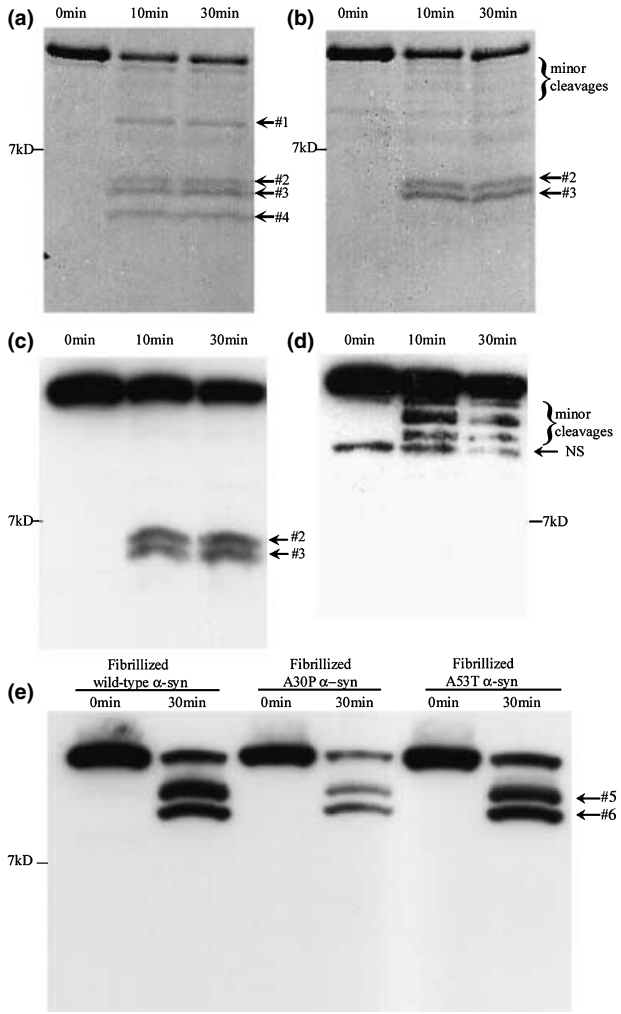

Fig. 5 Identification of calpain I generated fragments from mutated forms of α-syn. (a) Coomassie-stained SDS–polyacrylamide gels of A30P α-syn cleaved by calpain I identified four major fragments similar in size to those of wild-type α-syn. (b) In contrast, the Coomassie blue stain of SDS gels of A53T α-syn cleaved with calpain I indicated that only two (nos 2 and 3) of the four fragments identified in wild-type α-syn were present. (c) Immunolabeling of calpain I cleaved A53T α-syn with antibody Syn 303 confirmed the presence of fragments 2 and 3 as indicated by arrows. (d) Immunoblotting of A53T α-syn with antibody Syn 102 identified minor fragments just below full length. (e) Calpain I cleavage of fibrillized wild-type (lanes 1 and 2), A30P (lanes 3 and 4), and A53T α-syn (lanes 5 and 6) immunolabeled with N-terminal antibody Syn 303. Arrows indicate fragments produced from cleavage of fibrillized A30P and A53T that were similar in molecular mass to those observed in cleaved fibrillized wild-type α-syn. Molecular weight markers are indicated by lines. A non-specific (NS) band was recognized at the zero time point (d) and remains present through 30 min.

fragment (#4) and the C-terminal fragment (#1) were not identified by Coomassie blue or western blot analysis in the cleavage of A53T α-syn by calpain I using antibodies Syn 303 and 102. This suggested that a single cleavage was missing in the A53T α-syn (Figs 5c and d). Thus, both Coomassie blue stain and antibody results indicate that the predominant cleavage after amino acid 57 of wild-type α-syn does not occur in A53T α-syn. Antibody Syn 102 also demonstrated a small amount of N-terminal cleavage again likely representing calpain I cleavage at the repeat motifs (Fig. 5d).

