Cleavage of α-Synuclein by Calpain: Potential Role in Degradation of Fibrillized and Nitrated Species of α-Synuclein†

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ABSTRACT: α-Synuclein (α-syn) is a major protein component of the neuropathological hallmarks of Parkinson's disease and related neurodegenerative disorders termed synucleinopathies. Neither the mechanism of α-syn fibrillization nor the degradative process for α-syn has been elucidated. Previously, we showed that wild-type, mutated, and fibrillar α-syn proteins are substrates of calpain I *in vitro*. In this study, we demonstrate that calpain-mediated cleavage near and within the middle region of soluble α-syn with/without tyrosine nitration and oxidation generates fragments that are unable to self-fibrillize. More importantly, these fragments prevent full-length α-syn from fibrillizing. Calpain-mediated cleavage of α-syn fibrils composed of wild-type or nitrated α-syn generate C-terminally truncated fragments that retain their fibrillar structure and induce soluble full-length α-syn to co-assemble. Therefore, calpain-cleaved soluble α-syn inhibits fibrillization, whereas calpain-cleaved fibrillar α-syn promotes further co-assembly. These results provide insight into possible disease mechanisms underlying synucleinopathies since the formation of α-syn fibrils could be causally linked to the onset/progression of these disorders.

α-Synuclein (α-syn)[1] aggregation is the common neuropathological feature in several neurodegenerative diseases such as Parkinson's disease (PD), dementia with Lewy bodies (LBs), the LB variant of Alzheimer's disease, multiple system atrophy, and neurodegeneration with brain iron accumulation type I, collectively known as α-synucleinopathies (*1*). α-Syn is a 140 amino acid protein with an uncertain function. The N-terminal region contains four KTKEGV imperfect repeat motifs; the NAC (nonamyloid component of amyloid plaques) region contains two additional repeat motifs and comprises the middle region of the protein, amino acids 61−95; and the C-terminal region is rich in amino acids such as aspartate, glutamate, and proline (*2*). Soluble α-syn is natively unfolded, although it adopts an α-helical conformation in the presence of lipids and a *β*-sheet structure in the fibrillar form. While a hydrophobic stretch of amino acids (residues 71−82) within the NAC region is essential for fibrillization of α-syn (*3*), fibrillization is influenced by many factors including missense mutations (*4*−*14*), C-terminal truncation (*12*, *15*−*17*), molecular crowding (*18*, *19*), and oxidative post-translational modifications (*20*, *21*). Numerous *in vitro* studies demonstrate that α-syn assembles into 10−19-nm wide amyloid fibrils with similar biochemical and biophysical properties as the fibrils observed in human α-syn inclusions, such as LBs, and thus provide a basis for investigating molecular and chemical events that influence the process of α-syn fibril formation *in vivo* (*3*−*5*, *8*).

Although the mechanism of α-syn aggregation in disease is unknown, impaired degradation of α-syn by the proteasome and lysosome may be involved (*22*−*30*). However, metabolism of α-syn by the proteolytic enzyme calpain may also mediate the turnover of α-syn. Calpain cleaves α-syn *in vitro* and *in situ* (*13*, *31*−*33*). We previously demonstrated *in vitro* that soluble α-syn is cleaved by calpain after amino acids 57, 73, 74, and 83, while fibrillized α-syn is cleaved after amino acids 114 and 122 (*31*). Of the known degrading mechanisms for α-syn, only calpain produces limited C-terminal truncation. Calpain-cleaved α-syn fibrils *in vitro* are similar in molecular weight to C-terminally truncated α-syn fragments that accelerate α-syn fibrillization and lead to increased cell death (*12*, *15*−*17*, *34*). Our 1−122 amino acid calpain-generated fragment also has almost identical immunological and electrophoretic properties as an α-syn fragment identified in A53T α-syn transgenic mice (*31*). Moreover, Li et al. recently characterized a fragment of human α-syn found in brains of humans, in A53T human

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[1] Abbreviations: α-syn, α-synuclein; PD, Parkinson's disease; LBs, Lewy bodies; NAC, nonamyloid component of amyloid plaques; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; LMW, low molecular weight; HMW, high molecular weight; EM, electron microscopy.

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α-syn transgenic mice, as well as in neuronal cell lines, which has a putative cleavage site that correlates to our site of *in vitro* calpain cleavage after amino acid 122, providing additional support to our hypothesis that calpain may be an α-syn-degrading protease (*15*).

Oxidative injury has been implicated in the pathogenesis of many α-synucleinopathies (*35*). Reactive oxygen and nitrogen species produced *in vivo* from cellular reactions can form nitrating agents that convert tyrosine residues in proteins to 3-nitrotyrosine (*36*). Four tyrosine residues (Y39, Y125, Y133, and Y136) in α-syn are targets for nitration and oxidation, generating dityrosine covalently cross-linked dimeric and oligomeric α-syn (*37*, *38*). Antibodies specific for nitrated α-syn label LBs containing α-syn in several α-synucleinopathies (*35*). These data demonstrate that α-syn is nitrated in human diseases, although it is not clear whether nitration of α-syn occurs prior to or after fibrillization. Soluble nitrated α-syn does not fibrillize, while treatment of preformed fibrils with nitrating agents stabilizes these fibrils through dityrosine cross-linking (*39*, *40*). However, co-incubation of soluble nitrated α-syn at low concentrations with unmodified α-syn increases the rate of fibril formation as nitrated α-syn is incorporated into the fibrils (*40*). Although calpain cleaves nitrated α-syn *in vitro*, soluble nitrated α-syn is not cleaved as readily as unmodified wild-type α-syn, potentially accelerating fibril formation (*40*).

Thus, in the present study, we extended our previous findings by investigating the effect of nitration on proteolytic cleavage of soluble and fibrillar α-syn. In addition, we examined whether calpain-generated fragments could form fibrils and/or promote the fibrillization of soluble full-length α-syn and whether these processes were altered by the A53T mutation or exposure to peroxynitrite.

EXPERIMENTAL PROCEDURES

Expression and Purification of Wild-Type and Mutant α-Syn Protein Species. Recombinant human wild-type and mutant A53T α-syn were expressed and purified as previously described (*8*). cDNAs for each of the proteins were subcloned into the bacterial expression vector pRK172. After expression in BL21 (DE3) *E. coli*, bacterial pellets were resuspended in high-salt lysis buffer containing protease inhibitors, boiled to 100 °C for 10 min, and centrifuged at 20000*g* for 10 min. α-Syn proteins were further purified by Sephadex 200 gel filtration and Mono Q ion-exchange chromatography (Amersham Biosciences, Piscataway, NJ) with a 0−0.5 M NaCl gradient. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Exposure of α-Syn Proteins to Peroxynitrite. Peroxynitrite was synthesized from sodium nitrite and acidified hydrogen peroxide. Excess hydrogen peroxide was removed by treatment with manganese dioxide. Exposure of α-syn to peroxynitrite was described previously (*37*). Briefly, α-syn was diluted into nitration buffer (100 mM potassium phosphate, 25 mM sodium bicarbonate at pH 7.4, and 0.1 mM diethylenetriamine pentaacetic acid), and the concentration of peroxynitrite was determined spectrophotometrically at 302 nm in 2 N NaOH ($\lambda_{302} = 1670\ M^{-1}\ cm^{-1}$). Two boluses of peroxynitrite were added at 10-fold molar excess of protein immediately prior to each experiment.

Fibrillization of α-Syn Proteins. α-Syn proteins were incubated in 100 mM sodium acetate (pH 7.0) at various concentrations for 48 h at 37 °C with continuous shaking. Each sample was overlaid with ∼40 μL of mineral oil to prevent condensation of samples. After fibrillization, samples were centrifuged, and the pellet fraction was resuspended in 100 mM sodium acetate or nitration buffer for use in further studies.

Calpain-Mediated Cleavage of α-Syn Proteins. Calpain (Calbiochem, San Diego, CA) cleavage of purified recombinant human α-syn proteins (including soluble, fibrillized, and peroxynitrite-treated wild-type and mutant species) was carried out using methods similar to those described previously (*31*). Briefly, α-syn protein was incubated with calpain I in buffer containing 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5) and 5 mM dithiothreitol (DTT) at 37 °C. Calpain-mediated cleavage was initiated by the addition of calcium (1 mM final). In experiments involving full-length α-syn, α-syn protein was treated in parallel to cleaved proteins with the exception that calpain was omitted from the reaction buffer. The addition of 1 mM EDTA prior to the addition of calcium to the reaction buffer including calpain served as another control. For biochemical assays, aliquots were removed from the reaction mixture and added to an equal volume of 2× Invitrogen (Carlsbad, CA) sodium dodecyl sulfate (SDS) stop buffer at various time points, heated in a boiling water bath, and stored at −20 °C. Samples were then resolved on Tris-tricine gels and analyzed by immunoblotting using antibodies directed against wild-type and nitrated α-syn (*41*), followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and detected with enhanced chemiluminescence reagents (Pierce). For sedimentation and co-incubation studies, 1 mM EDTA was added to stop the reaction, and samples were then immediately subjected to centrifugal sedimentation or used in co-incubation experiments.

Chymotrypsin and Trypsin Cleavage of α-Syn Proteins. Chymotrypsin and trypsin (Sigma Chemical Co., St. Louis, MO) cleavage of α-syn proteins was performed as described previously (*32*). α-Syn was added to trypsin (100 fmol and 1 pmol) or chymotrypsin (2 pmol) reaction buffers containing 20 mM Tris-HCl (pH 7.5) and incubated at 37 °C. SDS sample buffer (2×) was added to each reaction at the indicated time points, and samples were then heated to 100 °C for 5 min and stored at −20 °C. These experiments were performed 3 times.

Antibodies. Monoclonal antibodies Syn 303 and Syn h75, which recognize epitopes at amino acids 2−4 and 5−20, respectively, were used in this study (*41*, *42*). In addition, nSyn 14, which specifically recognizes α-syn nitrated at tyrosine 39, was used to label nitrated α-syn (*35*). LB509 and NAC antibodies were used for immunoelectron microscopy recognizing the epitopes 115−122 and 75−91, respectively (*41*−*43*).

