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

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Abstract **α-Synuclein (α-syn) protein and a fragment of it, called NAC, have been found in association with the pathological lesions of a number of neurodegenerative diseases. Recently, mutations in the α-syn gene have been reported in families susceptible to an inherited form of Parkinson's disease. We have shown that human wild-type α-syn, mutant α-syn(Ala30Pro) and mutant α-syn(Ala53Thr) proteins can self-aggregate and form amyloid-like filaments. Here we report that aggregates of NAC and α-syn proteins induced apoptotic cell death in human neuroblastoma SH-SY5Y cells. These findings indicate that accumulation of α-syn and its degradation products may play a major role in the development of the pathogenesis of these neurodegenerative diseases.**

Key words: α-Synuclein; Parkinson's disease; Lewy body; Toxicity; Amyloid; Neurodegenerative disease

1. Introduction

α-Synuclein (α-syn) is a presynaptic protein which was originally identified as the precursor protein for the non-β-amyloid component (NAC) of Alzheimer's disease (AD) senile plaques [1]. NAC is a 35 amino acid peptide comprising amino acids 61–95 of the α-syn sequence and has been identified as the second major constituent in the plaques of AD brains [1]. It has been shown recently that during the progression of AD there is an increase in the levels of α-syn in the frontal cortex during the early stages of the disease [2]. α-Syn also accumulates in dystrophic neurites of the plaques as well as in the presynaptic terminals in AD brains [3,4], and is also found in AD hippocampal neuritic tangles [5].

Recently α-syn has been identified immunohistochemically and by immunoelectron microscopy studies as the major component in Lewy bodies (LBs) and Lewy neurites (LNs), the main pathological features of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [4,6–8]. Mutations in the α-syn gene have been reported to be associated with families susceptible to inherited forms of PD [9,10]. These mutations cause alterations in the amino acid sequence of α-syn (at residues Ala30Pro or Ala53Thr) in regions predicted to influence the secondary structure of α-syn. The substitutions may disrupt the structure of α-syn, rendering the protein more prone to self-aggregation and hence deposition in LBs and LNs [9–12].

Immunohistochemical studies have also shown that α-syn is also associated with pathological lesions in other neurodegenerative diseases, such as the glial cytoplasmic inclusions (GCIs) found in multiple system atrophy (MSA) [5,13,14]. GCIs are also found in other illnesses, including corticobasal degeneration and Steele-Richardson-Olszewski syndrome. α-Syn was also found in the astrocytes and Schwann cells in the spinal cords of patients with the motor neurone disorder amyotrophic lateral sclerosis (ALS) [5]. Taken together, all these findings suggest that accumulation of α-syn, and fragments of α-syn such as NAC, in the brain may be involved in the pathogenesis of these neurodegenerative diseases.

The roles of α-syn and NAC in the pathogenesis of these neurodegenerative diseases are unknown.

Here we report that aggregates of wild-type and mutant α-syn proteins, and the α-syn fragment NAC, induced apoptotic cell death in SH-SY5Y cells. These findings may provide some insight into the molecular pathogenesis of these neurodegenerative diseases.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesised on Rink polystyrene resins using Fmoc N-protection as previously described [15,16].

2.2. Preparation of α-syn proteins

Recombinant α-syn proteins were expressed in *Escherichia coli* and purified as previously described [17].

2.3. Preparation of aggregated 'aged' solutions of α-syn proteins and NAC peptides

α-Syn proteins were dissolved in PBS (phosphate-buffered saline pH 7.4) at a concentration of 110 μM, and the resulting solutions were aged for 7 days at 37°C. NAC and Aβ peptides were dissolved first in distilled water, then an equal volume of 2×PBS was added to give a final concentration of 550 μM and the resulting solutions were aged for 7 days at 37°C.

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Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid protein; ALS, amyotrophic lateral sclerosis; DLB, dementia with Lewy bodies; GCI, glial cytoplasmic inclusion; HD, Huntington's disease; LB, Lewy body; LN, Lewy neurite; MSA, multiple system atrophy; NAC, non-Aβ component of AD amyloid; PD, Parkinson's disease

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2.4. Thioflavine-S staining of the aggregated NAC peptides

The aggregated NAC peptides were formed as described above, 10 μl of the aggregate samples were mixed with 10 μl of thioflavine-S (Sigma), 20 mg/ml in water, which had been prepared freshly and filtered through a membrane filter (0.22 μm). The specimen was observed, and images were acquired with a Leica TCS-NT confocal laser scanning microscope (Leica, Milton Keynes, UK).