As A53T α-syn fibrillizes at faster rates than wild-type α-syn and A30P α-syn in most studies, we investigated whether fibrillized A53T α-syn was cleaved by calpain I and whether the cleavage sites differed from fibrillized wild-type and A30P α-syn. Both fibrillized A53T and A30P α-syn were substrates of calpain I and showed similar patterns of digestion to that of fibrillized wild-type α-syn as shown in Fig. 5(e). Immunolabeling by the N-terminal antibody, Syn 303, demonstrated two prominent fragments (5 and 6) in both proteins, with the same electrophoretic mobility as those produced from cleavage of fibrillized wild-type α-syn.

Transgenic mice

We then sought to establish whether the previously reported truncated forms of α-syn found in Tg mice represent fragments that could be generated by calpain I. Samples from the high salt fraction of mice overexpressing human wild-type α-syn or human A53T α-syn were analyzed by western blotting in parallel with *in vitro* calpain I-cleaved fibrillized α-syn (Fig. 6). Using antibody Syn 303, full-length α-syn was detected in samples from the cortex, cerebellum, and spinal cord from both mice models. A truncated form of α-syn was detected A53T Tg mice, and to a lesser extent in wild-type α-syn Tg mice, that had similar if not identical electrophoretic mobility with that of the calpain I cleavage product, fragment 5, from fibrillized recombinant α-syn. The truncated form was best detected in the cortex and cerebellum of A53T Tg mice and was faintly visible in the spinal cord of these mice as well as in the cortex and cerebellum of wild-type Tg mice.

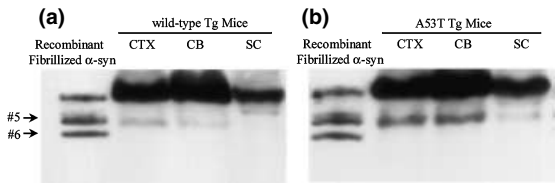

Fig. 6 Identification of putative calpain I α-syn fragment in Tg mice overexpressing human wild-type or A53T α-syn. The high salt fraction of transgenic mice tissue overexpressing human wild-type (a) or A53T α-syn (b) was analyzed by western blotting. Full-length α-syn and a truncated form are labeled with antibody Syn 303 in the cortex (CTX), cerebellum (CB), and spinal cord (SC) of wild-type Tg mice and A53T Tg mice. A truncated form of α-syn comigrated with a cleaved fragment of recombinant fibrillized α-syn. Fragments from recombinant α-syn are labeled as 5 and 6 in lane 1 indicated by the arrows.

Discussion

The data in the present study reveal that α-syn is a substrate of calpain I and that the A53T mutant and fibrillized forms of α-syn are cleaved differently from soluble wild-type α-syn (Fig. 7). Calpain I predominantly cleaves wild-type α-syn in the N-terminal segment after amino acid 57, and to a lesser degree within the NAC region. The location of the calpain I cleavage sites within the amino acid sequence of α-syn is consistent with the possibility that calpain I is involved in α-syn processing *in vivo* and may protect against the formation of pathological aggregation. The major cleavage site between amino acids 57 and 58 is not present in A53T mutant α-syn, and fibrillized α-syn (wild-type and mutants) is cleaved predominantly in the C-terminal region after amino acids 114 and 122.

The N-terminal and C-terminal fragments of wild-type α-syn produced by calpain I cleavages were identified by employing different methodologies that included the purification of the fragments by HPLC. To eliminate possible sequence matches, antibodies were used to identify unique epitopes within the sequence. The major cleavage site of soluble wild-type α-syn is located between amino acids 57 and 58, as these fragments are most readily detected by Coomassie stain, western blot and HPLC analysis (Fig. 3, samples F and I). Less prominent cleavages were identified between amino acids 73 and 74, 75 and 76, and 83 and 84. In addition, a possible cleavage between amino acids 31 and 32 at the third repeat motif, similar to the major cleavage after amino acid 57 at the fifth repeat motif, supports the western blot results indicating minor cleavages in the N-terminal segment of the protein.