Assessment of α-Syn Polymerization by Centrifugal Sedimentation Analysis. Reaction buffers containing 1 mg/mL of full-length or calpain-cleaved fibrillized α-syn proteins were centrifuged at 100000*g* for 20 min. Supernatants and pellets were separated, and samples were heated to 100 °C for 5 min in SDS sample buffer. Proteins were separated on

Tris-tricine gels, stained with Coomassie Blue R-250, and quantified by densitometry. Reaction buffers containing 1 mg/mL of soluble full-length or calpain-cleaved α-syn proteins were first exchanged into 100 mM sodium acetate and concentrated to 5 mg/mL using Centricon YM-3 centrifugal filters (Millipore, Bedford, MA). Samples were then incubated at 37 °C for 48 h with continuous shaking. After fibrillization, these samples were centrifuged and treated as described above.

Electron Microscopy. To examine the ultrastructure of α-syn fibrils, protein samples were adsorbed to 300-mesh carbon-coated copper grids, stained with 1% uranyl acetate, and visualized with a transmission electron microscope as described (*38*). Fibril width measurements were determined with Image-Pro Plus software (Media Cybernetics, Del Mar, CA). For immunoelectron microscopy, α-syn proteins were adsorbed onto 300-mesh carbon-coated copper grids, blocked with 1% bovine serum albumin for 5 min, and immunostained for 15 min with an affinity-purified antibody. Fibrils were decorated with anti-rabbit IgG or anti-mouse IgG antibodies conjugated to 5- or 10-nm gold particles and were negatively stained with 1% uranyl acetate.

Co-incubation Experiments. Full-length or calpain-cleaved soluble α-syn was exchanged from the reaction buffer into sodium acetate and concentrated to 2.5 mg/mL using Centricon YM-3 filters. These samples were co-incubated with 2.5 mg/mL of full-length α-syn as described above. Soluble full-length α-syn samples (at 2.5 and 5 mg/mL) were incubated in parallel with these samples. Proteins were subjected to electrophoresis, stained with Coomassie Blue R-250, and quantified by densitometry. The amount of full-length α-syn was compared to that in each sample.

Fibrillized calpain-cleaved α-syn proteins were exchanged from the calpain I reaction buffer into sodium acetate using Centricon YM-10 or YM-3 centrifugal filters to a final concentration of 1 mg/mL. Each sample was co-incubated with 1 mg/mL of soluble α-syn for 48 h at 37 °C with continuous shaking and centrifuged as described above. Samples were resolved on Tris-tricine gels and stained with Coomassie Blue R-250 for densitometric analysis. For calpain-cleaved α-syn experiments, the amount of full-length protein in the pellet of the calpain-cleaved α-syn samples was compared to the amount of full-length protein in the pellet of calpain-cleaved α-syn samples co-incubated with full-length α-syn. A total of 1 mg/mL of soluble full-length α-syn alone and 1 mg/mL of recombinant 1−125 α-syn co-incubated with full-length α-syn were incubated in parallel with these samples.

Statistics. All results were reported as mean ± standard deviation (SD). Unpaired two-tailed *t* tests were performed to define differences using Statview 5.01 software (SAS Institute Inc.). Significance was set at $p < 0.05$.

RESULTS

Proteolytic Cleavage of Soluble Nitrated α-Syn. Treatment of recombinant α-syn (wild type and A53T mutant) with peroxynitrite nitrates α-syn at tyrosine residues 39, 125, 133, and 136 (*37*, *38*) and results in the formation of tyrosine nitrated monomers as well as nitrated SDS-stable dityrosine cross-linked dimers and oligomers. Because calpain I and II cleaved soluble α-syn at the same sites (Figure S1 in the Supporting Information), calpain I was used for the present study. Activation of calpain decreased levels of both dimeric and monomeric α-syn and generated low molecular weight (LMW) fragments (Figure 1A). Immunoblotting with antibodies Syn 303 (epitope at amino acids 2−4) and nSyn 14 (epitope at nitrated tyrosine residue 39) revealed that the calpain-generated fragments were identical in molecular weight for unmodified and nitrated α-syn (Figure 1A). Cleavage of the nitrated protein also generated a species with an apparent 32-kD mass, likely a cleavage product of the oligomeric or dimeric species. Nitrated, mutant A53T α-syn was not cleaved after amino acid 57, as observed previously in nonnitrated A53T α-syn (Figure 1B) (*31*), but was otherwise cleaved similarly to wild-type α-syn.

To examine if nitration of α-syn changed the cleavage pattern of other proteases, nonnitrated and nitrated α-syn proteins were incubated with trypsin and chymotrypsin. Because trypsin cleaves proteins at numerous sites, the Syn h75 antibody that recognizes amino acids 5−20 of α-syn was used to detect the fragments. Consistent with a previous report (*37*), exposure to peroxynitrite did not change the cleavage pattern of α-syn exposed to 100 fmol/20 μL of trypsin (Figure 1C). Increasing the concentration of trypsin by 10-fold (1 pmol/20 μL) resulted in complete disappearance of full-length α-syn with production of unstable fragments by 5 min (data not shown). An over-exposed Western blot showed cleavage of full-length wild-type and nitrated α-syn into LMW fragments (Figure 1C). Some high molecular weight (HMW) species, although present in small levels compared to full-length protein, were detected by Syn h75 in the wild-type samples and were not readily degraded by trypsin. The abundant HMW species in the nitrated samples were degraded significantly.

Similarly, we investigated whether exposure to peroxynitrite changed the cleavage pattern of α-syn by chymotrypsin (Figure 1D). Chymotrypsin predominantly cleaves after phenylalanine, tyrosine, and tryptophan residues. Because chymotrypsin could cleave α-syn after Phe4 and Tyr39 residues, regions of the protein detected by antibodies Syn 303 and nSyn 14, respectively, the Syn h75 antibody, which recognizes amino acids 5−20 of α-syn, was used to identify the cleaved fragments. Treatment with chymotrypsin resulted in the disappearance of HMW species and full-length α-syn and caused the appearance of LMW fragments (Figure 1D). Therefore, the fragments observed are likely 1−94, 4−94, and 1−39 α-syn fragments based on the predicted cleavage after tyrosine residues 39, 125, 133, and 136 and after phenylalanine residues 4 and 94. Syn 303 detected fragments of the same molecular weight as the putative 1−94 and 1−39 α-syn fragments but not the putative 4−94 α-syn fragment (data not shown), showing that these were N-terminal fragments. Furthermore, antibody nSyn 14 detected fragments at the same molecular weight as 1−94 and 4−94 α-syn fragments. Both antibodies nSyn 14 and Syn h75 detected considerably smaller amounts of fragment 1−39 of nitrated α-syn, suggesting that the nitration of tyrosine 39 makes it a poor substrate for chymotrypsin cleavage, consistent with the previous observation that tyrosine nitration does not block but considerably slows digestion of proteins by chymotrypsin (*44*). In summary, nitration of soluble wild-type α-syn did not alter the cleavage pattern of

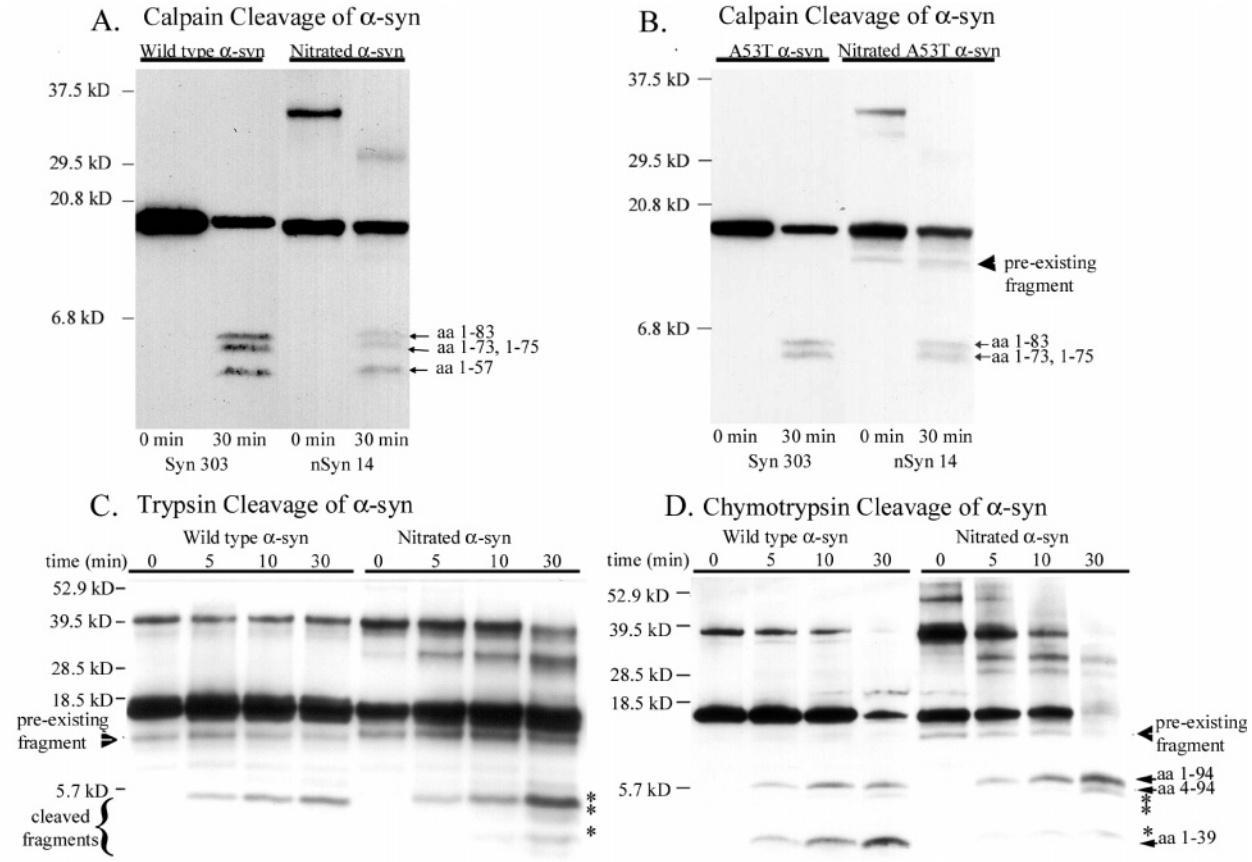

FIGURE 1: Nitration does not affect cleavage of soluble α-syn by proteases. (A) Representative Western blot of wild type (lanes 1 and 2) and nitrated wild-type α-syn (lanes 3 and 4) cleaved by calpain. Syn 303 detected wild-type full-length and calpain-generated fragments of α-syn after cleavage for 30 min. nSyn 14 detected dimeric, full-length, and calpain-generated fragments of α-syn after 30 min of cleavage. (B) Representative Western blot of A53T α-syn (lanes 1 and 2) and nitrated A53T α-syn (lanes 3 and 4) cleaved by calpain. Syn 303 detected nonnitrated A53T α-syn, and nitrated A53T α-syn was detected by nSyn 14. (C) Representative Western blot after trypsin cleavage of soluble and nitrated wild-type α-syn, immunolabeled with Syn h75. (D) Representative Western blot after chymotrypsin cleavage of soluble and nitrated wild-type α-syn, immunolabeled with Syn h75. Fragments of α-syn generated by chymotrypsin cleavage are marked with arrows. A pre-existing band in nitrated α-syn is labeled with an arrowhead. Asterisks show migration of calpain-cleaved α-syn fragments relative to trypsin- and chymotrypsin-cleaved fragments. Experiments were performed 3 times with similar results obtained in each experiment.

calpain and trypsin and only changed that of chymotrypsin slightly.