2.5. Electron microscopy (EM)

Samples of 10 μl were placed on carbon-coated copper grids, and incubated for 2 min. The droplet was then displaced with 10 μl of 0.5% (v/v) glutaraldehyde and incubated for an additional 2 min. The grid was then washed with five drops of water and wicked dry. Finally, the sample was stained with 10 μl of 2% (w/v) uranyl acetate solution for 2 min. This solution was wicked off, the grid was air-dried, then viewed on a JEOL TEMCX 100 II electron microscope and photographed.

2.6. Circular dichroism (CD)

Spectra were recorded at 21°C over a wavelength range of 190–250 nm on a JASCO J720 spectropolarimeter. An aliquot of the peptide solution (about 130 μl) was placed in a quartz cell (0.2 mm pathlength). All spectra were corrected by subtracting the baseline of the solvent. Results are expressed as molar ellipticity, [θ], in units of deg cm^2 $dmol^{-1}$.

2.7. Cell culture

The following culture conditions are critical for the success of the cytotoxic assay. SH-SY5Y cells (European Collection of Cell Cultures, Porton Down, UK) were routinely cultured in Dulbecco's MEM/Nutrient Mix F-12 (1:1) (Gibco BRL) containing 1% penicillin-streptomycin, 15% foetal calf serum, 1% minimal essential medium amino acid supplement, and 2 mM freshly prepared glutamine (Gibco BRL) and maintained at 37°C in a humidified incubator with 5% CO_2/95% room air. For cytotoxicity studies, before plating, medium was removed and fresh medium was gently added and the cell layer dissociated by trituration. Cells were used for a maximum of 20 passages.

2.8. Cytotoxicity assay

The cytotoxic effect of α-syn proteins or NAC peptides was assessed by measuring cellular redox activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) obtained from Sigma. Cells were plated at a density of 7500 cells per well on 96-well plates in 100 μl of fresh medium. After 24 h the medium was exchanged with 100 μl of OPTI-MEM (Gibco BRL) serum-free medium and 10 μl of α-syn proteins or NAC peptides were added at the concentration indicated, and cells were incubated for an additional 2 days. 10 μl of stock MTT in PBS was added to a final concentration of 0.5 mg/ml, and the incubation was continued for another 4.5 h. Cell lysis buffer (100 μl per well; 15% SDS/50% *N*,*N*-dimethylformamide, pH 4.7) was added and incubated overnight at 37°C in a humidified incubator. Absorbance values at 590 nm were determined with an automatic plate reader.

2.9. Apoptosis

The cell nuclei were visualised using Hoechst 33342 (Sigma). The cells were grown on a 96-well plates as described above, after the treatment with 10 μM of α-syn proteins or NAC peptides for 2 days. A solution of Hoechst 33342 in water (100 μg/ml) was added to give a final concentration in each well of 2.5 μg/ml. The samples were then incubated for 10 min at room temperature and examined under a microscope equipped for epifluorescence.

3. Results

3.1. Thioflavine-S staining studies

Fig. 1A,B demonstrates that the thioflavine-S positive aggregates were formed from aged NAC(1–35) and NAC(1–18) solutions, indicative of β-pleated sheet conformation characteristic of amyloid-like filaments. No aggregates were detected when incubation was carried out with NAC(19–35) and NAC(18–1) peptides under the same conditions.

