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The Formation of Highly Soluble Oligomers of α-Synuclein Is Regulated by Fatty Acids and Enhanced in Parkinson's Disease

Ronit Sharon, Ifat Bar-Joseph, Matthew P. Frosch, Dominic M. Walsh, James A. Hamilton, and Dennis J. Selkoe*
Center for Neurologic Diseases
Harvard Medical School
Brigham and Women's Hospital
Boston, Massachusetts 02215

Summary

Accumulation of misfolded proteins as insoluble aggregates occurs in several neurodegenerative diseases. In Parkinson's disease (PD) and dementia with Lewy bodies (DLB), α-synuclein (αS) accumulates in insoluble inclusions. To identify soluble αS oligomers that precede insoluble aggregates, we probed the cytosols of mesencephalic neuronal (MES) cells, normal and αS-transgenic mouse brains, and normal, PD, and DLB human brains. All contained highly soluble oligomers of αS whose detection was enhanced by delipidation. Exposure of living MES neurons to polyunsaturated fatty acids (PUFAs) increased αS oligomer levels, whereas saturated FAs decreased them. PUFAs directly promoted oligomerization of recombinant αS. Transgenic mice accumulated soluble oligomers with age. PD and DLB brains had elevated amounts of the soluble, lipid-dependent oligomers. We conclude that αS interacts with PUFAs in vivo to promote the formation of highly soluble oligomers that precede the insoluble αS aggregates associated with neurodegeneration.

Introduction

A central unresolved question in the study of human neurodegeneration is how normally soluble neuronal proteins such as tau and α-synculein (αS) become converted into highly insoluble, potentially cytotoxic neuronal inclusions such as the neurofibrillary tangles of Alzheimer's disease and the Lewy bodies of Parkinson's disease (PD). A related issue concerns whether such pathological conversions arise in some way from the normal functions of these proteins or rather represent entirely distinct toxic properties. Here, we have attempted to address these questions in the case of αS, which has been implicated at the levels of both genetics and biochemical pathology in the pathogenesis of familial and sporadic PD.

αS was first associated with PD when a missense mutation (A53T) in the gene encoding this 140 residue cytosolic protein was linked to a rare, dominant form of PD (Polymeropoulos et al., 1997). Shortly thereafter, αS was identified biochemically and immunohistochemically as the major constituent of the pathognomonic Lewy bodies and the associated neuritic abnormalities referred to as Lewy neurites (Baba et al., 1998; Spillantini et al., 1997, 1998). A second missense mutation (A30P) was then found in another PD family (Kruger et al., 1998). Despite the rarity of these αS mutations, their recognition has opened up the field of PD research to molecular attack. Overexpression of wt human αS in some transgenic mouse lines results in the development of cytoplasmic αS inclusions, loss of dopaminergic synapses, and associated motoric impairment (Masliah et al., 2000). Overexpression of mutant human A53T αS in other transgenic lines produces severe motor deficits leading to paralysis and death, in addition to the appearance of insoluble αS and Lewy-like inclusions (Giasson et al., 2002; Lee et al., 2002). Expression of wt or mutant human αS in *Drosophila melanogaster* leads to Lewy-like inclusions in dopaminergic neurons, associated with neuronal loss and impaired climbing (Feany and Bender, 2000). Coexpression in *Drosophila* of αS and the protein chaperone HSP70 reduces the vulnerability of the dopaminergic neurons (Auluck et al., 2002).

Despite this compelling evidence for a key role of αS in the onset of PD pathology in animal models, there is very little information about its physiological function in the brain and how a portion of it is gradually converted to highly insoluble inclusions in dopaminergic neurons destined to degenerate. αS is abundant in the brain, estimated to constitute ~1% of total brain proteins, and is localized in part to presynaptic terminals. Developmental studies in songbirds suggest that its expression in certain neurons is associated with the acquisition of learning and may thus play a role in synaptic plasticity (George et al., 1995). In αS knockout mice, a role as a negative regulator of dopamine neurotransmission was suggested for αS (Abeliovich et al., 2000). Other studies have suggested that αS may function as a chaperone protein, based on its abundance in cytosol, its natively unfolded structure, and its prevention of protein aggregation induced by heat shock or chemical treatment (Souza et al., 2000).

Although αS is principally a soluble, cytosolic protein, a portion of it is reversibly associated with membranes (Davidson et al., 1998; McLean et al., 2000; Perrin et al., 2000; Sharon et al., 2001). The interaction with membranes appears to be mediated by the 6–7 imperfect repeats in its N-terminal region that are homologous to the apolipoprotein α-helical region (Perrin et al., 2000). In vitro studies suggest that αS binds acidic phospholipid vesicles in a way that markedly alters its secondary structure (Davidson et al., 1998). Moreover, binding and oligomerization of αS were enhanced when acidic phospholipid vesicles were enriched with long-chain PUFAs (Perrin et al., 2001). In cells treated with high fatty acid (FA) concentrations, wild-type (wt) and A53T, but not A30P, αS accumulated on phospholipid monolayers surrounding triglyceride-rich lipid droplets (Cole et al., 2002). It was suggested that wt αS, but not A53T or A30P, may protect stored triglycerides from hydrolysis. Recently, we obtained evidence that αS shares some properties with the family of fatty acid binding proteins (FABP). We identified regional homologies within αS to a FABP signature motif and showed the ability of purified,

*Correspondence: dselkoe@rics.bwh.harvard.edu

recombinant αS to bind radiolabeled oleic acid in a specific and stochiometric manner, although with a lower affinity than classic members of the FABP family (Sharon et al., 2001). Moreover, we detected a pool of cytosolic αS that occurs in high-molecular-weight, lipid-associated complexes that are detectable only upon lipid removal. Interestingly, LBs have been shown to contain lipids (den Hartog Jager, 1969; Gai et al., 2000), in addition to αS and other proteins. It is therefore important to examine the potential role of lipids in the genesis and modulation of PD-type neuropathology.

We now describe the existence of highly soluble αS oligomers in human αS-expressing mesencephalic neuronal cells, in the brains of normal and αS transgenic mice, and in the brains of humans with PD and the related disorder, Lewy body dementia (DLB). We show that the oligomerization process is directly regulated by cellular fatty acid levels, with specific enhancement by polyunsaturated FAs and inhibition by saturated FAs. The oligomers are of disease relevance in that they are augmented in the neuronal cytoplasm in both PD and DLB patients, compared to age-matched control subjects. Moreover, they appear earlier and accumulate to a greater extent in A53T than wt αS-expressing mesencephalic neuronal cells, and they accumulate with age in transgenic mouse brains. We conclude that soluble, metastable oligomers of αS that are induced by certain FAs and exist in the high-speed cytosol represent a very early aggregated form of the protein that occurs normally in small amounts but can gradually transition in vivo to much larger, insoluble polymers.

Results

Stable Oligomers of αS Occur in a Lipid-Associated Form in the High-Speed Cytosol of Brain Tissue

αS is a highly soluble, ~17 kDa cytoplasmic protein that nevertheless forms insoluble, high-molecular-weight (MW) aggregates in the neurons of older humans. We reasoned that this conversion would begin with the formation of small, diffusible oligomers of αS that are entirely soluble. On this basis, we previously searched for and identified stable αS-immunoreactive species migrating at ~35 kDa and above in the very high speed (post-370,000 × g) cytosols (S370) of mesencephalic (MES) neuronal cells and human and mouse brains (Sharon et al., 2001). We designate these αS-reactive species as buffer-soluble oligomers to distinguish them from soluble protein complexes containing αS and other proteins (Xu et al., 2002) and from buffer-insoluble, highly aggregated αS species (Baba et al., 1998; Hashimoto et al., 2001; Kahle et al., 2001; Spillantini et al., 1998). To further elucidate the relationship of these soluble oligomers to brain lipids, we directly extracted whole mouse brains in chloroform/methanol (2:1), a method previously used to purify myelin basic protein and other lipid-associated proteins from brain (Eylar et al., 1969; Oshiro and Eylar, 1970). This extraction of lipids revealed both the 17 kDa αS monomer and abundant higher MW αS-immunoreactive bands migrating at 35 kDa and above in the residual, water-soluble material (Figure 1A, lane 2). In contrast, extraction with a physiologic HEPES/$MgCl_2$ buffer yielded only the 17 kDa monomer in the water-soluble fraction (Figure 1A, lane 1). The αS banding pattern after chloroform/methanol extraction of whole brain is highly similar to that observed in the S370 cytosols after extraction of lipids by either chloroform/methanol or the fatty acid binding resin, Lipidex1000, or after heat treatment at 65°C (Sharon et al., 2001). The detection of these larger αS species directly after lipid removal by chloroform/methanol but not in aqueous buffer extracts (Figure 1A) suggests that αS oligomers exist in complexes with lipids in the brain but cannot be detected by Western blotting unless the αS epitopes are first exposed by lipid removal.