Proteolytic Cleavage of α*-Syn Fibrils.* Calpain cleaves fibrillized α-syn in the C terminus, resulting in two major N-terminal fragments 1−114 and 1−122 α-syn (lanes 1 and 2 in parts A and B of Figure 2) (*31*). Nitration/oxidation of preformed fibrils did not alter their cleavage by calpain, demonstrated by the presence of two C-terminally truncated fragments that migrated at the same molecular weight as those fragments of cleaved, nonnitrated fibrils (lanes 3 and 4 in parts A and B of Figure 2).

We also investigated whether nitration of preformed α-syn fibrils changed the proteolytic susceptibility to chymotrypsin and trypsin. Nonnitrated fibrillized α-syn was cleaved in the C terminus by chymotrypsin, resulting in two major fragments after 30 min, identified by antibody Syn h75 (Figure 2C). Similarly, trypsin cleaved fibrillized α-syn, producing one major N-terminal fragment that migrated just below full-length α-syn (Figure 2D). Nitration of fibrils did not affect the cleavage pattern by trypsin or chymotrypsin. Interestingly, both proteases cleaved full-length α-syn (lanes 1 and 2 in parts A and B of Figure 2; lanes 1−4 in parts C and D of Figure 2) but did not readily degrade nitration-stabilized dimeric and oligomeric forms of α-syn (lanes 3 and 4 in parts A and B of Figure 2; lanes 5−8 in parts C and D of Figure 2).

Characteristics of Calpain-Generated Fragments from Soluble α*-Syn.* To determine whether the calpain-generated fragments of soluble α-syn proteins were able to form fibrils, full-length or calpain-cleaved α-syn proteins were incubated at 5 mg/mL for 48 h with continuous shaking. After sedimentation, the majority of full-length wild-type and A53T α-syn proteins were recovered in the pellet fraction, consistent with the formation of fibrils (Figure 3; *38*). In contrast, the mixture of soluble nitrated α-syn monomers, dimers, and oligomers did not fibrillize, consistent with previous studies (*38, 40*). Under the same conditions, calpain-cleaved fragments from all soluble α-syn forms were recovered in the supernatant fraction (Figure 3), indicating that cleavage after amino acid 57 and within the NAC region generates fragments that do not readily form fibrils.

Characteristics of Calpain-Generated Fragments Generated from α*-Syn Fibrils*. Sedimentation analysis and electron microscopy (EM) were used to determine whether the calpain-cleaved fragments of fibrillized α-syn remained fibrillar or became soluble. Centrifugal sedimentation analysis of full-length fibrillized α-syn detected full-length α-syn fibrils of wild-type (59.7 ± 10.9%), A53T (61.3 ± 9.5%),

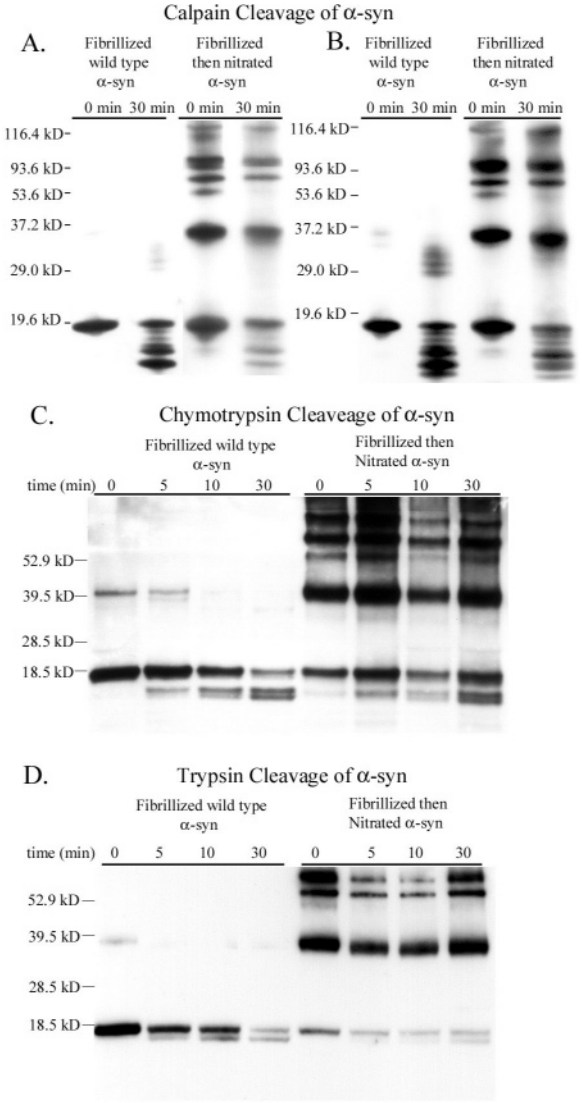

FIGURE 2: Nitration does not affect cleavage of preformed fibrils of α-syn by proteases. (A) Representative Western blot of fibrillized and fibrillized then nitrated α-syn cleaved by calpain at 0 and 30 min after cleavage. Syn 303 immunolabeled fibrillized wild-type α-syn and nSyn 14 immunolabeled fibrillized then nitrated α-syn fibrils. (B) Representative Western blot of fibrillized and fibrillized then nitrated α-syn cleaved by calpain and immunolabeled with Syn h75. (C) Representative Western blot of fibrillized nonnitrated and nitrated α-syn cleaved by chymotrypsin, immunolabeled with Syn h75. (D) Representative Western blot of fibrillized nonnitrated and nitrated trypsin-cleaved α-syn fibrils, immunolabeled with Syn h75. Experiments were performed 3 times with similar results obtained in each experiment.

and fibrillized then treated with peroxynitrite (57.2 ± 14.6%) α-syn in the pellet (P) as identified by Coomassie Blue stain of the Tris-tricine gels (parts A−C of Figure 4). After incubation with activated calpain, calpain-cleaved truncated fragments were recovered in the pelleted fraction after sedimentation for wild-type (87.2 ± 4.6%), A53T (75.9 ± 4.6%), and fibrillized then nitrated (76.3 ± 8.9%) α-syn (Figure 4D). Truncated forms were not noted in the soluble fraction (S). The truncated fragments in the pellets of these samples were cleaved in the C terminus as determined by recognition with an N-terminal antibody but not a C-terminal antibody (data not shown, see ref *31*). A significantly greater amount of wild-type α-syn was recovered in the pellet fraction following calpain cleavage as compared to full-length α-syn (Figure 4D). These data show that calpain-cleaved α-syn fibrils were pelletable.

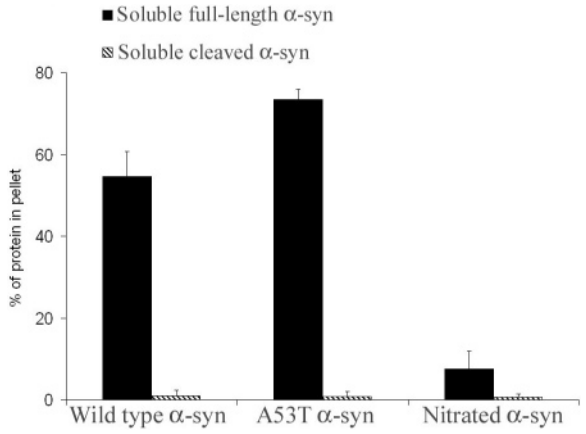

FIGURE 3: Calpain-generated fragments of soluble α-syn do not fibrillize under conditions favorable for full-length α-syn fibrillization. Quantitative sedimentation analysis of full-length and calpain-cleaved α-syn incubated at 5 mg/mL at 37 °C for 48 h with continuous shaking. Data are results from 2 to 4 experiments with similar results.

Electron Microscopic Evaluation of the Calpain-Cleaved α-Syn Fibrils. Direct observation by EM of the calpain-cleaved α-syn fibrils recovered in the pellet fraction following sedimentation confirmed that the fibrils retained a fibrillar structure. The increase in the amount of insoluble α-syn following calpain-mediated cleavage raised the possibility that treatment with calpain might alter the structure of the fibrils as well as its sedimentation properties. Fibrils generated from full-length wild-type α-syn fibrils had a mean width of 11.42 ± 0.35 nm (mean ± SEM; $n = 50$) (parts A and C of Figure 5), whereas morphologically similar calpain-cleaved fibrils had a significantly smaller width, 8.92 ± 0.25 nm (mean ± SEM; $n = 66$) (parts B and C of Figure 5). The stability of the calpain-cleaved fibrils exposed to peroxynitrite was also confirmed by EM analysis. However, in contrast to calpain-cleaved nonnitrated fibrils, the mean width of calpain-cleaved nitrated preformed fibrils was 11.54 ± 0.2 nm (mean ± SEM; $n = 99$), comparable to that of both unmodified full-length and nitrated α-syn fibrils 11.96 ± 0.19 nm (mean ± SEM; $n = 124$) (parts D−F of Figure 5). Therefore, although nitration did not affect the cleavage pattern of fibrillized α-syn, it did change the fibril ultrastructure following cleavage by calpain.