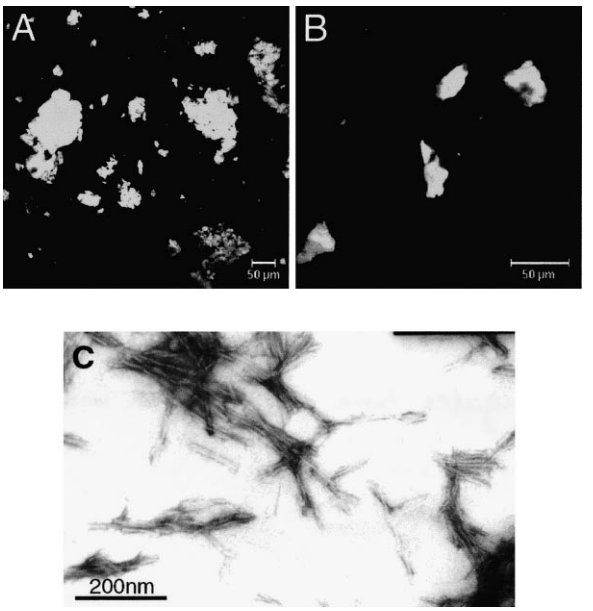

Fig. 1. Analysis for aggregation of NAC peptides. A: Thioflavine-S staining of the aggregated NAC(1–35). B: Thioflavine-S staining of the aggregated NAC(1–18). C: Representative negatively staine-d electron micrograph (EM) of filaments obtained from aged NAC(1-35) solution. The EM showed different morphology of filaments including short 4–8 nm wide filaments. EM scale bar, 200 nm.

3.2. Electron microscopy studies

We used EM to examine uranyl acetate-stained filament preparations, made from fresh and aged samples. EM examination revealed filaments of aged NAC peptides, apart from NAC(19–35) and NAC(18–1) peptides. NAC(1–35) and NAC(1–18) formed clumps of short irregular filaments of variable length, mainly of diameter 4–8 nm. A representative example is shown for NAC(1–35) (Fig. 1C). Individual clumps of amorphous material were also sometimes present.

3.3. Circular dichroism studies

CD spectra were recorded from fresh and aged solutions in PBS. The effects of ageing on the CD spectra of NAC peptides are shown in Fig. 2. Freshly dissolved NAC peptides are characteristic of random coil, whereas after 7 days the spectra for NAC(1–35) and NAC(1–18) indicated a conversion to β-sheet (Fig. 2A). In contrast, there was no change in the NAC(19–35) and NAC(18–1) spectra upon ageing for 7 days (Fig. 2B).

3.4. Cytotoxicity studies

Human dopaminergic neuroblastoma SH-SY5Y cells were exposed for 2 days to freshly prepared or aged solutions of α-syn proteins or NAC peptides. Unrelated amyloidogenic peptide Aβ(25–35), which is known to be toxic to cells [18–22], and reverse sequence peptides Aβ(35–25) and NAC(18–1) were used as positive and negative controls respectively for the cytotoxicity assay. After 2 days, cell viability was evaluated using the MTT assay and compared with that of cells treated with vehicle only. In cells which were treated with freshly prepared α-syn proteins at 10 μM concentration there was only about 20% loss of cell viability compared to control conditions (Fig. 3A). By contrast, there was a greater loss of about 55% of cell viability upon exposure to aged α-syn pro-

teins at 10 μM concentration (Fig. 3A). Cell toxicity for aged α-syn proteins was first detected at a concentration of 1 μM and was statistically significant at 5 μM (Fig. 3A). Under the same conditions, fresh and aged NAC(1–35) and NAC(1–18) peptides were also toxic to cells (Fig. 3B). Toxicity of aged NAC(1–35) was first detected at a concentration of 0.001 μM, whereas that of fresh NAC(1–35) first arose at 0.1 μM. In contrast, NAC(1–18) was toxic at the same concentration whether prepared fresh or aged, as was the amyloidogenic control peptide Aβ(25–35) (Fig. 3B).

3.5. Apoptosis studies

We also investigated the mechanism involved in the toxicity induced by α-syn proteins and NAC peptides. Morphological examination of cell nuclei, stained with the DNA-binding fluorochrome Hoechst 33342, showed that cells exposed to aged solutions of wild-type or mutant α-syn proteins or NAC(1–35) and NAC(1–18) peptides presented a typical apoptotic morphology, including condensation of chromatin and nuclear fragmentation. Representative examples are shown in Fig. 4A,B.