Determination of the apparent molecular weights of the ladder of αS-reactive bands strongly suggested that they are, in fact, oligomers. The bands migrate on denaturing SDS gels at 17 kDa (monomer), 35 kDa (predicted dimer, ~34 kDa), 53 kDa (trimer, ~51 kDa), 71 kDa (tetramer, ~68 kDa), 84 kDa (pentamer, ~85 kDa), and 105 kDa (hexamer, ~102 kDa) (Figure 1B). Importantly, this fine delineation of the individual bands is only observed when samples have first been exposed to delipidating agents such as chloroform/methanol or Lipidex1000, in combination with heat treatment at 65°C. In the case of living cells, delipidation can also be achieved by growing the cells in the absence of serum for 16 hr (e.g., Figure 1B). Without delipidation, the bands run as a poorly focused immunoreactive smear throughout the middle and upper portions of the gel. To obtain further support for their identity as oligomers, we attempted to separate the individual bands by isolelectric focusing on two-dimensional gels. Both the monomer and the higher species migrated at the same isoelectric point of ~4.0 (the calculated isoelectric point for αS is 4.6) on SDS denaturing gels (Figure 1C), strongly supporting the conclusion that the modification of αS that produces these additional species is oligomerization.

The αS Oligomers Occur In Vivo

The detection of the soluble αS oligomers appears to depend on delipidation using chloroform/methanol, heat, or Lipidex1000 combined with heat (above data and Sharon et al., 2001). In addition to delipidating proteins, such treatments can partially denature proteins, and this could conceivably lead to some oligomerization occurring in vitro. It was therefore important to confirm the in vivo existence of the soluble oligomers in neuronal cells and brain without the use of potentially denaturing conditions. To this end, we carried out size exclusion chromatography (SEC) of the high-speed soluble fractions (S370) of αS-expressing MES cells and fresh mouse brains under entirely nondenaturing conditions, i.e., in physiological buffer. The Sephadex G75 columns used gave linear elution profiles of a set of protein standards having MWs of ~1 to ~70 kDa (Figures 2A and 2B). The largest amount of αS species in the S370 cytosol eluted in SEC fractions corresponding to ~40–65 kDa; smaller amounts were present in flanking fractions, yielding a full size range of ~30 kDa to >70 kDa (the void volume) (Figures 2C–2F). When each SEC fraction was directly run on SDS-PAGE gels and Western blotted (without heating), almost all of the αS-immunoreactive

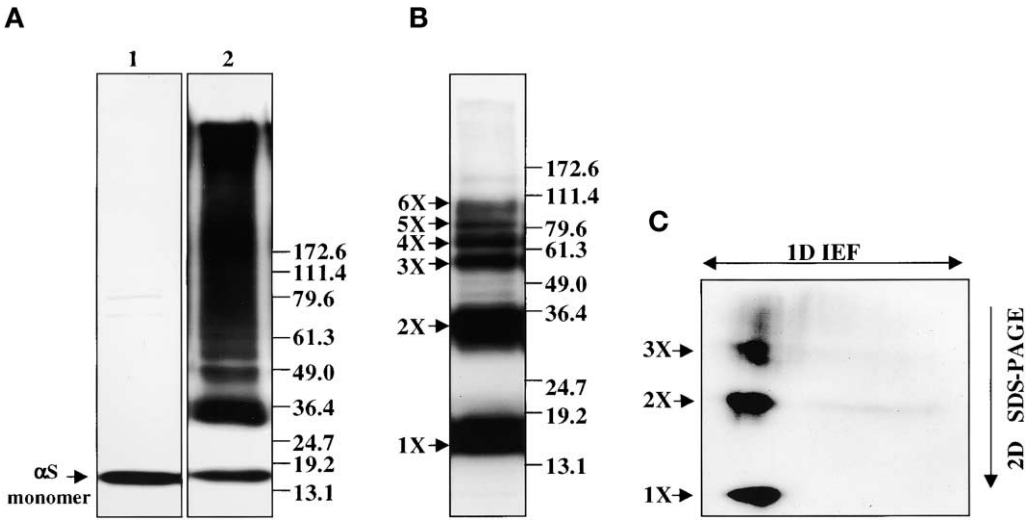

Figure 1. αS Occurs in Lipid-Associated, Soluble Oligomers in Normal Mouse Brain and in αS-Transfected MES Cells

(A) Chloroform/methanol extraction reveals αS-reactive oligomeric species in whole mouse brain. Normal mouse brain (20 μg) was extracted in HEPES-$MgCl_2$ buffer containing 1% NP-40 (lane 1) or in chloroform/methanol (2:1) (lane 2), and the aqueously soluble fractions in each case were analyzed by Western blotting with H3C. The three very faint bands migrating at ~70–100 kDa in lane 1 are nonspecific. The vast majority of αS immunoreactivity throughout lane 2 is αS specific, i.e., disappears upon antibody absorption (not shown).

(B) Apparent MWs of the cytosolic αS species suggest that they are oligomers. Human wt αS-transfected MES neurons were conditioned in the absence of serum and presence of FA-free BSA (to assist delipidation). Samples of high-speed cytosol (~15 μg) were incubated at 65°C overnight and then Western blotted with LB509. The relative migration of αS-specific bands was calculated using a BenchMark prestained protein ladder and AlphaEase (version 5.5) software. 2X through 6X indicate multiples of the monomer (1X).

(C) αS monomers and higher MW species migrate to the same isoelectric point, supporting the identity of the later as oligomers. High-speed cytosol (10 μg) of human wt αS transfected MES cells was incubated at 65°C overnight and than subjected to isoelectric focusing (IEF) (pH gradient, 3 to 10) followed by SDS-PAGE (8%–16% Tris-Glycine gradient). The 2D gel was blotted with LB509.

protein appeared at the monomer position (~17 kDa) on the gel (Figures 2C and 2E). However, if these blots were incubated at 65°C just before development, the SEC fractions were shown to contain αS dimers (~35 kDa) and some higher oligomers, in addition to the monomers; the detection of the latter was also markedly enhanced (Figures 2D and 2F). The S370 fractions from mouse brain tended to have slightly larger αS species (range ~30 to >70 kDa) than those of MES cells (range ~20–45). We obtained closely similar results by performing SEC on pure, recombinant human αS (data not shown). These SEC fractionation and sizing results demonstrate that dimers and higher oligomers of αS occur in the high-speed soluble fraction of both αS-expressing MES cells and normal mouse brain in the absence of a denaturing treatment. Moreover, soluble αS oligomers that have been gel-separated and fixed onto membranes are not detected well unless the membranes are exposed to heat, a treatment that may remove αS-associated lipids to expose αS epitopes (see Discussion). When we heated the SEC fractions at 65°C *before* gel electrophoresis/Western blotting, the heating again increased the amounts of oligomers as well as monomers detectable on the blots, similar to the result obtained if the unheated fractions were run and then the blots were heated (data not shown). Thus, the invariant increase, rather than decrease, in monomer levels that accompanies the increase in oligomers upon heat treatment indicates that the soluble αS oligomers do not result from in vitro aggregation of the monomer but rather exist as such in the high-speed cytosols of MES neurons and brain tissue.