Immuno-EM of Calpain-Cleaved α-Syn Fibrils. To identify additional ultrastructural changes created by exposure to peroxynitrite or calpain-mediated cleavage, we used immuno-EM of α-syn fibrils with antibodies to the C terminus (LB509) and the NAC region of α-syn. LB509 decorated the fibrils in all samples (parts A−D of Figure 6), indicating that the epitope of amino acids 115−122 of α-syn was exposed. The NAC antibody also labeled the full-length fibrils of nonnitrated and nitrated fibrillized α-syn (parts E and G of Figure 6). Although this antibody also labeled calpain-cleaved, nitrated preformed fibrils, this antibody did not label the fibrils of calpain-cleaved unmodified wild-type α-syn (parts F and H of Figure 6). These results suggest that this epitope (amino acids 75−91) is less accessible in the calpain-cleaved fibrils of nonnitrated α-syn than in other α-syn fibrils. This correlates with the decreased fibril width of nonnitrated calpain-cleaved α-syn fibrils (parts B and C

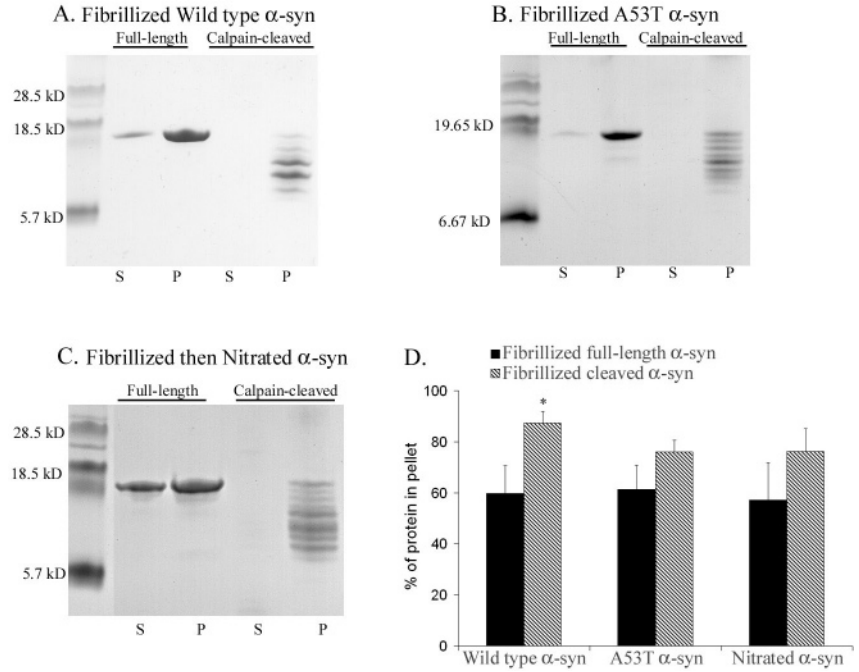

FIGURE 4: Fibrillized forms of full-length and calpain-cleaved α-syn are recovered in the pellet fraction following sedimentation. Sedimentation analysis of fibrillized wild-type α-syn, fibrillized A53T α-syn, and fibrillized then nitrated α-syn in the presence (calpain-cleaved) and absence (full-length) of calpain. Supernatants (S) and pellets (P) were resolved on Tris-tricine gels and stained with Coomassie Blue R-250 stain. (A) Coomassie Blue stained gel of fibrillized wild-type α-syn, calpain-cleaved, and full-length samples. (B) Coomassie Blue stained gel of fibrillized A53T α-syn, calpain-cleaved, and full-length samples. (C) Coomassie Blue stained gel of fibrillized nitrated α-syn, calpain-cleaved, and full-length samples. The bar graph in D shows percentages of pelletable α-syn proteins recovered after centrifugation. (*) $p = 0.048$ compared to fibrillized full-length wild-type α-syn. Data are results from 2 to 5 experiments with similar results.

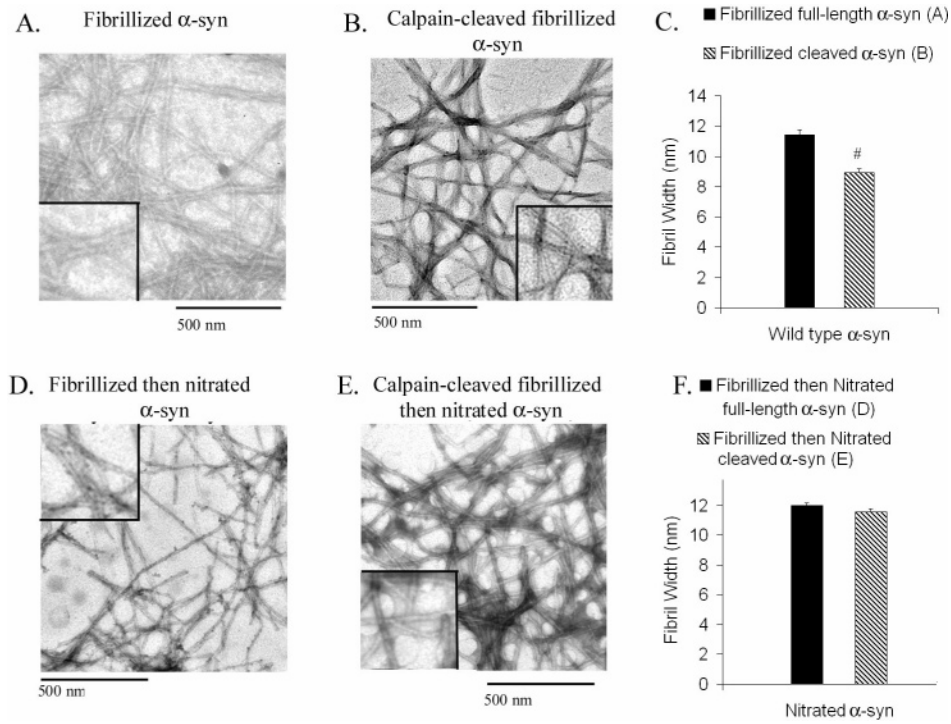

FIGURE 5: Calpain-cleaved fibrils have fibrillar ultrastructure. EM was used to assess fibril formation of (A) full-length and (B) calpain-cleaved wild-type α-syn after incubation with shaking for 48 h. The bar graph in C shows fibril width (in nanometers) of full-length and calpain-cleaved α-syn fibrils. (#) $p < 0.0001$. (D) Assessment of nitrated full-length preformed α-syn fibrils by EM. (E) Assessment of calpain-cleaved nitrated preformed α-syn fibrils. The bar graph in F shows fibril width (in nanometers) of full-length and calpain-cleaved nitrated preformed α-syn fibrils. Scale bar equals 500 nm. The inserts are 4× software magnification of individual fibrils. Data are mean ± SEM of results from 50 to 124 measurements.

of Figure 5) and suggests that the decrease in fibril width lessened the accessibility of this region of α-syn.

Calpain-Cleaved Soluble α-Syn Co-incubated with Full-Length α-Syn. We then investigated whether calpain-cleaved

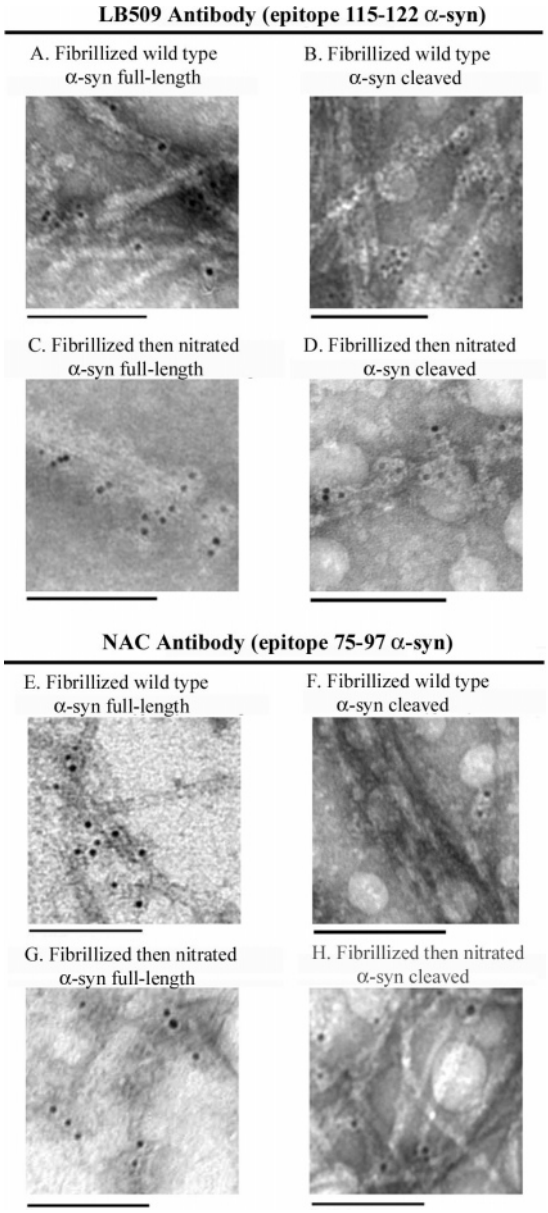

FIGURE 6: Immunoelectron microscopy reveals accessibility of the NAC and C-terminal regions. Antibody LB509 was used to label (A) full-length fibrillized wild-type α-syn, (B) calpain-cleaved fibrillized wild-type α-syn, (C) full-length fibrillized nitrated α-syn, and (D) calpain-cleaved fibrillized nitrated α-syn. The NAC antibody was used to label (E) full-length fibrillized wild-type α-syn, (F) calpain-cleaved fibrillized wild-type α-syn, (G) full-length fibrillized nitrated α-syn, and (H) calpain-cleaved fibrillized nitrated α-syn. Scale bar equals 100 nm. Gold particles (5 or 10 nm) conjugated to the secondary antibody on the fibrils appear as black dots.

soluble α-syn fragments promoted the fibrillization of full-length α-syn. As shown in Figures 3 and 7, 5 mg/mL of calpain-cleaved soluble α-syn did not form fibrils indicated by recovery of the LMW fragments in the supernatant fraction. In contrast, 2.5 or 5 mg/mL concentrations of full-length α-syn protein alone fibrillized as demonstrated by recovery of 62.9 ± 9.2 and 54.6 ± 5.8% of the total protein, respectively, in the pellet fraction following sedimentation (Figure 7). These data are consistent with other reports demonstrating statistically similar sedimentation results after 48 h (*38*, *45*). However, when full-length α-syn was co-incubated with calpain-cleaved soluble α-syn in a 1:1 ratio, a significantly smaller amount of full-length α-syn protein was recovered in the pellet fraction compared to full-length α-syn alone. This suggested that co-incubation of calpain-cleaved fragments of soluble α-syn did not promote fibrillization of full-length α-syn but instead inhibited fibrillization. This effect was greatest with the co-incubation of calpain-cleaved soluble A53T and full-length wild-type α-syn (Figure 7B and bar graph).