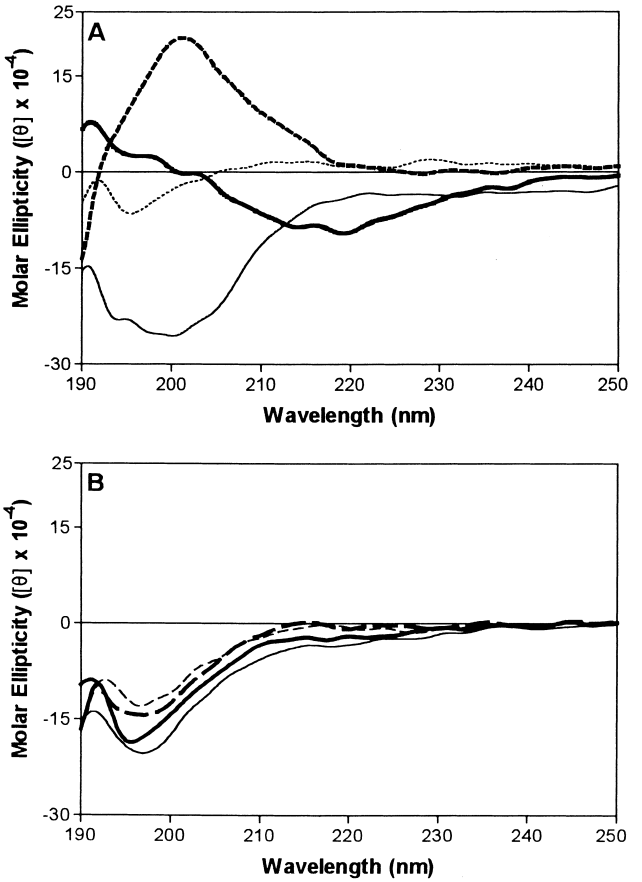

Fig. 2. CD spectra. A: Effect of ageing on the CD spectra of solutions of NAC(1–35) and NAC(1–18) peptides. CD spectra of solutions of NAC(1–35) (100 μM) and NAC(1–18) (100 μM) in PBS were obtained immediately upon preparation and after incubation at 37°C for 7 days: NAC(1–35) at time 0 (light solid line); NAC(1–35) after 7 days (heavy solid line); NAC(1–18) at time 0 (light dotted line); NAC(1–18) after 7 days (heavy dotted line). B: Effect of ageing on the CD spectra of solutions of NAC(19–35) and NAC(18–1) peptides. CD spectra of solutions of NAC(19–35) (100 μM) and NAC(18–1) (100 μM) in PBS were obtained immediately upon preparation and after incubation at 37°C for 7 days: NAC(19–35) at time 0 (light dashed line); NAC(19–35) after 7 days (heavy dashed line); NAC(18–1) at time 0 (light solid line); NAC(18–1) after 7 days (heavy solid line).