Polyunsaturated Fatty Acids Promote and Saturated Fatty Acids Inhibit the Formation of Soluble αS Oligomers in Living Mesencephalic Neurons

Both chloroform/methanol and Lipidex extractions reveal the SDS-stable αS oligomers in brain tissue, suggesting the association of the oligomers with lipids. Moreover, we previously identified sequences near the N and C termini of αS that are homologous with a conserved motif of the fatty acid binding protein (FABP) family (Sharon et al., 2001). These findings led us to ask whether the formation and stability of soluble αS oligomers are regulated by FAs in intact cells. We supplemented the conditioned medium of MES cells stably expressing wt human αS with specific FAs. In these experiments, BSA was used as a vehicle to introduce the FAs into the cells (at a BSA:FA ratio of 1:5) (Bonen et al., 1999; Schaffer and Lodish, 1994). MES cells grown overnight in serum-free medium supplemented with FA-free BSA served as the control condition to establish basal levels of αS oligomers in the high-speed (S370) cytosol. In parallel, sister cultures were grown overnight in serum-free medium supplemented with BSA plus specific FAs. After overnight incubation, the S370 samples were blotted for αS oligomer content. Because the total FA concentration in normal human plasma is about 500 μM, we initially used this concentration for either FA mixtures or individual FAs. As this would represent a high concentration for an individual FA, we subsequently used concentrations as low as 35 μM and obtained indistinguishable results. Analysis of the αS pattern in wt αS transfected cells indicated that while monounsaturated fatty acids (oleic acid [18:1] or eicosenoic acid

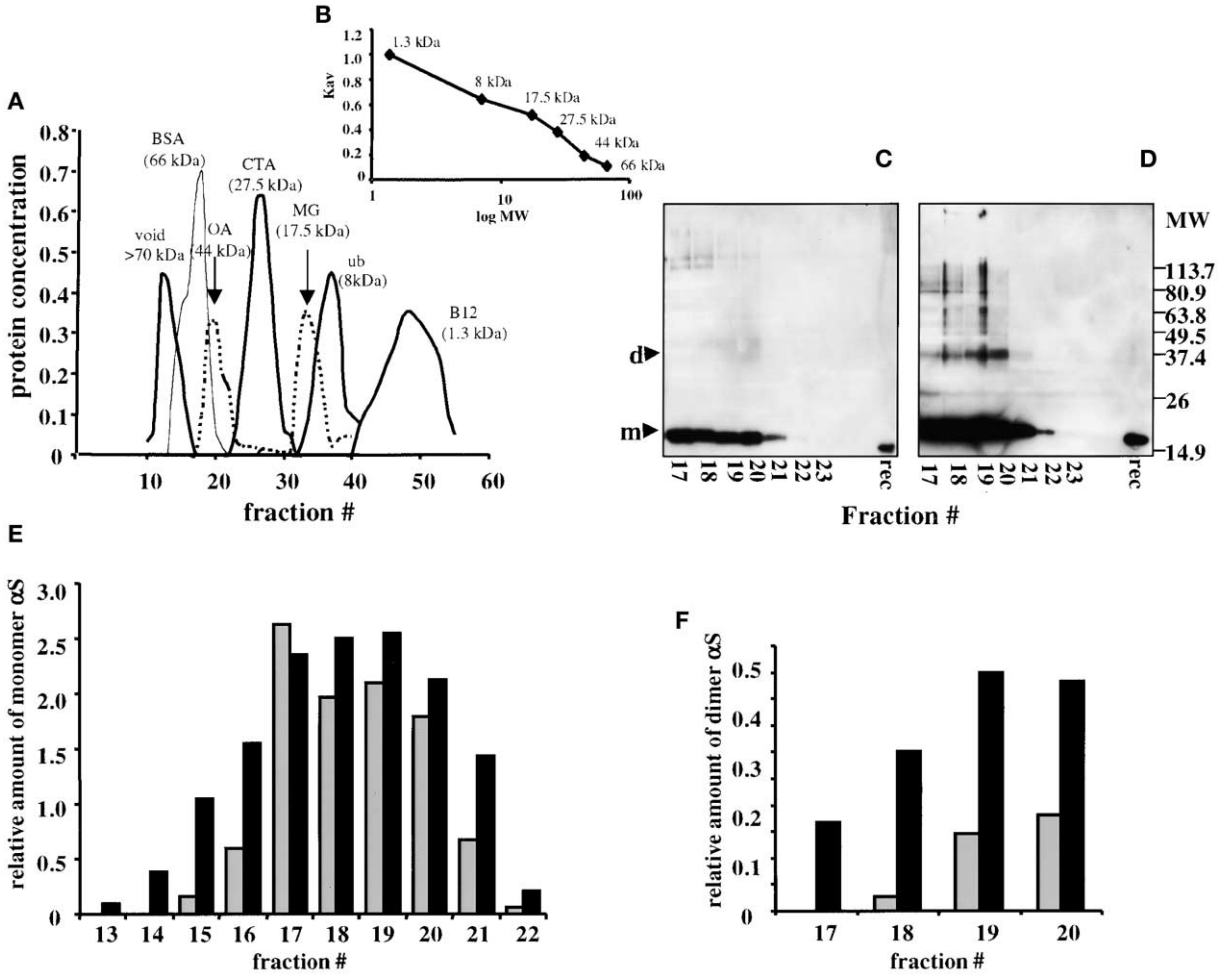

Figure 2. Soluble αS Oligomers Occur In Vivo

(A) Elution pattern of a Sephadex G75 SEC column established using the indicated proteins as size markers. The column was eluted with 50 mM ammonium acetate (pH 7.4), at 15 ml/hr. One milliliter fractions were collected and assayed for protein content. Protein size markers: void >70 kDa, fractions 10–15; bovine serum albumin (BSA) 66 kDa, fractions 16–19; ovalbumin (OA) 44 kDa, fractions 19–22; chymotrypsinogen A (CTA) 27.5 kDa, fractions 25–29; myoglobin (MG) 17.5 kDa, fractions 32–35; ubiquitin (ub) 8 kDa, fractions 36–40; vitamin B12 (B12) 1.3 kDa, fractions 45–53.

(B) The elution pattern of the protein size markers was linear on a semi-log plot, indicating efficient column separation. SEC conditions and protein size markers as in (A). Graph presented as log MW to Kav (Ve − Vo/Vt − Vo, where Ve = elution vol, Vo = void vol, Vt = total column vol).

(C) αS reactivity of SEC fractions of S370 cytosol (1.5 mg protein starting sample) from normal mouse brain. Fractions were loaded on 8%–16% Tris-Glycine SDS-PAGE without any heat treatment and the PVDF blot was stained with ab H3C. m, monomer; d, dimer.

(D) αS reactivity of same SEC fractions as in (C), but the PVDF blot was incubated at 65°C before αS immunostaining. Note that oligomers are now detectable in the ~40–65 kDa SEC fractions (lanes 21–17), and monomer detection is also enhanced.

(E) Blots shown in (C) and (D) were quantified for the relative amount of αS monomer in each fraction normalized to a standard amount (50 ng) of purified, recombinant human αS run on the same blot (far right lanes in C and D). Gray bars, results from standard Western blots (C); black bars, results from blots preincubated at 65°C before immunostaining (D).

(F) As in (E), but quantification of the αS dimer alone.

[20:1]) had no effect on the levels of αS oligomers in the S370 cytosol compared to BSA alone, conditioning the cells in saturated FAs (stearic acid [18:0] or arachidic acid [20:0]) resulted in a striking and consistent decrease in the levels of αS oligomers, without appreciably changing monomer levels (Figure 3A). In contrast, otherwise identical conditioning in polyunsaturated FAs (PUFAs) (α-linolenic acid [ALA] [18:3] or eicosapentaenoic acid [20:5]) dramatically enhanced the levels of αS oligomers, including high MW species that extended into gel-excluded material (Figure 3A). When carbon chain length was held constant, there was a direct correlation between the degree of unsaturation (number of double bonds) and the level of αS oligomerization; the more unsaturated the FA was, the more effective it was in promoting the appearance of αS oligomers (Figure 3A).

Longer carbon chains were also associated with increasing levels of αS oligomers. PUFAs ranging from 18 to 22 carbons were added to the media of the wt αS-expressing MES cells at concentrations of 500 μM.