Calpain-Cleaved Fibrillized α-Syn Co-incubated with Full-Length Soluble α-Syn. The ability of calpain-cleaved fibrillar fragments to promote co-assembly of soluble full-length α-syn was also examined. In this experiment, a concentration of soluble full-length α-syn was used (1 mg/mL) that does not polymerize after 48 h at 37 °C with continuous shaking (13.2 ± 2.6% of the total protein recovered in the pellet; parts D and F of Figure 8). This protein was co-incubated in a 1:1 ratio with calpain-cleaved wild-type, A53T, or nitrated (preformed) fibrillar fragments, in which ≤20% of full-length α-syn remained following cleavage (not significantly different than the amount of full-length α-syn in 1 mg/mL incubations). After 48 h, the samples were centrifuged and the amount of insoluble full-length α-syn was measured by densitometry (parts A−E of Figure 8). In this assay, under conditions in which full-length α-syn does not polymerize alone, incubation of full-length soluble α-syn with calpain-cleaved fibrillar fragments increased the amount of full-length α-syn in the pellet fraction compared to the amount in the incubation of calpain-cleaved α-syn alone. This suggests that the calpain-cleaved fibrillar fragments promoted the co-assembly of full-length α-syn. This effect was noted for calpain-cleaved wild-type, A53T, and nitrated fibrils. Additionally, incubation of full-length soluble α-syn with fibrillized recombinant 1−125 α-syn also promoted the co-assembly of soluble full-length α-syn (parts E and F of Figure 8). Full-length fibrils also promoted the co-assembly of soluble full-length α-syn when co-incubated (Figure 8F and Figure S2 in the Supporting Information). Although this experiment was designed as a qualitative rather than quantitative observation, fibrillized full-length α-syn promotes soluble full-length α-syn assembly slightly more than calpain-cleaved fibrillized α-syn ($p = 0.041$; Figure 8G). The percent of full-length wild-type and A53T α-syn in the pellet fraction of the co-incubation conditions did not significantly differ from full-length fibrillized α-syn alone (Figure S2 in the Supporting Information). This observation indicates that fibrillized α-syn-induced polymerization of soluble α-syn reaches a steady-state phase in which the fibrils and soluble α-syn are at equilibrium. In contrast, co-incubation of nitrated preformed fibrils with full-length soluble α-syn resulted in a significantly smaller percentage of the total protein recovered in the pellet fraction ($p = 0.037$) compared to nitrated preformed fibrils alone (Figure S2 in the Supporting Information). This suggests that nitration of preformed full-length α-syn fibrils inhibits the ability to polymerize soluble α-syn protein.

DISCUSSION

In the present study, proteolytic cleavage by calpain *in vitro* alters α-syn fibrillogenesis in a manner that could modulate the pathophysiology of synucleinopathies. Calpain-generated fragments of soluble α-syn (nonnitrated and nitrated) do not fibrillize when incubated alone and inhibit

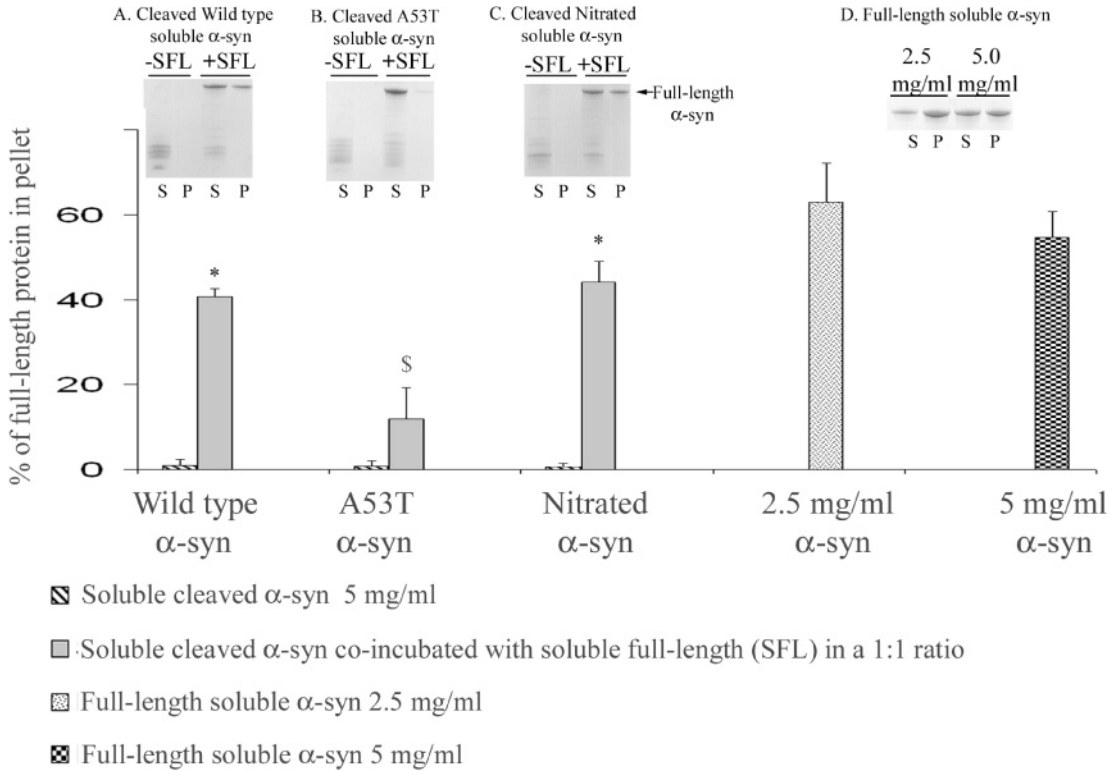

FIGURE 7: Calpain-cleaved soluble fragments inhibit fibrillization of soluble full-length wild-type (SFL) α-syn. A total of 2.5 mg/mL of calpain-cleaved soluble (A) wild-type, (B) A53T, and (C) nitrated α-syn alone (−SFL) or in a 1:1 ratio with soluble full-length α-syn (+SFL) was incubated for 48 h at 37 °C with continuous shaking. Each sample was fractionated into supernatants (S) and pellets (P) by sedimentation at 100000*g* for 20 min and resolved on Tris-tricine gels that were stained with Coomassie Blue R-250. (D) Full-length soluble α-syn incubated at 2.5 and 5 mg/mL alone in parallel. The bar graph depicts the percent of pelleted full-length α-syn from each sample. (*) $p < 0.05$. ($) $p < 0.0001$ compared to full-length soluble α-syn at 2.5 mg/mL. Data are results from 2 to 5 experiments with similar results.

fibrillization of full-length α-syn. In contrast, the calpain-mediated cleavage of fibrillized α-syn generates fragments 1−114 and 1−122 that remain fibrillar as examined using sedimentation and EM analysis. Moreover, the fibrillar fragments generated by calpain induce co-assembly of soluble full-length α-syn, increasing the fraction of insoluble protein in the pellet after centrifugation. These results show that calpain cleaves soluble α-syn in a manner that blocks fibrillization, while cleaving fibrillar α-syn in a manner that promotes further co-assembly of soluble full-length α-syn (Figure 9). Because fibril formation is a potential disease-generating event in synucleinopathies, these results are important for understanding the pathophysiology of these disorders.

Calpain-mediated cleavage of soluble α-syn generates fragments of α-syn that inhibit the fibrillization of soluble α-syn, particularly fragments produced from A53T α-syn. Because A53T α-syn lacks the major cleavage site after amino acid 57, this suggests that even partial destruction of the NAC region alone can interfere with the fibrillization process. As suggested in other studies, the NAC region may form the core of both *in vitro* and *in vivo* α-syn fibrils and is resistant to proteinase K digestion (*3*, *46*). In addition, mutant α-syn protein species lacking the NAC region do not fibrillize or promote co-assembly with full-length α-syn (*3*).

Understanding the biochemical properties of nitrated α-syn is important, because nitrated α-syn has been identified in the inclusions of synucleinopathies (*35*). Like calpain-mediated cleavage, nitration differentially affects the formation and stability of α-syn oligomers and fibrils. Exposure of soluble α-syn to nitrating species promotes oligomerization primarily through dityrosine cross-linking but inhibits the formation of fibrils (Figure 3; *37*−*40*, *47*). It does not alter the proteolytic degradation pattern by calpain, trypsin, or chymotrypsin, although it slows the rate of cleavage (*40*). Nitration could increase the half-life of α-syn, leading to greater amounts of soluble α-syn capable of fibrillization with unmodified or nitrated monomeric α-syn (*40*). Exposure of preformed fibrils to nitrating species such as peroxynitrite stabilizes the fibril structure through dityrosine cross-linking (*37*, *38*). In this study, both full-length and calpain-cleaved nitrated preformed fibrils were recovered in the pellet fraction following sedimentation (Figure 4). Although nitration did not affect the proteolytic cleavage of preformed fibrils, it did alter the ultrastructure of the cleaved fibrils in comparison to the nonnitrated cleaved fibrils. Calpain-cleaved wild-type α-syn fibrils have a reduced fibril width and sequestered central region of the fibril. However, nitration of the preformed fibrils abolished this reduction in width and increased the antibody binding to the NAC region of α-syn (Figure 6). This suggests that the calpain-mediated cleavage pattern of fibrils is not affected by nitration, but this modification does prevent the structural change induced by calpain in wild-type α-syn fibrils.