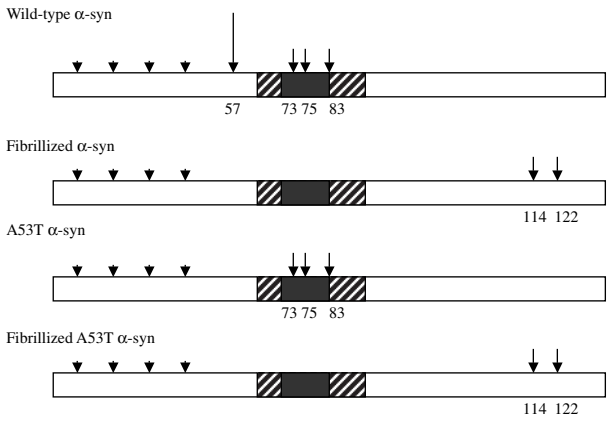

Fig. 7 Calpain I cleavage of α-syn. The major cleavage of wild-type α-syn was indicated by the large arrow after amino acid 57. Cleavages within the NAC region are indicated by smaller arrows after amino acids 73, 75, and 83. Fibrillized wild-type and A53T α-syn are cleaved predominantly in the C-terminus after amino acids 114 and 122. The arrowheads indicate minor cleavages. The striped region indicates the NAC region (amino acids 61–95); the solid area indicates the amino acids crucial for fibrillization.

The difference in cleavage sites between wild-type native α-syn and fibrillized wild-type α-syn suggests a pathophysiological role of calpain I cleavage in mechanisms of α-syn fibrillization. Fibrillized α-syn is a substrate of calpain I; however, the predominant cleavage occurs not in the N-terminal region or middle of the protein but in the C-terminal region after amino acids 114 and 122, producing C-terminally truncated forms of the protein. C-terminal truncation of α-syn may play a role in the pathogenesis of LBs and selective loss of dopaminergic neurons in PD. C-terminally truncated α-syn more readily changes conformation into the β-sheet conformation and forms fibrils at a faster rate than the full-length protein (Crowther *et al*. 1998; Serpell *et al*. 2000). Truncation also enhances the vulnerability of dopaminergic cells to oxidative damage (Kanda *et al*. 2000). The region of α-syn that is essential for filament assembly is located in the NAC region from amino acids 71–82 (Giasson *et al*. 2001). Based on our results, calpain I-mediated cleavage of native wild-type α-syn could protect against fibrillization by cleaving within the NAC region, altering the structure of this region, whereas cleavage of fibrillized α-syn by calpain I in the C-terminal region could further enhance the fibrillization process.

The truncated forms identified in this study match closely to the molecular mass of the C-terminally cleaved fragments produced in human LBs (∼14–16 kDa; Baba *et al*. 1998). A C-terminally truncated form of α-syn, which has almost identical immunoreactive and electrophoretic properties as fragment 5 from this study (Fig. 6), was detected in Tg mice overexpressing human α-syn (Giasson *et al*. 2002). In addition, another study of α-syn Tg mice (Lee *et al*. 2002), identified a truncated form of α-syn (12 kDa) in all Tg mice overexpressing human α-syn. Moreover, a lower molecular mass form of α-syn (10 kDa) was identified in affected A53T Tg mice (those that developed progressive motor dysfunction) in brain regions correlating with neuropathology (Lee *et al*. 2002). It is possible that these truncated fragment(s) detected in the Tg mice are generated by calpain I, but this remains to be demonstrated directly. Nevertheless, if these fragments are generated by calpain I, it would suggest that there might be some 'protofibrillar' or small aggregate forms of α-syn in these mice as calpain I cleavage resulting in fragment 5 *in vitro* was predominantly observed for fibrillized α-syn. Alternatively, other chemical modifications might alter the cleavage of α-syn by calpain I to include C-terminal cleavage. Further studies will be needed to ascertain these possibilities.