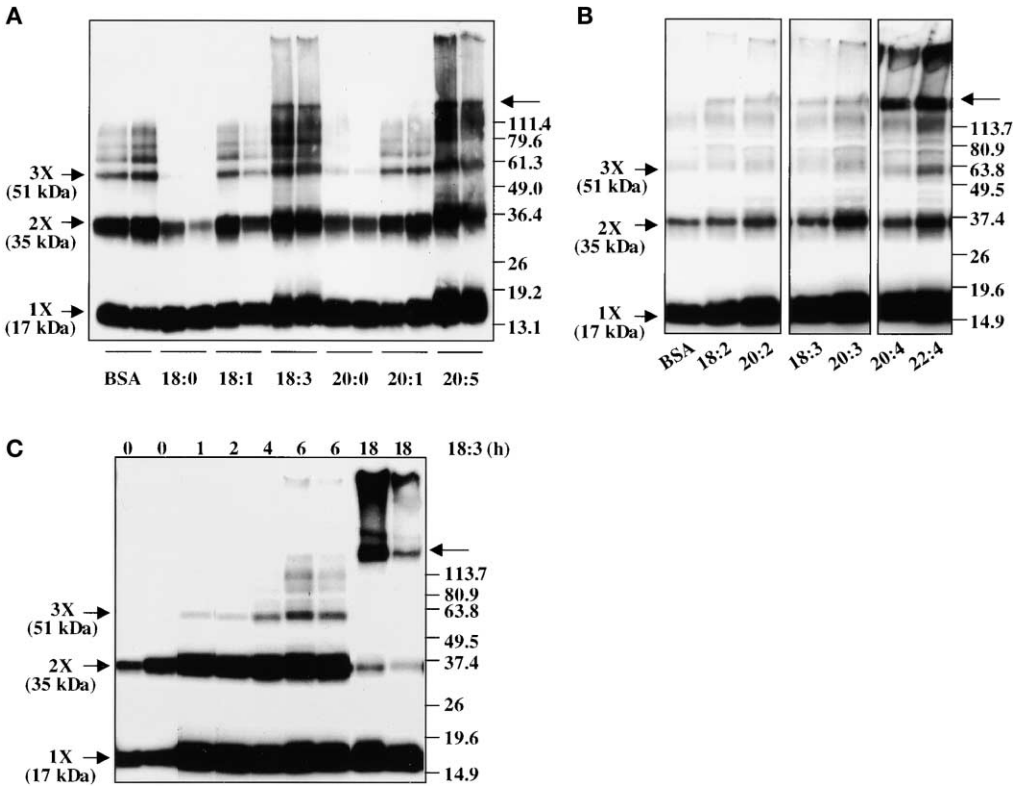

Figure 3. FAs Regulate the Extent of αS Oligomerization in the Cytosol of Living MES Cells

(A) αS oligomerization is differentially affected by the degree of FA saturation. High-speed cytosols (15 μg protein) of human wt αS stable MES cells conditioned in the presence of the indicated FA/BSA complexes (500/100 μM) in serum-free medium. Samples were treated at 65°C overnight prior to gel loading and blotting with LB509. Samples are run in duplicate; each lane represents a separate dish (sister culture) treated and processed in parallel. 18:0, stearic acid; 18:1, oleic acid; 18:3, α-linolenic acid; 20:0, arachidic acid; 20:1, eicosenoic acid; 20:5, eicosapentaenoic acid. Right arrow, position of the resolving/stacking gel interface.

(B) Longer carbon chains are more effective in promoting αS oligomerization. High-speed cytosols (15 μg) of human wt αS transfected cells conditioned and analyzed as in (A). 18:2, linoleic acid; 20:2, eicosadienoic acid; 18:3, α-linolenic acid; 20:3, eicosatrienoic acid; 20:4, arachidonic acid; 22:4, docosatetraenoic acid.

(C) Dynamic nature of αS oligomerization in MES cells conditioned with α-linolenic acid (18:3). Human wt αS-transfected MES cells were conditioned with 18:3 for the times (h) indicated at the top of each lane and analyzed as in (A). "0" hr lanes are sister cultures conditioned in BSA alone (i.e., FA-free) for the longest incubation time tested (18 hr). The stable MES clone used in (C) was under cultivation longer than the one used in (A), resulting in increased high MW polymeric αS in the former (see Figure 5 and Results).

Comparisons were made of the following pairs: 18:2 versus 20:2; 18:3 versus 20:3; and 20:4 versus 22:4 (Figure 3B). In each case, the longer chain PUFA enhanced the levels of soluble dimers and oligomers more potently. The effect of carbon chain length was less pronounced than that of degree of saturation. Nevertheless, densitometric analyses confirmed that the oligomer levels rose about 2-fold within each pair we compared (not shown).

The Effect of Fatty Acids on αS Oligomerization Is Time Dependent

In cells, FAs are bound mainly to membranes and to FABPs, when these are present (Hamilton and Kamp, 1999). Free cytosolic FA concentrations are generally low, i.e., in the nM range. A principal force keeping them low is the formation of a thioester linkage between the FA carboxyl group and the thiol group of Coenzyme A (yielding fatty acyl-CoA) within minutes after a FA enters a cell (Nelson and Cox, 2000). The esterified FAs are then consumed for energy production by mitochondria and peroxisomes or else used for synthesis of lipids. FAs are assumed to cross cell membranes continuously, either actively by protein transporters or via flip-flop of the FA through the membrane (Hamilton, 1999). We therefore asked how soon after a cell is exposed to extracellular FA can the enhancement of αS oligomerization be observed. We assumed that a rapid response might suggest that αS was reacting to the FA itself, whereas a delayed response might suggest that it was reacting to a FA metabolite or complex lipid molecule. Cells were incubated with α-linolenic acid (ALA [18:3]) in their conditioned media for the time periods indicated in Figure 3C. We initially used a high concentration (500 μM) and then examined low concentrations (35 μM), with the latter yielding closely similar results. Cells were harvested, and equal protein amounts of S370 cytosols were incubated at 65°C and Western blotted. The levels of both αS monomers and oligomers, particularly dimers, rose within 1 hr after adding PUFA to the cultures (Figure 3C). A further increase in the amount of αS oligomers occurred with time up to 6 hr. However, an oppo-

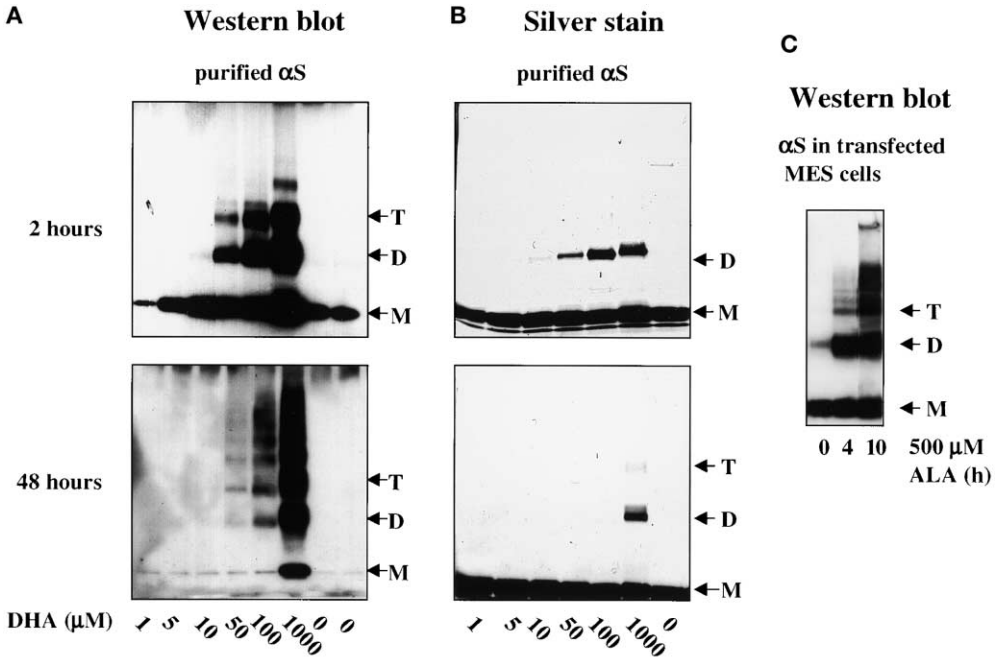

Figure 4. Polyunsaturated FAs Induce the Oligomerization of Purified, Recombinant Human Wild-Type αS In Vitro

(A) 2.0 μM purified αS was incubated without or with docosahexaenoic acid (DHA, 22:6) at the indicated concentrations at 37°C for 2 or 48 hr. Samples (75 ng protein) were Western blotted with H3C without any 65°C treatment.

(B) Same as in (A), but analysis by silver staining of samples of 500 ng protein each.

(C) αS oligomerization in response to PUFA treatment in stably transfected MES cells is closely similar to that of pure αS protein in vitro (A). Human wt αS MES cells were conditioned with 500 uM ALA (20:3) for the indicated times (h). High-speed cytosols were treated and analyzed by Western blot as per Figure 3A. M, monomer; D, dimer; T, trimer.

site trend was found in cells incubated with ALA for 18 hr. We observed a reduction in the intensity of the αS oligomers, while the amount of very high MW and gel-excluded immunoreactive material rose sharply. The gel-excluded material represents high MW aggregated αS species that are no longer SDS soluble. Thus, αS responds in a dynamic fashion to the introduction of a specific FA into the MES cell, with the initial rapidity of the response raising the possibility of a direct FA effect on αS (see below).