In contrast, C-terminally truncated fibrils of wild-type, nitrated, and A53T α-syn generated by calpain promote co-assembly of full-length soluble α-syn (Figure 8). Analysis of full-length fibrils and calpain-cleaved fibrils co-incubated with soluble full-length α-syn suggests that fibrillized full-

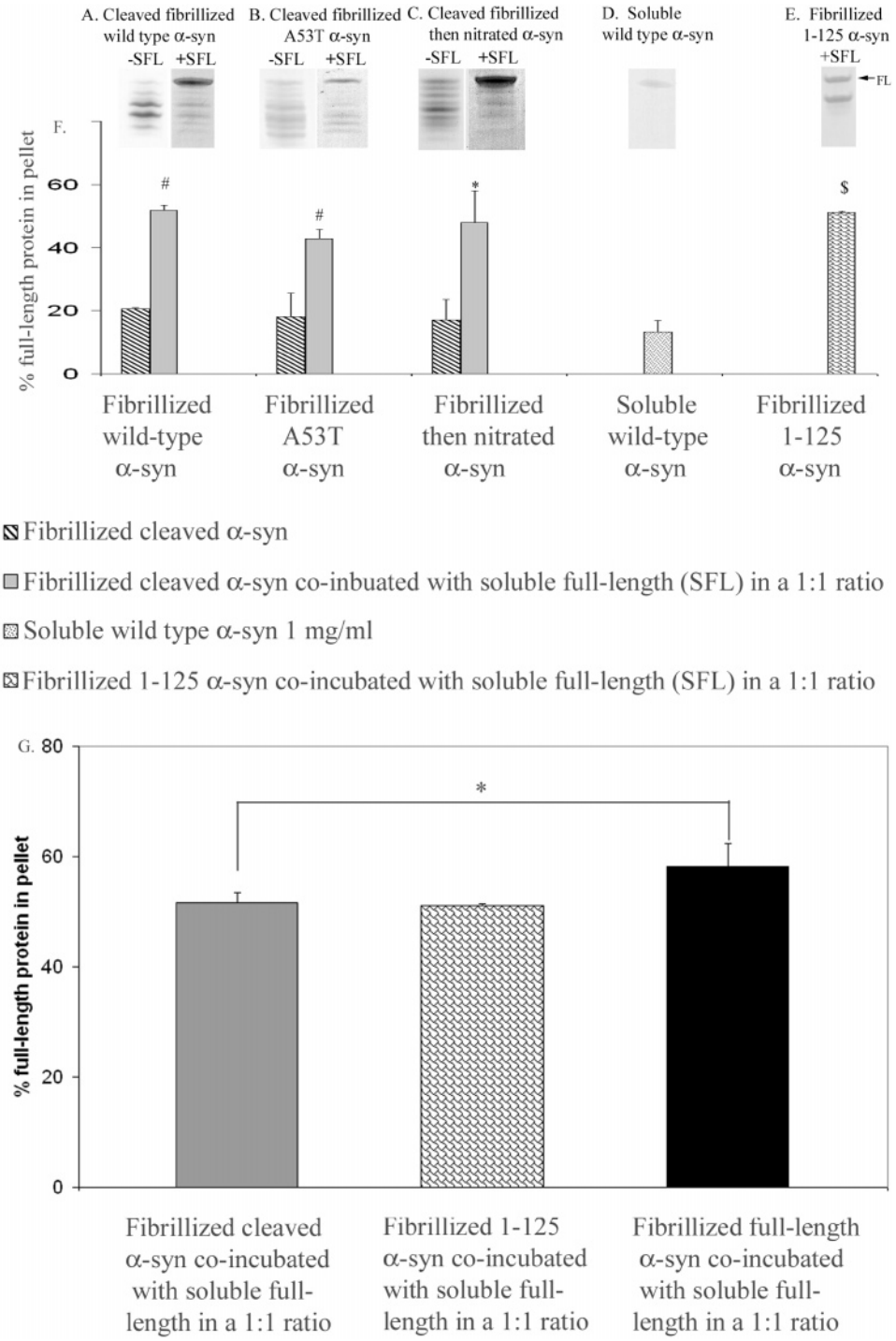

FIGURE 8: Calpain-cleaved fibrillized α-syn promotes polymerization of soluble full-length α-syn (SFL). Calpain-cleaved preformed fibrils of (A) wild-type, (B) A53T, or (C) nitrated α-syn were generated as described in the Experimental Procedures. These proteins were incubated alone (−SFL) or in a 1:1 ratio with soluble full-length α-syn (+SFL). Samples were fractionated by sedimentation at 100000*g* for 20 min and resolved on Tris-tricine gels that were stained with Coomassie Blue R-250. Representative lanes of pellet sample for each experiment are shown. (D) Full-length soluble α-syn incubated at 1 mg/mL alone in parallel with co-incubated samples. (E) Recombinant fibrillized 1−125 α-syn co-incubated with soluble full-length α-syn. (F) Bar graph represents the percentage of pelleted full-length α-syn from each sample. (#) $p < 0.0001$. (*) $p = 0.033$ compared to respective calpain-cleaved α-syn sample alone. ($) $p = 0.0003$ compared to 1 mg/mL soluble full-length α-syn incubated alone. (G) Bar graph represents the percentage of pelleted full-length α-syn from each sample. (*) $p = 0.041$. Data are results from 2 to 8 experiments with similar results.

length α-syn fibrils may result in a slightly larger increase in total amounts of assembled α-syn than calpain-cleaved truncated α-syn fibrils. Li et al. report that in a cell-free *in vitro* assembly experiment, an increase in the total amount of aggregated full-length α-syn is enhanced by the presence of the α-syn fragment 1−123 compared to the full-length α-syn assembly alone (*15*). The discrepancy between that study and ours may reflect the difference in starting material (soluble versus fibrillized α-syn) between the studies. Further studies evaluating the rate of assembly as well as the effect of varying protein concentrations under these different conditions may help to elucidate these differences. Impor-

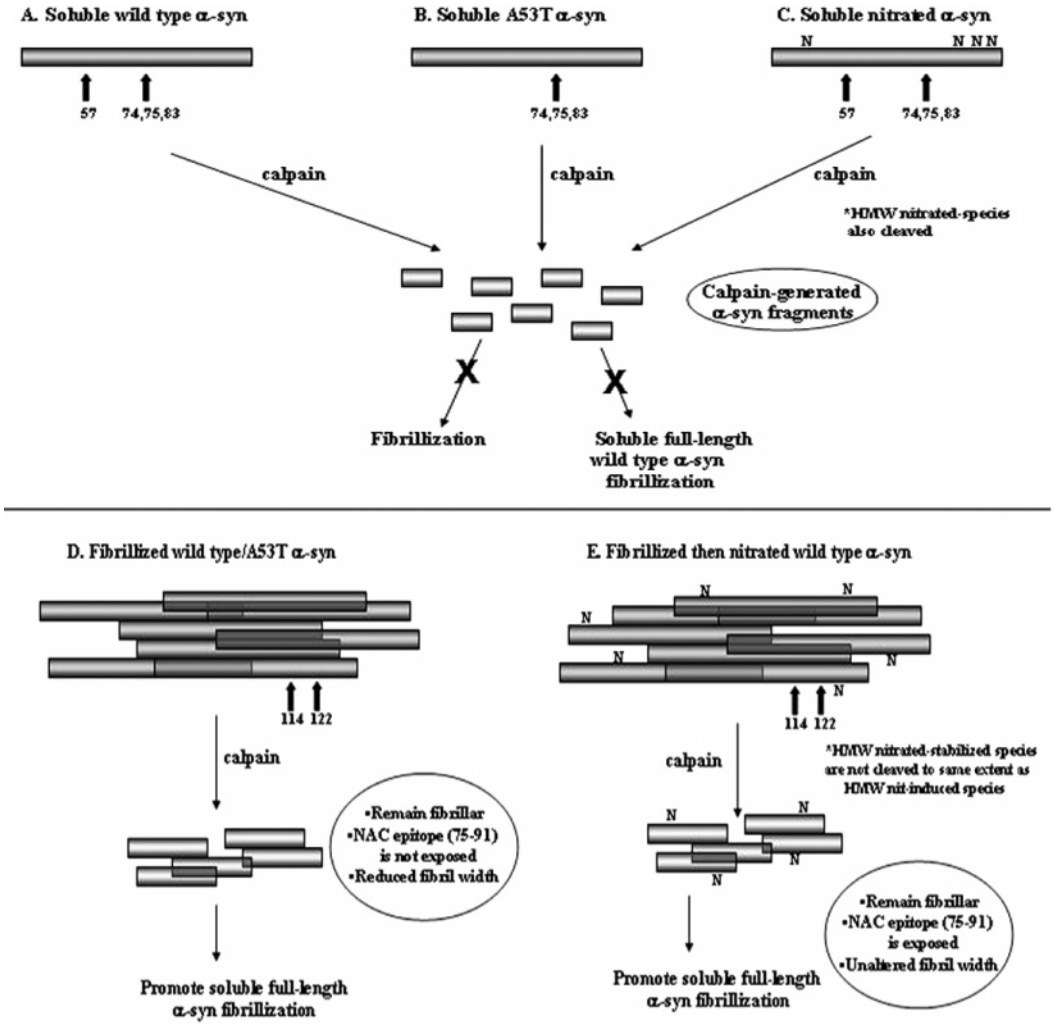

FIGURE 9: Potential effects of calpain-mediated cleavage and nitration on α-syn fibrillization. Calpain-mediated cleavage of soluble (A) wild-type, (B) A53T, and (C) nitrated α-syn near and within the NAC region abolishes it ability to fibrillize and inhibits fibrillization of soluble full-length α-syn. Nitration of soluble α-syn does not affect proteolytic cleavage, and calpain can cleave nitration-induced α-syn dimers. Nitrated calpain-cleaved α-syn fragments, like nitrated full-length α-syn, do not fibrillize and inhibit the fibrillization of full-length α-syn. (D) Calpain-mediated cleavage of fibrillized α-syn after amino acids 114 and 122 generate stable C-terminally truncated fibrils with a reduced fibril width compared to full-length α-syn fibrils. Similar to full-length wild-type and A53T α-syn fibrils, calpain-cleaved α-syn fibrils promote polymerization of full-length soluble α-syn. (E) Nitration of preformed fibrils does not affect proteolytic cleavage sites but does reduce the amount of calpain-mediated cleavage of nitrated-stabilized HMW α-syn species. Nitrated preformed α-syn fibrils do not show a reduction in fibril width shown in nonnitrated calpain-cleaved α-syn fibrils. While full-length nitrated preformed fibrils do not promote soluble full-length α-syn polymerization, C-terminal truncation by calpain abolishes this inhibition and promotes polymerization of soluble full-length α-syn.

tantly, these studies are in agreement that C-terminal cleavage of α-syn in the amino acid region of residue 122 promotes co-assembly of full-length α-syn, providing additional evidence that this modification of α-syn plays an important role in the aggregation of this protein.