Autosomal dominant PD is linked to two mutations in α-syn, A53T, and A30P (Polymeropoulos *et al*. 1997; Krüger *et al*. 1998). In this study, *in vitro* cleavage by calpain I showed no difference in breakdown products produced by cleavage of A30P α-syn compared with wild-type. This matches evidence from a recent study showing a lack of significant pathology or abnormal fragmentation of A30P

α-syn Tg mice (Lee *et al*. 2002). However, a different cleavage pattern was identified for A53T α-syn in our *in vitro* study. Notably, the major C-terminal fragment beginning at amino acid 58 (fragment 1) and the corresponding N-terminal fragment (#4) were not produced by calpain I cleavage in the A53T α-syn mutant. These results indicate that, unlike wild-type α-syn, A53T α-syn is not cleaved at the predominant cleavage site after amino acid 57 while the less prominent cleavages do occur. The A53T mutation, which is located between the fourth and fifth KTKEGV repeats, could change the local microenvironment of α-syn, rendering the cleavage site at the fifth repeat (amino acid 57) inaccessible to calpain I, an enzyme that frequently recognizes repeat structures in its substrate specificity (Johnson and Guttmann 1997; Melloni *et al*. 1998). An analogous change in calpain I sensitivity in mutant tau is associated with frontotemporal dementia, in which disease-causing mutations alter the accessibility of calpain I cleavage sites (Yen *et al*. 1999).

The accumulation of wild-type α-syn into proteinaceous inclusions has been widely studied, particularly with regard to the involvement of proteasomal pathways. This is still controversial, although several *in vitro* studies have suggested that the proteasomal pathway is involved in α-syn degradation (Bennett *et al*. 1999; Gai *et al*. 2000; Chung *et al*. 2001; McLean *et al*. 2001; Shimura *et al*. 2001). In addition, one study found a functional impairment of the 20/26S proteasome in the substantia nigra of PD brains (McNaught and Jenner 2001) while several other studies have observed ubiquitin staining of some, but not all, α-syn stained LBs (Spillantini *et al*. 1998a, 1998b; Sharma *et al*. 2001). In contrast, other *in vitro* studies failed to show any relationship between α-syn and the proteasome (Ancolio *et al*. 2000; Paxinou *et al*. 2001). The present results do not exclude a role of proteosomal degradation of α-syn (Tofaris *et al*. 2001; Liu *et al*. 2003). Inhibition of protein degradation via the proteasome pathway, regardless of whether α-syn is involved, would increase the level of protein and can result in α-syn fibril formation (Uversky *et al*. 2001; McNaught *et al*. 2002; Shtilerman *et al*. 2002). Alternatively, calpain I degradation of α-syn *in vivo* could act in parallel with actions of the proteasome, such that impairment of the proteosome could increase the need for degradation through calpain I-mediated pathways. Future studies may better address these possibilities.

It is possible that overactivation of calpain could deplete the levels of full-length α-syn, resulting in impaired functions that can include vesicular transport (Cabin *et al*. 2002) and chaperone activity (Souza *et al*. 2000). On the other hand, our results predict that calpain I may play a protective role against the aggregation of α-syn by cleaving within the NAC region, which contains 12 amino acids essential for fibrillization (Giasson *et al*. 2001). The A53T mutation in α-syn prevents calpain I cleavage after residue 57 and this paucity of proteolysis may lead to increased stability of the mutant protein, thus increasing the propensity of accumulating α-syn inclusions that can lead to disease in transgenic mice models (Giasson *et al*. 2002; Lee *et al*. 2002) and in humans (Duda *et al*. 2002). Therefore, calpain I activity may have a pathologic role in the formation of LB as well as the death of nigral neurons in PD. However, regardless of whether or not calpain I plays a dominant or subsidiary role in the metabolism of α-syn, further insights in the proteolytic processing of α-syn and the proteases for which it might be a substrate in the normal and diseased brain will likely clarify the cascade of events that result in the fibrillization of α-syn and the aggregation of insoluble α-syn fibrils into LBs. Such studies may also reveal whether such processing leads to the onset and/or progression of α-synucleinopathies in the same manner that advances in understanding how the aberrant processing of APP and Aβ peptides contribute to mechanisms underlying senile plaque formation in AD.

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