By quantitative Western blotting, the levels of both αS monomers and total αS immunoreactive species (including gel-excluded aggregated material) were markedly increased in the cytosol of MES cells after overnight exposure to PUFAs. The average increase was ~300-fold and ranged from ~50- to ~500-fold, depending on the particular clonal αS cell line being examined and its time under cultivation (note that total αS oligomer content changes with the time of cultivation [see below]). We hypothesized that these PUFA-induced rises were due to the increased formation of soluble oligomers and/or to conformational changes in monomers and oligomers that enhance antibody recognition. To exclude the alternative possibility of transcriptional activation by the PUFAs, we conducted quantitative Northern blotting for αS. This revealed no changes whatsoever in cellular αS mRNA levels in response to overnight PUFA incubation (data not shown).

Because PUFAs are a target for lipid peroxidation, we asked whether the oligomer-promoting effect of FAs was direct or was a side product of FA peroxidation. As one method of assessing this, we used a naturally occurring oxidized PUFA, 13-hydroxy octadecadienoic acid (13-S-HODE), an enzymatically oxidized form of linoleic acid (18:2). 13-S-HODE was no more potent in inducing oligomers than the parental, nonoxidized 18:2 (data not shown). As another approach, the MES cells were conditioned with or without PUFAs under either oxidizing conditions (33 or 100 μM H_2O_2) or reducing conditions (33 or 100 μM thioctic [α-lipoic] acid). We observed no difference in the oligomer-promoting effect of a PUFA (18:3) between these two conditions (data not shown).

Fatty Acids Bind to Purified αS and Directly Promote Its Oligomerization In Vitro

The striking effects of physiological levels of PUFAs on the amount of soluble αS oligomers we observed in living cells raised the question of whether the FAs have direct or indirect effects on αS. We previously found that purified, recombinant human αS could bind radiolabeled oleic acid (^{14}C 18:1) in a stochiometric manner in vitro (Sharon et al., 2001). We now asked whether the direct binding of αS to FAs promoted its oligomerization. Purified, recombinant human wt αS (2.0 μM) was incubated at 37°C with increasing concentrations of decosohexanoic acid (DHA; 22:6), and aliquots were analyzed by SDS-PAGE after varying intervals of incubation (Figure

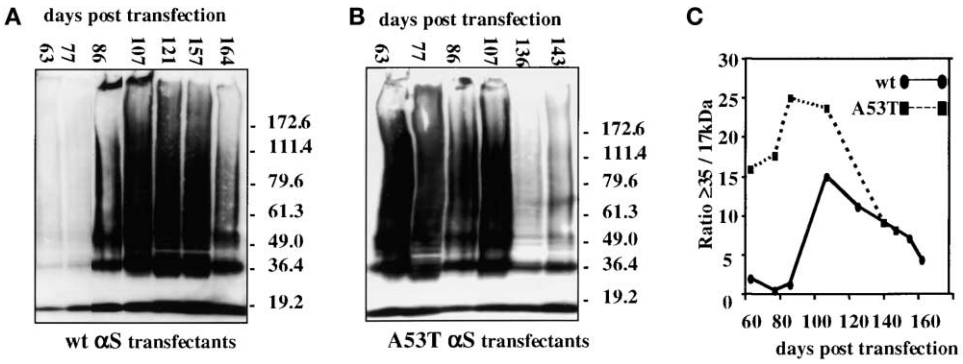

Figure 5. Mutant A53T αS-Expressing MES Cells Accumulate Soluble Oligomers Earlier than Do Wild-Type αS Transfectants

(A) Wild-type αS expressing MES cells. Samples of high-speed cytosols (15 μg protein) collected at increasing intervals post DNA-transfection were treated at 65°C overnight and Western blotted with LB509.

(B) A53T αS expressing MES cells were processed identically as in (A).

(C) Higher relative levels of soluble αS oligomers accumulate in A53T than in wt transfectants over time. Blots from (A) and (B) were scanned and quantified (Sharon et al., 2001). Values at the *y* axis represent the ratios of quantitative densitometry of αS oligomers (migrating at 35 kDa and higher) to monomers (migrating at 17 kDa) for each time point.

4). After 2 hr incubation without FA, the αS was only detectable in monomeric form, as confirmed on both silver stains and Western blots (Figures 4A and 4B, extreme right lanes). In contrast, a 2 hr incubation with DHA clearly promoted oligomerization of pure αS at concentrations of the PUFA as low as 10 μM, whereas incubations in 1 μM or 5 μM were without significant effect. αS oligomerization was enhanced by DHA in a dose-dependent fashion between 10 and 1000 μM (Figures 4A and 4B). Importantly, in the case of purified αS, there was no need for heat treatment in order to detect the oligomers by Western blotting. The dimer (35 kDa) of pure αS observed by silver staining after incubation with DHA at >10 μM migrated at the same position as the presumptive dimers we had identified by Western blotting in intact αS-transfected MES cells incubated with PUFA (Figure 4C). Incubation of pure αS with PUFA not only promoted its oligomerization but was also associated with the detection of increased (rather than decreased) levels of monomeric αS by Western blotting (Figure 4A). In addition, SDS sample preparation and subsequent SDS-PAGE never led to in vitro oligomerization of pure αS monomer (e.g., Figures 4A and 4B, extreme right lanes). These findings again support the conclusion that the highly soluble αS oligomers we detect in the cytosols of MES neurons and brains exist in vivo.

We next examined the effects on the oligomer pattern of incubating pure αS in DHA for prolonged periods. After 48 hr, the overall level of oligomeric species detected by Western blotting was increased (compared to 2 hr) in samples containing 100 or 1000 μM DHA (Figure 4A). In particular, more high MW (≥35 kDa) oligomers extending up the running gel were observed, and this change was accompanied by less monomers and dimers than were seen at 2 hr. These Western blot changes were generally reflected by the less sensitive method of silver staining, in which the dimer was far less visible at 48 hr than at 2 hr at DHA concentrations of 10, 50, and 100 μM (Figure 4B). At the highest FA concentration (1000 μM), the trimer band became visible by silver staining at 48 hr. Several different PUFAs were tested for their ability to mediate the oligomerization of pure αS. In general, the longer and more unsaturated FAs were the most potent, with a rank order of 22:6 > 20:4 > 18:3 > 18:0 (data not shown), reflecting the results obtained with living MES cells (Figure 3).

We also conducted in vitro FA incubation studies on pure αS that had first been subjected to size exclusion chromatography. Beginning with the SEC-purified monomer alone, we were able to show unequivocally that it oligomerizes in the presence of PUFAs, thereby excluding the remote possibility that the PUFAs were disaggregating some hypothetical higher MW αS aggregates (data not shown).

Soluble αS Oligomers Accumulate More Rapidly in Cells Expressing A53T Mutant Human αS

To explore the relevance of the soluble αS oligomers described above to the pathogenesis of α-synucleinopathies like PD, we compared MES neuronal lines stably expressing wt or A53T mutant human αS. Five different matched pairs of wt and A53T clones were compared for soluble high MW αS immunoreactivity accumulating with time in culture; a representative pair of clones is shown in Figure 5. The clones were matched for closely similar expression levels of αS monomer and were maintained identically under normal serum conditions from the time of initial DNA transfection through selection of stable clones and then serial passage in culture. S370 was prepared at increasing time points posttransfection; equal protein aliquots were incubated at 65°C, electrophoresed in parallel, and Western blotted for αS monomers and oligomers. Early during clonal lifetime (63 days posttransfection), the content of human αS oligomers was very low in the wt clones but was already appreciable in the matched A53T clones (compare Figures 5A and 5B). Thereafter, both the wt and A53T clones showed first a time-dependent accumulation and then a loss of oligomers. We quantified the ratio of all αS-reactive species ≥35 kDa (oligomers) to the 17 kDa

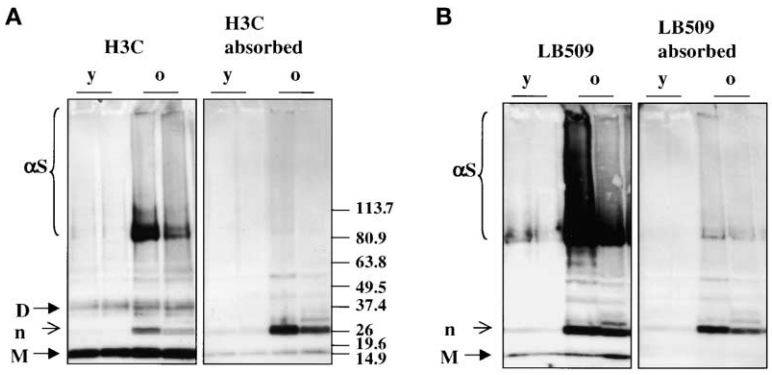

Figure 6. Age-Dependent Accumulation of Soluble αS Oligomers of Both Transgenic Human αS and Endogenous Mouse αS in Brain