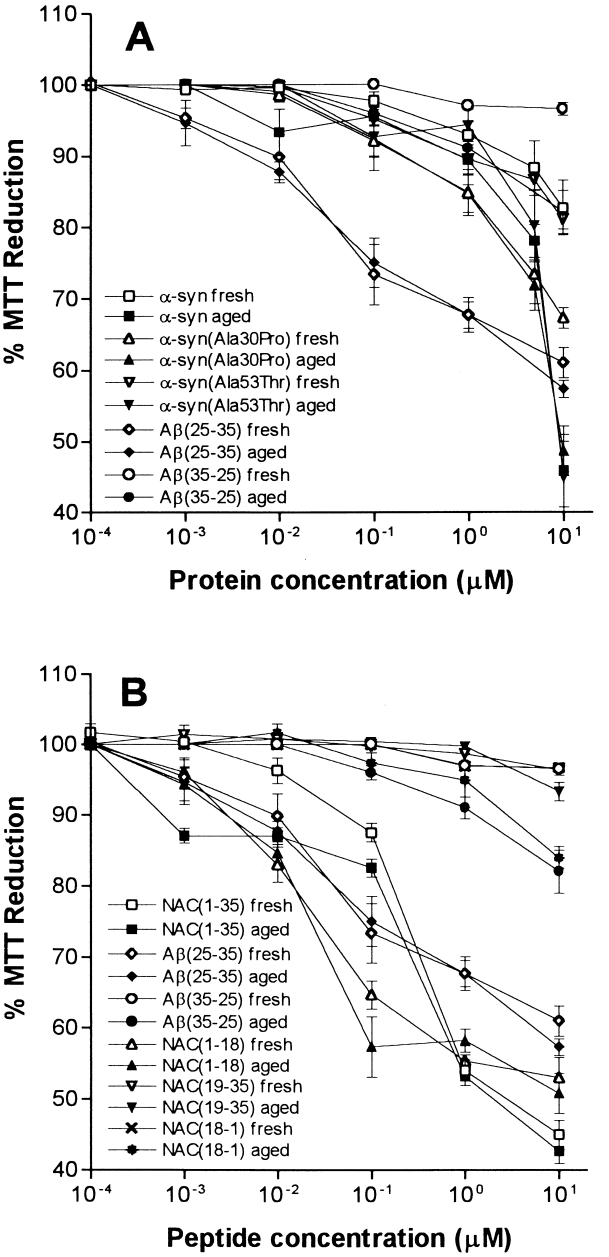

Fig. 3. Comparison of freshly prepared and aged solutions at different concentrations of (A) α-syn proteins and (B) NAC peptides, for their ability to inhibit MTT reduction by SH-SY5Y cells. Aβ(25–35) and the reverse sequence peptide Aβ(35–25) were included in the assay for both α-syn proteins and NAC peptides as positive and negative controls respectively. Data shown are expressed as percentage of control values (vehicle alone) from at least three independent experiments, where each experiment was performed in triplicate. Standard errors of the mean are shown for each point as bars above and below the mean; in some cases the spread is smaller than the symbol used and bars are therefore not visible. It should be noted that the symbols overlap in some cases due to the close proximity of the % MTT reduction values.

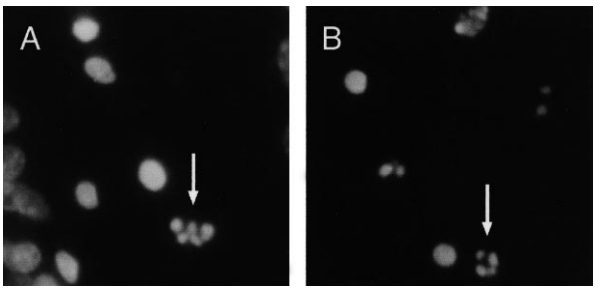

Fig. 4. Representative fluorescence photomicrographs showing apoptosis of SH-SY5Y cells treated with aged solutions of 10 μM α-syn proteins or NAC peptides. Nuclei were stained with Hoechst 33342 which is a fluorescent marker of DNA; the arrow indicates a nucleus with typical apoptotic features. A: Cells were treated with aged 10 μM α-syn(Ala^{53}Thr). B: Cells were treated with 10 μM NAC(1–18) peptide.

4. Discussion

Conformation-dependent neurotoxicity of amyloid proteins is a common mechanism and is an emerging theme in neurodegenerative disorders such as AD [19], prion diseases [23] and Huntington's disease (HD) [24]. The enhanced in vitro toxicity of amyloid peptides observed after in vitro ageing is correlated with a prominent increase in both β-sheet structure of the aged proteins and filament formation ([18,19,23,25], reviewed in [26,27]). On this basis, we hypothesised that neuronal death in PD, DLB, AD, MSA and ALS may be due to abnormal accumulation of α-syn and/or degradation products, such as NAC, as amyloid-like filaments in the brain. Previously, we have shown that wild-type and mutant α-syn proteins can self-aggregate and form amyloid-like filaments (El-Agnaf et al., manuscript submitted). It has also been reported that NAC(1–35) can aggregate and form amyloid-like filaments upon ageing in solution [28,29]. We have been able to identify that the N-terminus (1–18) of NAC(1–35) is the amyloidogenic region, which drives β-sheet formation, and hence aggregation and deposition of NAC(1–35) [30]. Therefore, we investigated some of the biophysical properties of NAC(1–35), NAC(1–18), NAC(19–35) and reverse NAC(18–1) peptides, to correlate their biophysical properties with their toxic effects on the viability of dopaminergic human neuroblastoma SH-SY5Y cells. Furthermore, we investigated the toxic properties of recombinant human wild-type α-syn and the PD-linked mutants α-syn(Ala30Pro) and α-syn(Ala53Thr).

The formation of NAC peptides in a crossed β-pleated sheet conformation was assessed by thioflavine-S staining (Fig. 1A,B). Only the aged samples of NAC(1–35) and NAC(1–18) were thioflavine-S positive, indicative of the presence of amyloid-like filaments [31]. Indeed, EM studies on the aged samples of NAC(1–35) and NAC(1–18) showed the presence of filaments which were similar in size to those already reported [28–30]. These results confirm our previous report that NAC(1–18) is the amyloidogenic region of NAC(1–35) [30].