In this study, calpain-cleaved fibrillized nitrated α-syn but not full-length fibrillized nitrated α-syn fibrils promote co-assembly of soluble full-length α-syn in co-incubation studies. This outcome is consistent with the proposed role of the C terminus in α-syn aggregation and suggests that this region, containing three of the four nitrated tyrosine residues, is responsible for the inhibition of co-assembly caused by full-length nitrated fibrils. The cleavage of the region by calpain containing the nitrated tyrosine residues does not lead to a reduction in fibril width as observed in wild-type cleaved fibrils, suggesting that the presence of the nitrated tyrosine residue 39 may play a role in this inhibition of fibril width reduction. The presence of only tyrosine residue 39 (by mutation of the C-terminal tyrosine residue to phenylanine) is sufficient for the formation of nitrating agent-induced α-syn dimers *in vitro* (*38*). In the present study, the retention of nitrated tyrosine 39 may be sufficient to block the calpain-induced reduction in fibril width. However, our data still suggest that calpain-mediated C-terminal cleavage of nitrated preformed fibrils can abolish the inhibitory effect that nitration exerts on α-syn fibril formation (*37*−*40*, *47*).

The turnover of α-syn and the events that lead to α-syn accumulation remain controversial. α-Syn transgenic mice as well as human PD brains contain truncated α-syn fragments such as those produced by calpain (*48*−*50*). The recent study by Li et al. also identified a fragment of α-syn present in human brains, A53T human α-syn transgenic mice, and SH-SY5Y cells (*15*). The authors identify the fragment as a C-terminally truncated fragment with a possible cleavage site between amino acids 122 and 123 or in very close

proximity to these amino acids. At present, calpain is the only known protease that generates limited C-terminal cleavage of α-syn to produce fragments similar in molecular weight to those observed in human brains and α-syn transgenic mice models. Unlike other proteases, calpain usually modifies but does not fully degrade its substrates (*51*). Therefore, calpain may act with other protein degradation mechanisms, including the proteasome (*22*, *25*−*27*), the lysosome (*28*−*30*), or the ubiquitin-ligase E3 parkin (*33*), to degrade α-syn. In some systems calpain, activated by parkin, cleaves α-syn and prevents α-syn-induced cell death (*33*). Moreover, calpain activation is increased in the substantia nigra of PD brains compared to controls, suggesting that pathological activation of calpain may lead to neuropathology (*52*). In addition, calpain-mediated cleavage of various proteins has been implicated in several neurodegenerative diseases including Alzheimer's disease (*53*−*55*), Huntington's disease (*56*−*58*), frontal temporal dementia and parkinsonism linked to chromosome 17 (*59*), and PD (*52*, *60*, *61*).

Regardless of the exact role of calpain *in vivo*, the present study further defines the structural components of α-syn and its breakdown products that modulate fibril formation and stability. Depending on the solubility of α-syn (nonfibrillized versus fibrillized), both calpain-mediated cleavage and nitration of α-syn will affect fibril development. This study provides evidence that the effect of protein modifications and proteolytic processing of proteins are interdependent, which may be relevant in elucidating the mechanism of protein aggregation.

SUPPORTING INFORMATION AVAILABLE

Western blot analysis of wild-type α-syn cleaved with calpain II and sedimentation analysis of full-length fibrillized α-syn co-incubated with full-length soluble α-syn. This information is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Galvin, J. E., Lee, V. M.-Y., and Trojanowski, J. Q. (2001) Synucleinopathies, *Arch. Neurol. 58*, 186−190.
2. Clayton, D. F., and George, J. M. (1999) Synucleins in synaptic plasticity and neurodegenerative disorders, *J. Neurosci. Res*. *58*, 120−129.
3. Giasson, B. I., Murray, I. V. J., Trojanowski, J. Q., and Lee, V. M.-Y. (2001) A hydrophobic stretch of 12 amino acid residues in the middle of α-synuclein is essential for filament assembly, *J. Biol. Chem. 276*, 2380−2386.
4. Conway, K. A., Harper, J. D., and Lansbury, P. T., Jr. (1998) Accelerated *in vitro* fibril formation by a mutant α-synuclein linked to early-onset Parkinson disease, *Nat. Med. 4*, 1318−1320.
5. Conway, K. A., Harper, J. D., and Lansbury, P. T., Jr. (2000) Fibrils formed *in vitro* from α-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid, *Biochemistry 39*, 2552−2563.
6. El-Agnaf, O. M., Jakes, R., Curran, M. D., Middleton, D., Ingenito, R., Bianchi, E., Pessi, A., Neill, D., and Wallace, A. (1998a) Aggregates from mutant and wild-type α-synuclein proteins and NAC peptide induce apoptotic cell death in human neuroblastoma cells by formation of *β*-sheet and amyloid-like filaments. *FEBS Lett. 440*, 71−75.
7. El-Agnaf, O. M., Jakes, R., Curran, M. D., and Wallace, A. (1998b) Effects of the mutations Ala^{30} to Pro and Ala^{53} to Thr on the physical and morphological properties of α synuclein protein implicated in Parkinson's disease, *FEBS Lett. 440*, 67−70.
8. Giasson, B. I., Uryu, K., Trojanowski, J. Q., and Lee, V. M.-Y. (1999) Mutant and wild-type human α-synucleins assemble into elongated filaments with distinct morphologies *in vitro*, *J. Biol. Chem. 274*, 7619−7622.
9. Narhi, L., Wood, S. J., Steavenson, S., Jiang, Y., Wu, G. M., Anafi, D., Kaufman, S. A., Martin, F., Sitney, K., Denis, P., Louis, J.-C., Wypych, J., Biere, A. L., and Citron, M. (1999) Both familial Parkinson's disease mutations accelerate α-synuclein aggregation, *J. Biol. Chem. 274*, 9843−9846.
10. Li., J., Uversky, V. N., and Fink, A. L. (2003) Effect of familial Parkinson's disease point mutations, *Biochemistry 40*, 11604−11613.
11. Kessler, J. C., Rochet, J.-C., and Lansbury, P. T., Jr. (2003) The N-terminal repeat domain of α-synuclein inhibits *β*-sheet and amyloid fibril formation, *Biochemistry 42*, 672−678.
12. Crowther, A., Jakes, R., Spillantini, M. G., and Goedert, M. (1998) Synthetic filaments assembled from C-terminally truncated α-synuclein, *FEBS Lett. 436*, 309−312.
13. Greenbaum, E. A., Graves, C. L., Mishizen-Eberz, A. J., Lupoli, M. A., Lynch, D. R., Englander, S. W., Axelsen, P. H., and Giasson, B. I. (2005) The E46K mutation in α-synuclein increases amyloid fibril formation, *J. Biol. Chem. 280*, 7800−7807.
14. Choi, W., Zibaee, S., Jakes, R., Serpell, L. C., Davletov, B., Crowther, R. A., and Goedert, M. (2004) Mutation E46K increases phospholipids binding and assembly into filaments of human α-synuclein, *FEBS Lett. 576*, 363−368.
15. Li, W., West, N., Colla, E., Pletnikova, O., Troncoso, J. C., Marsh, L., Dawson, T. M., Jäkälä, P., Hartmann, T., Price, D. L., and Lee, M. K. (2005) Aggregation promoting C-terminal truncation of α-synuclein is a normal cellular process and is enhanced by the familial Parkinson's disease-linked mutation, *Proc. Natl. Acad. Sci. U.S.A. 102*, 2162−2167.
16. Serpell, L. C., Berriman, J., Jakes, R., Goedert, M., and Crowther, R. A. (2000) Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-*β* conformation, *Proc. Natl. Acad. Sci. U.S.A. 97*, 4897−4902.
17. Murray, I. V. J., Giasson, B. I., Quinn, S. M., Koppaka, V., Axelsen, P. H., Ischiropoulos, H., Trojanowski, J. Q., and Lee, V. M.-Y. (2003) Role of α-synuclein carboxy-terminus on fibril formation *in vitro*, *Biochemistry 42*, 8530−8540.
18. Uversky, V. N., Cooper, E. M., Bower, K. S., Li, J., and Fink, A. L (2001) Accelerated α-synuclein fibrillization in crowded milieu, *FEBS Lett. 515*, 99−103.
19. Shtilerman, M. D., Ding, T. T., and Lansbury, Jr. P. T. (2002) Molecular crowding accelerates fibrillization of α-synuclein: Could an increase in the cytoplasmic protein concentration induce Parkinson's disease? *Biochemistry 41*, 3855−3860.
20. Hashimoto, M., Hsu, L. J., Xia, Y., Takeda, A., Sundsmo, M., and Masliah, E. (1999a) Oxidative stress induces amyloid-like aggregate formation of NACP/α-synuclein *in vitro*, *NeuroReport 10*, 717−721.
21. Hashimoto, M., and Masliah, E. (1999b) α-Synuclein in Lewy body disease and Alzheimer's disease, *Brain Pathol. 9*, 707−720.
22. Bennett, M. C., Bishop, J. F., Leng, Y., Chock, P. B., Chase, T. N., and Mouradian, M. M. (1999) Degradation of α-synuclein by proteasome, *J. Biol. Chem. 274*, 33855−33858.
23. Ancolio, K., Alves da Costa, C., Uéda, K., and Checler, F. (2000) α-Synuclein and the Parkinson's disease-related mutant Ala53Thr-α-synuclein do not undergo proteasomal degradation in HEK293 and neuronal cells, *Neurosci. Lett. 285*, 79−82.
24. Gai, W. P., Yuan, H. X., Li, X. Q., Power, J. T. H., Blumbergs, P. C., and Jensen, P. H. (2000) *In situ* and *in vitro* study of colocalization and segregation of α-synuclein, ubiquitin, and lipids in Lewy bodies, *Exp. Neurol. 166*, 324−333.
25. Chung, K. K. K., Dawson, V. L., and Dawson, T. M. (2001) The role of the ubiquitin-proteasomal pathway in Parkinson's disease and other neurodegenerative disorders, *Trends Neurosci. 24*, S7−S14.
26. McLean, P. J., Kawamata, H., and Hyman, B. T. (2001) α-Synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons, *Neuroscience 104*, 901−912.
27. Shimura, H., Schlossmacher, M. G., Hattori, N., Frosch, M. P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K. S., and Selkoe, D. J. (2001) Ubiquitination of a new form of α-synuclein by parkin from human brain: Implications for Parkinson's disease, *Science 293*, 263−269.
28. Lee, H.-J., Khoshaghideh, F., Patel, S., and Lee, S.-J. (2004) Clearance of α-synuclein oligomeric intermediates via the lysosomal degradation pathway, *J. Neurosci. 24*, 1888−1896.