Duplicate samples of high-speed supernatants of young (4 week, n = 3) or old (18 month, n = 3) mice were incubated at 65°C and then blotted with (A) H3C (20 μg protein) for total αS or (B) LB509 (50 μg) for human αS only. To verify specificity, each antibody was absorbed with 10 μg purified recombinant αS. Monomer (M), dimer (D), and immunoreactive oligomers of ≥85 kDa (bracket) are all αS specific. n, nonspecific band.

species (monomers) (Figure 5C). This ratio was far higher in the A53T- than wt-expressing clones already at the earliest posttransfection time point we measured (63 days). (Earlier time points could not be taken, because it took ~60 days to select stable clones after DNA transfection.) There was further accumulation of oligomers over time in both the wt and A53T clones, but the maximal oligomer:monomer ratio was reached somewhat earlier in the case of the A53T clones. After ~100 days in culture, we observed a striking decline in total αS levels, with the oligomers declining more rapidly than the monomers, resulting in falling ratios. This decline is related to the overall loss of αS expression that we reported previously in stable MES transfectants (Sharon et al., 2001). We did not detect a significant difference between wt and A53T clones as regards the time interval until complete loss of αS expression (for monomers as well as oligomers).

Age-Dependent Accumulation of Soluble αS Oligomers in Mouse Brain

To determine whether the above data in mesencephalic neurons reflect changes in αS that occur in vivo, we assessed soluble oligomer levels over time in human αS transgenic (tg) mouse brains (Sharon et al., 2001) and compared these to the pattern of endogenous αS in the same mice. Equal protein aliquots of S370 supernatants from young and old tg brains were incubated at 65°C and then analyzed by Western blotting. To detect solely the human αS protein, we used antibodies LB509 and 15G7, whereas for total αS (endogenous mouse plus transgenic human), we used antibodies H3C and Syn-1. Comparisons with non-tg littermates revealed that the level of brain expression of human αS was about a half of that of endogenous mouse αS (not shown). We found that brain levels of αS monomers were similar for young (4 week) and old (18 month) mice. Using H3C antibody, we detected a modest accumulation of the dimer in old compared with young mice (Figure 6A). The dimer was not observed with antibody LB509, probably due to its low abundance (Figure 6B). However, substantial differences between young and old mice were detected in the higher MW αS species. In the older mice, we observed accumulation of soluble αS oligomers, specifically accumulation of a ~85 kDa band and a higher immunoreactive smear migrating up to the gel-excluded region. This pattern was observed for human wt tg αS (LB509) as well as for endogenous mouse αS (H3C) (Figure 6). We confirmed the specificity of this high MW αS reactivity, by showing the same patterns with the additional antibodies 15G7 (human-specific) and syn-1 (both mouse and human αS) (data not shown). We also preabsorbed each antibody with purified, recombinant αS (Figures 6A and 6B). Both the dimer and the high MW smear at ~85 kDa and above was αS-specific, whereas a ~25 kDa band appearing in the older mice was nonspecific.

Accumulation of Soluble αS Oligomers in Human Brains with Synucleinopathies

Finally, we asked whether the accumulation of soluble αS oligomers in MES neurons and mouse brains described above was reflected in similar changes in human brain. Cerebral cortex from five brains each of PD, DLB, and age-matched normal subjects (15 brains total) were analyzed by quantitative Western blotting. We assayed the high-speed (S370) cytosols and also a lipid-rich, low MW "floating" fraction, both prepared as previously described (Sharon et al., 2001). To adjust for varying absolute levels of αS among the different brain samples, we expressed the results as a ratio between the levels of 35 kDa (dimeric) and 17 kDa (monomeric) αS measured in the same sample (i.e., the same gel lane). In the high-speed cytosols, αS monomers, dimers, and some higher MW oligomers were detected after incubation at 65°C (Figure 7A). We observed a significant ~2-fold increase in the 35/17 kDa αS ratio in the cytosols of both PD and DLB brains, compared to that in simultaneously analyzed controls (Figure 7D) ($p < 0.005$ for PD and $p < 0.01$ for DLB). The detection of the higher MW oligomers was strongly enhanced by incubating the cytosols with the fatty acid binding resin, Lipidex1000, at 65°C and analyzing the material that was bound to the Lipidex, as previously reported (Figure 7B; Sharon et al., 2001). In the case of the lipid-rich, low-speed fraction of brain homogenates that floats above a sucrose cushion, we extracted this fraction in chloroform/methanol (2:1) and then Western blotted the residual water-soluble material. Here, we observed very high MW αS-immunoreactive material migrating only in the stacking gel down to its interface with the resolving gel, and this was consistently present in the PD and DLB samples but absent in the controls (Figure 7C).

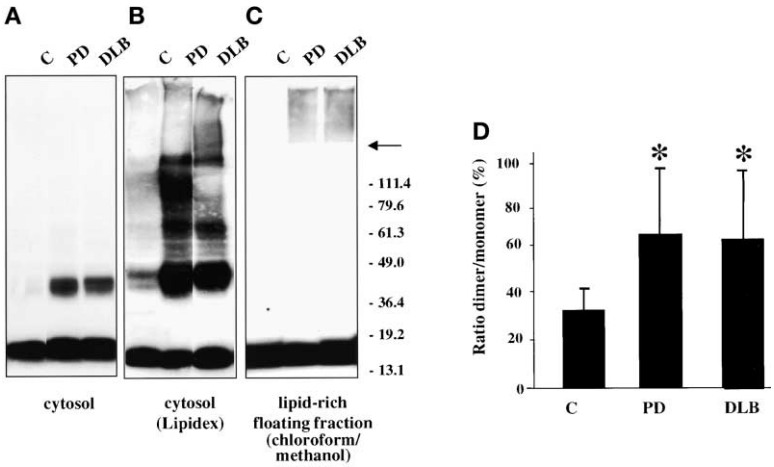

Figure 7. Accumulation of Soluble αS Oligomers in PD and DLB Human Brains

(A) Soluble αS oligomers in human brain. High-speed supernatant samples (20 μg) from a representative case each of control (n = 5), PD (n = 5), and DLB (n = 5) brains were incubated at 65°C and blotted with LB509.

(B) Detection of soluble αS oligomers is enhanced by the FA binding resin, Lipidex1000. Supernatant samples as in (A), but treated with Lipidex1000 (Sharon et al., 2001). The material that bound to Lipidex was then blotted with LB509.

(C) Insoluble (gel-excluded) αS aggregates in PD and DLB brains. Chloroform/methanol extraction of the lipid-rich, low-speed fraction (Sharon et al., 2001) of the brains shown in (A) and (B). Equal volumes (50 μl of a total 500 μl suspension) were extracted in chloroform/methanol (2:1). The interface between the organic (lower) and aqueous (upper) fractions was resuspended in 2X Laemmli buffer (Sharon et al., 2001) and blotted with LB509. Arrow, position of the resolving/stacking gel interface. Insoluble αS aggregates are present in the stacking gel only and do not enter the resolving gel.

(D) Dimer levels in PD and DLB brains are significantly more abundant than in controls. Densitometry ratios of 35 kDa dimer to 17 kDa monomer for control, PD, and DLB brains (n = 5 each). Control to PD comparison, $p < 0.005$; control to DLB comparison, $p < 0.01$ (two-tailed equal variance t test).

Discussion

Numerous human neurodegenerative diseases are defined pathologically by the presence of highly insoluble inclusions of protein aggregates within characteristic neuronal populations. In the case of PD, DLB, multiple systems atrophy, and related disorders, the prototypical inclusions are the Lewy bodies, composed in large part of insoluble filaments of αS. For many years, there has been controversy about whether such inclusions are a cause or an effect of the neuronal degeneration. This debate has recently begun to be resolved by the recognition that the large inclusions are in equilibrium with much smaller, diffusible oligomeric assemblies that may be able to induce cell injury. If this emerging hypothesis, which appears relevant to PD, Alzheimer's disease, Huntington's disease, and several other disorders, is correct, then a major mechanistic and therapeutic objective is to identify and inhibit the earliest soluble oligomers of the protein, arising well before the formation of insoluble, microscopically visible aggregates.