CD spectroscopy was used to study the conformational preferences of the NAC peptides. CD spectra have been used to distinguish between proteins or peptides displaying predominantly α-helical, β-sheet or random coil conformations. The first are characterised by a maximum at 192 nm and minima at 208 and 222 nm and the second by a maximum at 195 nm and single minimum at 218 nm. In both cases, the maximum has greater intensity than the minimum. Random conformations are characterised by a minimum at 197 nm. Only NAC(1–35) and NAC(1–18) peptides revealed a conformation transition from random coil structure to predominantly β-sheet conformation upon ageing for 7 days in PBS (Fig. 2A). In contrast, NAC(19–35) and NAC(18–1) peptides did not develop β-sheet upon ageing under the same conditions (Fig. 2B). Our results would suggest that this structural transition must occur as a prelude to aggregation, as has been reported for other amyloid peptides (reviewed in [26,27]). Such a transition in secondary structure may well be a general prelude to the formation of toxic filaments by amyloidogenic peptides, as has been suggested previously (reviewed in [26,27]).

While the α-syn fragment NAC (1–35) is known to be present extracellularly in the plaques of AD brains [1], there is as yet no evidence to support the presence of the full length α-syn protein in extracellular form. If α-syn accumulates in neurones, which eventually die, one wonders whether the aggregates could leak out of the dead neurone and spread the disease to the neighbouring cells [5]. On these bases we have investigated the in vitro toxicity of wild-type and mutant α-syn proteins and NAC peptides.

An early indicator of toxicity is the inhibition of cellular MTT reduction to MTT formazan, a widely used assay for measuring cell viability [20,22]. We used this assay to investigate the toxicity of α-syn and NAC peptides towards human neuroblastoma SH-SY5Y cells as shown in Fig. 3. Aged solutions of α-syn and NAC(1–35) are more toxic than fresh. In contrast, NAC(1–18) and Aβ(25–35) were toxic to cells whether prepared fresh or aged. We interpret the toxicity either from fresh or aged solutions to be due to their ability to form β-sheet and aggregate immediately upon solubilisation [20,21,30]. By contrast, neither fresh nor aged solutions of the reverse peptides Aβ(35–25) and NAC(18–1), nor NAC(19–35), affected cell survival compared to controls (Fig. 3B).

These results indicate that the toxicity of α-syn and NAC is sequence specific and increased by ageing in solution for 7 days, meaning that it is dependent on aggregation and formation of filaments. A similar mechanism was reported for other amyloid peptides [18,19,23,25]. It is also apparent that the amyloidogenic portion of α-syn fragment NAC(1–18) mediated the toxic effect of α-syn protein and NAC(1–35) peptide. The toxicity of α-syn proteins and NAC peptides is due to the induction of apoptotic cell death as revealed by staining of cell nuclei with the DNA-binding fluorochrome Hoechst 33342 (Fig. 4A,B). Similar findings have been reported for other amyloid proteins [18,23,32].

In general, amyloid toxicity is closely allied to the processes of aggregation and fibril formation. Recently, it has been shown that different amyloid proteins share the same mechanism of toxicity to cells in vitro [18,19,23,25]. This mechanism may involve reactive oxygen species and elevation of intracellular calcium ion levels [33]. Thus it has been proposed that the ability of amyloid proteins to undergo a transition to β-sheet structure is a prerequisite to filament formation and subsequent pathological consequences (reviewed in [26,27]). It is not known whether amyloid filaments are directly toxic or produce damage by mechanical disruption of tissue. Alternatively, the toxic state may consist of a soluble conformation-

ally altered filament precursor that induces cell death directly or indirectly.

In summary, this study provides evidence that a neurotoxic mechanism is potentially responsible for neuronal cell loss in the neurodegenerative diseases PD, DLB, AD, MSA and ALS. This mechanism is based on the accumulation of α-syn and possibly also its degradation products, such as NAC, in the neurones. Similar mechanisms could be relevant for neuronal death in other neurodegenerative disorders. Accordingly, efforts to elucidate the pathological roles of α-syn may lead to improved strategies for the development of novel therapeutic agents for the treatment of these neurodegenerative disorders.

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