29. Paxinou, E., Chen, Q., Weisse, M., Giasson, B. I., Norris, E. H., Rueter, S. M., Trojanowski, J. Q., Lee, V. M.-Y., and Ischiropoulos, H. (2001) Induction of α-synuclein aggregation by intracellular nitrative insult, *J. Neurosci. 21*, 8053−8061.
30. Bahr, B. A., and Beniske, J. (2002) The neuropathogenic contributions of lysosomal dysfunction, *J. Neurochem. 83*, 481−489.
31. Mishizen-Eberz, A. J., Guttmann, R. P., Giasson, B. I., Day, G. A., Hodara, R., Ischiropoulos, H., Lee, V. M.-Y., Trojanowski, J. Q., and Lynch, D. R. L (2003) Distinct cleavage patterns of normal and pathological forms of α-synuclein by calpain I *in vitro*, *J. Neurochem. 86*, 836−847.
32. Paik, S. R., Leem, J.-H., Kim, D.-H., Chang, C.-S., and Kim, J. (1997) Aluminum-induced structural alterations of the precursor of the non-Aβ component of Alzheimer's disease amyloid, *Arch. Biochem. Biophys. 344*, 325−334.
33. Kim, S. J., Sung, J. Y., Um, J. W., Hattori, N., Mizuno, Y., Tanaka, K., Paik, S. R., Ki, J., and Chung, K. C. (2003) Parkin cleaves intracellular α-synuclein inclusions via the activation of calpain, *J. Biol. Chem. 278*, 41890−41899.
34. Kanda, S., Bishop, J. F., Eglitis, M. A., Yang, Y., and Mouradian, M. M. (2000) Enhanced vulnerability to oxidative stress by α-synuclein mutations and C-terminal truncation, *Neuroscience 97*, 279−284.
35. Giasson, B. I., Duda, J. E., Murray, I., Chen, Q., Souza, J. M., Hurting, H. I., Ischiropoulos, H., Trojanowski, J. Q., and Lee, V. M.-Y. (2000) Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synucleinopathy lesions, *Science 290*, 985−989.
36. Souza, J. M., Daikhin, E., Yudoff, M., Raman, C. S., and Ischiropoulos, H. (1999) Factors determining the selectivity of protein tyrosine nitration, *Arch. Biochem. Biophys. 371*, 169−178.
37. Souza, J. M., Giasson, B. I., Chen, Q., Lee, V. M.-Y., and Ischiropoulos, H. (2000) Dityrosine cross-linking promotes formation of stable α-synuclein polymers, *J. Biol. Chem. 275*, 18344−18349.
38. Norris, E. H., Giasson, B. I., Ischiropoulos, H., and Lee, V. M.-Y. (2003) Effects of oxidative and nitrative challenges on α-synuclein fibrillogenesis involve distinct mechanisms of protein modifications, *J. Biol. Chem. 278*, 27230−27240.
39. Yamin, G., Uversky, V. N., and Fink, A. L. (2003) Nitration inhibits fibrillization of human α-synuclein *in vitro* by formation of soluble oligomers, *FEBS Lett. 542*, 147−152.
40. Hodara, R., Norris, E. H., Giasson, B. I., Mishizen-Eberz, A. J., Lynch, D. R., Lee, V. M.-Y., and Ischiropoulos. H. (2004) Functional consequences of α-synuclein tyrosine nitration: Diminished binding to lipid vesicles and increased fibril formation, *J. Biol. Chem. 279*, 47746−47753.
41. Giasson, B. I., Jakes, R., Goedert, M., Duda, J. E., Leight, S., Trojanowski, J. Q., and Lee, V. M.-Y. (2000) A panel of epitope-specific antibodies detects protein domains distributed throughout human α-synuclein in Lewy bodies of Parkinson's disease, *J. Neurosci. Res. 59*, 528−533.
42. Duda, J. E., Giasson, B. I., Mabon, M. E., Lee, V. M.-Y., and Trojanowski, J. Q. (2002) Novel antibodies to synuclein show abundant striatal pathology in Lewy body diseases, *Ann. Neurol. 52*, 205−210.
43. Jakes, R., Crowther, A., Lee, V. M.-Y., Trojanowski, J. Q., Iwatsubo, T., and Goedert, M. (1999) Epitope mapping of LB509, a monoclonal antibody directed against human α-synuclein, *Neurosci. Lett. 269*, 13−16.
44. Souza, J. M., Choi, I., Chen, Q., Weisse, M., Daikhin, E., Yudkoff, M., Obin, M., Ara, J., Horwitz, J., and Ischiropoulos, H. (2000) Proteolytic degradation of tyrosine nitrated proteins, *Arch. Biochem. Biophys. 380*, 360−366.
45. Giasson, B. I., Forman, M. S., Higuchi, M., Golbe, L. I., Graves, C. L., Kotzbauer, P. T., Trojanowski, J. Q., and Lee, V. M.-Y. (2003) Initiation and synergistic fibrillization of tau and α-synuclein, *Science 300*, 636−640.
46. Miake, H., Mizusawa, H., Iwatsubo, T., and Hasegawa, M. (2002) Biochemical characterization of the core structure of α-synuclein filaments, *J. Biol. Chem. 277*, 19213−19219.
47. Takahashi, T., Yamashita, H., Nakamura, T., Nagano, Y., and Nakamura, S. (2002) Tyrosine 125 of α-synuclein plays a critical role for dimerization following nitrative stress, *Brain Res. 938*, 73−80.
48. Giasson, B. I., Duda, J. E., Quinn, S. M., Zhang, B., Trojanowski, J. Q., and Lee, V. M.-Y. (2002) Neuronal α-synucleinopathy with severe movement disorder in mice expressing A53T human α-synuclein, *Neuron 34*, 521−533.
49. Lee, M. K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A. S., Dawson, T. M., Copeland, N. G., Jenkins, N. A., and Price, D. L. (2002) Human α-synuclein-harboring familial Parkinson's disease-linking Ala53Thr mutation causes neurodegenerative disease with α-synuclein aggregation in transgenic mice, *Proc. Natl. Acad. Sci. U.S.A. 99*, 8968−8973.
50. Baba, M., Nakajo, S., Tu, P.-H., Tomita, T., Nakaya, K., Lee, V. M.-Y., Trojanowski, J. Q., and Iwatsubo, T. (1998) Aggregation of α-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies, *Am. J. Pathol. 152*, 879−884.
51. Perrin, B. J., and Huttenlocher, A. (2002) Calpain, *Int. J. Biochem. Cell Biol. 34*, 722−725.
52. Crocker, S. J., Smith, P. D., Jackson-Lewis, V., Lamba, W. R., Hayley, S. P., Grimm, E., Callaghan, S. M., Slack, R. S., Melloni. E., Przedborski, S., Robertson, G. S., Anisman, H., Merali, Z., and Park, D. S. (2003) Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease, *J. Neurosci. 23*, 4081−4091.
53. Nixon, R. A. (2003) The calpains in aging and aging-related diseases, *Ageing Res. Rev. 2*, 407−418.
54. Lee, M.-S., Kwon, Y. T., Li, M., Peng, J., Friedlander, R. M., and Tsai, L.-H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain, *Nature 405*, 360−364.
55. Patzke, H., and Tsai, L.-H. (2002) Calpain-mediated cleavage of the cyclin-dependent kinase-5 activator p39 to p29, *J. Biol. Chem. 277*, 8054−8060.
56. Gafni, J., and Ellerby, L. M. (2002) Calpain activation in Huntington's disease, *J. Neurosci. 22*, 4842−4849.
57. Gafni, J., Hermel, E., Young, J. E., Wellington, C. L., Hayden, M. R., and Ellerby, L. M. (2004) Inhibition of calpain cleavage of huntingtin reduces toxicity: Accumulation of calpain/caspase fragments in the nucleus, *J. Biol. Chem. 279*, 20211−20220.
58. Bizat, N., Hermel, J.-M., Boyer, F., Jacquard, C., Créminon, C., Ouary, S., Escartin, C., Hantraye, P., Krajewski, S., and Brouillet, E. (2003) Calpain is a major death effector in selective striatal degeneration induced *in vivo* by 3-nitropropionate: Implications for Huntington's disease, *J. Neurosci. 23*, 5020−5030.
59. Yen, S., Easson, C., Nacharaju, P., Hutton, M., and Yen, S.-H. (1999) FTDP-17 tau mutations decrease the susceptibility of tau to calpain I digestion, *FEBS Lett. 461*, 91−95.
60. Mouatt-Prigent, A., Karlsson, J. O., Agid, Y., and Hirsch, E. C. (1996) Increased calpain expression in the mesencephalon of patients with Parkinson's disease but not in other neurodegenerative disorders involving the mesencephalon: A role in nerve cell death? *Neuroscience 73*, 979−987.
61. Chera, B., Scjaecjer, K. E., Rocchini, A., Imam, S. Z., Sribnick, E. A., Ray, S. K., Ali, S. F., and Banik, N. L. (2004) Immunofluorescent labeling of increased calpain expression and neuronal death in the spinal cord of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, *Brain Res. 1006*, 150−156.

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