Here, we define and characterize a normal cellular pool of entirely soluble αS oligomers whose formation and stability are regulated by saturated and polyunsaturated fatty acids. Importantly, the soluble αS oligomers are detectable in the very high-speed cytosols of mesencephalic neuronal cells, mouse brains, and human brains. The detection of these SDS-stable, soluble intermediates by Western blotting depends on protocols that include a delipidation step. In this regard, we applied the chloroform/methanol extraction method (Sharon et al., 2001) to remove lipids from αS-oligomers and enable their detection by antibodies. We further show that the soluble αS oligomers accumulate with time in the cytoplasm of cultured mesencephalic dopaminergic cells and that this accumulation is accelerated by expressing the PD-causing A53T mutation. In accord, the appearance of soluble αS oligomers in mouse brain was found to be age-dependent, in that younger mice had substantially less oligomers than older mice. This trend obtained for both transgenic human αS and endogenous mouse αS. Moreover, in the human synucleinopathies PD and DLB, there is a pronounced accumulation both of soluble αS oligomers in high-speed brain supernatants and of gel-excluded αS aggregates that are revealed by extracting the lipid-rich fraction of brain with chloroform/methanol. The various properties of the oligomers described here suggest that they represent an early, still soluble but detergent-stable assembly form of αS that has direct relevance to human synucleinopathies.

We use the term soluble oligomers based on their presence in a very high-speed (post-370,000 × g) supernatant. Protofibrils or fibrils of αS would not be present in the S370, because these would sediment at lower forces (<100,000 × g), as has been reported for both αS protofibrils (Kahle et al., 2001) and Aβ protofibrils (Walsh et al., 2002).

We performed size exclusion chromatography to demonstrate that the soluble αS oligomers exist in vivo and do not result from in vitro aggregation of αS monomer induced by the 65°C treatment and SDS-PAGE that we use to immunodetect them. Both dimers and higher oligomers were separated by SEC performed in physiological buffer, and they eluted in fractions clearly corresponding to sizes greater than monomer (Figure 2). Furthermore, after the SEC fractions were electrophoresed (without any heating) and fixed to PVDF membranes, heating these membranes to 65°C enhanced the detection of *both* oligomers and monomers in a step that could not allow further aggregation of the monomers. Heat and delipidation (Lipidex; chlorform/methanol) treatments that reveal the soluble oligomers are invariably associated with detection of more, rather than less, monomers, further obviating the possibility that these treatments are inducing αS aggregation in vitro. Moreover, SDS solubilization of S370 samples and denaturing

gel electrophoresis was never found to induce the oligomerization of either the natural or recombinant αS monomers. Rather, SDS-PAGE of fractions eluting from the SEC column at ≥35 kDa (dimers and oligomers) appears to induce partial depolymerization of these metastable oligomers to release monomers (Figure 2).

In view of the ability of lipid extraction to markedly enhance the detection of soluble αS oligomers in the cytosol, we asked whether their formation and/or stability were regulated by the fatty acid content of a cell. Conditioning intact MES neurons with physiological (plasma) levels of different fatty acids revealed that PUFAs promote the appearance of oligomers in the cytosol in a time-dependent fashion. Increasing the extent of unsaturation of the FA strikingly enhanced the amounts of soluble αS oligomers detected in the treated cells. Conversely, MES cells conditioned with fully saturated FAs consistently showed lower amounts of soluble αS dimers and oligomers than those exposed to monounsaturated FAs or to the BSA carrier protein alone. We obtained evidence that these regulatory effects represent, at least in part, a direct action of the FAs on αS by incubating purified human αS in vitro with DHA. This resulted in the dose- and time-dependent formation of SDS-stable oligomers of the pure protein, similar to those observed for native αS in the cytosols of the PUFA-treated MES cells, in αS transgenic mouse brains, and in the brains of humans with PD or DLB.

In our in vitro assays, we observed the de novo appearance of dimers and trimers of pure αS within 2 hr of incubation with PUFA (Figure 4), and in intact dopaminergic mesencephalic cells, the oligomers began accumulating within 1 hr after supplementing the medium with PUFAs (Figure 3C). Further, our results indicate that the transition from soluble to insoluble (gel-excluded) oligomers is also FA dependent. At early time points (1–6 hr) of incubation with ALA, there was clear enhancement of the soluble oligomers (migrating at 35 to ~120 kDa), but after longer incubation times (18 hr), there was a major reduction in these oligomers but an increase in αS-reactive gel excluded material (Figures 3A–3C). We hypothesize that this gradual progression in the size and insolubility of αS assemblies represents an analog of the changes in αS solubility and aggregation state reported in PD and other synucleinopathies. In this regard, we document abnormally elevated amounts of both soluble and gel-excluded forms of lipid-associated αS in PD and DLB brains (Figure 7).

Of direct relevance to our findings is the recent report that purified human αS oligomerized in the presence of phospholipid vesicles that contained long-chain PUFAs and that this was detected as early as 12 hr after adding the vesicles (Perrin et al., 2001). However, in this in vitro study, αS assembly state was not altered by a PUFA (arachidonic acid [AA; 20:4]) at physiological concentrations, only at a very high concentration (1000 μM) that is above the critical micelle concentration (20 μM) of AA (Richieri et al., 1992). In our experiments, oligomerization of pure αS was observed at concentrations as low as 10 μM for DHA. Indeed, the lowest concentration of AA we tested in our in vitro oligomerization studies was 10 μM, and this effectively induced αS oligomerization (data not shown). The discrepancy may be due to differences in the detection protocols. For example, we included a pretreatment (Lipidex1000 extraction) aimed at removing lipids, especially FAs, from the purified recombinant αS protein prior to its incubation with FAs. This may make αS more able to bind FAs at lower concentrations and at a faster rate. The oligomerization of αS has also been observed in cellular experiments utilizing chemical crosslinking with the reducible crosslinker DTBP (Cole et al., 2002). On this basis, Cole and coworkers suggested that αS exists in cells in an equilibrium of monomers and oligomers and that the crosslinking stabilized existing oligomers. These findings are compatible with our own, although we did not require crosslinking to detect a population of SDS-metastable oligomers in our cell and brain cytosols. One difference between the two studies is the cell lines used: we studied MES cells with dopaminergic properties, whereas Cole and coworkers examined HEK293 cells. This difference raises the possibility that certain cell-specific factors in dopaminergic neurons might help to promote or stabilize αS oligomers. In this regard, recent findings suggest a positive role for PUFAs in the maintenance of dopamine levels and the amounts of dopamine-containing lipid vesicles in neurons (Zimmer et al., 1998, 2000).

Based on our collective findings, we speculate that αS monomers interact in the neuronal cytoplasm with FAs, and that certain FAs, particularly longer and more unsaturated molecules, help mediate the formation of early, soluble oligomers. The oligomers appear to exist normally in small amounts; however, over time (i.e., during aging) or with prolonged exposure to PUFAs in the neuronal cytoplasm, a portion of them may aggregate into much larger, insoluble assemblies, as we observed in both mesencephalic neurons and transgenic mice. According to this hypothetical model, the ability of αS to interact with FAs in the cytoplasm, during which a portion of αS is converted into highly soluble, lipid-associated oligomers, may constitute part of the physiological function of the protein. We base the latter assumption on the following observations: (1) soluble oligomers of endogenous αS appear in normal mouse brain as early as 3 weeks of age; (2) soluble oligomers respond very rapidly to changes in the lipid environment, being induced in intact MES neurons after just 1 hr of incubation in physiological extracellular concentrations of PUFAs; and (3) the soluble oligomerization process is reversible, in that there is inhibition and apparent disassembly of oligomers when αS-expressing cells are exposed to physiological levels of saturated FAs.

It is likely that the accumulation of soluble oligomers beyond a certain level becomes harmful for neurons. In the three systems we examined here, we found support for the hypothesis that the time-dependent accumulation of first soluble and then insoluble oligomers is harmful and that neurons use a mechanism of aggregating αS oligomers that are potentially cytotoxic. First, in stably αS-transfected MES neurons, there was loss of αS expression shortly after the cells accumulated high levels of soluble oligomers (Figure 5C). Second, in mouse brains, lipid-associated soluble oligomer levels rose with age, associated with the appearance of gel-excluded, insoluble αS aggregates (Figures 6A and 6B). Third, in human brains, both the soluble oligomers found in the high-speed cytosols and the insoluble oligomers found in the lipid-rich floating fraction were detected in

substantially higher levels in ten synucleinopathy than in five control subjects. In this regard, we have recently detected a positive correlation between the accumulation of αS oligomers in α-synucleinopathy brains and their cytosolic PUFA levels (R.S. and D.J.S., unpublished data). For example, significantly higher levels of DHA (22:6) were observed in PD and DLB than control brains ($p < 0.001$). A potential therapeutic corollary of our findings is that the pharmacological regulation of brain lipid levels, particularly those of PUFAs, could serve to decrease the tendency of αS, a highly abundant neuronal protein, to assemble into diffusible oligomers that could promote neurotoxicity.

Experimental Procedures

Reagents

Fatty acids (sodium salts) were obtained from NU-CHEK PREP, Inc. (Elysian, MN). Stock solutions (50 mM) were prepared in 10% ethanol in DDW. Aliquots were kept at −20°C in the dark under argon. 13-S-HODE (in ethanol) was from Cayman Chemicals (Ann Arbor MI), and Lipidex1000 (Hydroxyalkoxypropyl Dextran, Type VI) was from Sigma (St. Louis, MO).

Cultured Cells and Transgenic Mice

The rodent mesencephalic neuronal cell line, MES 23.5, which has dopaminergic properties (Crawford et al., 1992), was maintained and transfected as described (Sharon et al., 2001). Tg mice (heterozygous) expressing human wt αS under the control of the platelet-derived growth factor B, were kindly provided by Drs. M.S. Goldberg and J. Shen and generated routinely (Sharon et al., 2001).

Human Brains

Frozen tissue samples (control, nondemented cases, n = 5; PD, n = 5; DLB, n = 5) were obtained from the neuropathology cores of the Massachusetts Alzheimer Disease Research Center at the Brigham and Women's Hospital, the Massachusetts General Hospital, and the Massachusetts Institute of Technology, Morris K. Udall Center for Excellence in PD Research. All cases were evaluated neuropathologically by current diagnostic criteria. Frontal cortex was sampled from Broadmann area 8 in the middle frontal gyrus at the level of the anterior border of the caudate. To calculate p values for comparisons between controls and PD or controls and DLB, we used a t test with 2-tailed, paired distribution.

Fractionation Protocol

A fractionation procedure (Sharon et al., 2001) was used to obtain very high-speed cytosols (post-370,000 × g × 1 hr) of homogenates of MES cells or brain. The same protocol yielded a lipid-rich fraction: the initial low-speed (post-170 × g × 15 min) pellet of the homogenate was loaded on a 2.15 M sucrose cushion and spun at 60,000 × g × 1 hr. The lipid-rich fraction is taken from the top of the cushion.

Chloroform/Methanol Extraction of Whole-Mouse Brain

The procedure was modified from one used to isolate and purify myelin basic protein from bovine brain (Eylar et al., 1969; Oshiro and Eylar, 1970). Whole-mouse brain (~0.5 g) was ground with a pestle in LN_2. Twenty milliliters chloroform/methanol (2:1) were added, followed by extensive mixing and addition of 80 ml. After rocking 30 min at 4°C, the blend was filtered (Whatmann 3 MM paper). The filtrate was transferred to a clean beaker and 100 ml fresh chloroform/methanol were added. The blend was rocked at 4°C for 30 min and filtered again. The chloroform/methanol-soluble extract (containing lipids) was discarded. The filtered material was completely evaporated (4 hr) and extracted in 10 ml of 0.01 N NaOH (pH 11), for 20 hr at 4°C. The extract was spun at 10,000 × g for 10 min. The clear supernatant was transferred to a clean tube and neutralized with Tris HCl (pH 8.0). The resultant precipitate was removed by centrifugation, and the supernatant transferred to a clean tube. Protein concentration determined by the Bradford procedure (Bradford, 1976).

Conditioning Living Cells with FAs

Subconfluent (~75%) cultures of MES cells expressing human wt αS were cultured in DMEM containing the essential nutrients for MES cells (Crawford et al., 1992), but without serum. BSA/FA complexes were added to the medium just before applying it to the cultures. These complexes were prepared by mixing BSA (FA-free; Sigma) with the indicated FA (at a molar ratio of 1:5) in binding buffer (10 mM Tris HCl [pH 8.0], 150 mM NaCl) at 37°C for 30 min. We first used 500 μM FA and 100 μM BSA to prepare the complexes. Later, we used much lower (physiological) concentrations: 35 μM FA and 7 μM BSA (see Results). Sister cultures, used as controls, were incubated in parallel with BSA but without FA. In the case of experiments using 13-S-HODE, the ethanol was evaporated under argon flow to a minimal volume of 100 μl. Parallel cultures treated with either BSA alone or a BSA-PUFA (18:2) mixture contained ethanol to the same final concentration of 0.3%. The final FA concentration in this experiment was 100 μM, which is in the range of FA concentrations we found effective (500–35 μM). Cells were then collected, and S370 cytosols prepared as above.

Quantitative Western Blotting

Quantitative Western blotting was performed as described (Sharon et al., 2001). Antibodies used were as follows: human αS specific, LB509 (Zymed, San Francisco, CA) and 15G7 (Q Biogene, La Jolla, CA); mouse and human αS, H3C (George et al., 1995) and Syn-1 (Transduction Labs, Lexington, KY); and anti-ubiqutin (MBL, Nagoya, Japan). In experiments that included heat treatment of PVDF membranes, incubation of the blots at 65°C for 16 hr in PBS was performed right after the protein transfer step.

In Vitro Oligomerization

Purified, recombinant human αS (gift of Dr. P.T. Lansbury) was resuspended in binding buffer (10 mM Tris HCl [pH 8.0], 150 mM NaCl) and incubated with Lipidex1000 (10% w/v) at 37°C with rocking for 30 min. The suspension was spun at 10,000 × g and the supernatant removed to a clean tube. αS protein (2.0 μM) was incubated with the indicated FAs at indicated concentrations in binding buffer at 37°C for the indicated times. For detection by Western blotting, we loaded 75 ng protein, and for detection by silver staining (Invitrogen, Carlsbad, CA), we loaded 500 ng; these aliquots were from the same reaction.

Isoelectric Focusing

We used the IPGphor isoelectric focusing system (Amersham/Pharmacia Biotech). High-speed cytosol (10 μg protein) was heated at 65°C overnight and brought to 200 μl with sample rehydration buffer (7 M urea, 2 M thiourea, 2% Chapso, 0.5% IPG buffer [Amersham Pharmacia Biotech], 0.3% DTT, bromophenol blue) and applied on an 11 cm 1D ready-strip (Bio-Rad) with a pH gradient of 3–10. The strip was overlaid with mineral oil to eliminate drying. Sample was subjected to the following steps: rehydration for 12 hr, isoelectirc focusing at 500 V for 30 min followed by 1000 V for another 30 min and 8000 V for 7 hr. As focusing proceeded, the current across the strip was dropped to 35 μA. The 1D strip was then equilibrated in 10 ml equilibration buffer (50 mM Tris HCl [pH 8.8], 6 M urea, 30% glycerol, and 10% DTT) for 15 min, applied to a precast 2D SDS-PAGE gel (Novax, San Diego CA) and run for 2 hr at 100 V. The resultant gels were Western blotted as described (Sharon et al., 2001).

Molecular Weight Estimations of αS Oligomers

Human wt αS transfected MES cells were incubated overnight in DMEM containing the essential nutrients for MES (Crawford et al., 1992) but without serum. Medium was supplemented with 0.1 mM BSA (FA free). High-speed cytosols were prepared (above), analyzed by Western blotting, and scanned by Umax Magic Scan (Eastman Kodak). The relative migration of αS-specific bands was analyzed with a standard BenchMark prestained protein ladder (Invitrogene) using the AlphaEase version 5.5 software (Alpha Innotech Corp., San Leandro, CA)

Size Exclusion Chromatography

Bed volumes of 40 ml of Sephadex G-75 superfine gel (Amersham Pharmacia Biotech, Uppsala, Sweden) were packed into borosilicate Econo-columns (Bio-Rad, Hercules, CA). S370 samples (1.5 mg) from mouse brains or wt-αS transfected MES cells, or 1.0 mg of purified recombinant αS protein, were loaded on the columns. Elution was at a linear flow rate of ~15 ml/hr in 50 mM ammonium acetate (pH 7.4), using a peristaltic pump. Fractions (1 ml) were collected, and 100 μl of the indicated fractions were lyophilized, resuspended in HEPES-$MgCl_2$ buffer, and analyzed by Western blotting for αS.

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