# α-Synuclein Adopts an α-Helical Conformation in the Presence of Polyunsaturated Fatty Acids To Hinder Micelle Formation†

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ABSTRACT: α-Synuclein is a small cytosolic protein involved in the pathogenesis of Parkinson's disease and other neurodegenerative disorders. Recent studies suggested a lipid-related function for this brain-enriched protein. Since the brain carries a high level of docosahexaenoic acid (DHA) and since the extent of α-synuclein gene expression increases in response to DHA intake, we have investigated the interaction of α-synuclein with this essential omega-3 fatty acid. We show that α-synuclein allows DHA to be present in a soluble rather than micellar form. Upon interaction with DHA, the normally unstructured α-synuclein rapidly adopts an α-helical conformation. Prolonged exposure to DHA, however, gradually converts α-synuclein into amyloid-like fibrils. These results identify a potential biological function for α-synuclein and define an omega-3-linked pathway leading to α-synuclein aggregation.

Parkinson's disease (PD)[1] is the most common movement disorder, affecting more than 1% of the population over the age of 65 (*1*). Most cases of PD occur sporadically, with advancing age being an important risk factor. Abundant filamentous inclusions in the form of Lewy bodies and Lewy neurites constitute the defining neuropathological characteristics of PD and the related condition, dementia with Lewy bodies (DLB) (*2*). These inclusions are made of the protein α-synuclein (*3*−*5*). The relevance of α-synuclein dysfunction for the etiology and pathogenesis of PD and DLB is demonstrated by the existence of familial forms of these diseases that are caused by missense mutations (A30P, E46K, and A53T) in the α-synuclein gene (*6*−*8*). Furthermore, duplications and triplications of the region on chromosome 4 that contains the α-synuclein gene also lead to familial PD/DLB, indicating that overproduction of the wild-type protein is sufficient to cause disease (*9*−*11*).

Recombinant α-synuclein is an unfolded protein in solution (*12*) which acquires an α-helical structure when it interacts with phospholipid membranes (*13*−*15*). Phospholipid binding has been linked to the neuroprotective role of α-synuclein (*16*). α-Synuclein has also been implicated in fatty acid uptake and metabolism (*17*−*20*). In line with these findings, dietary intake of polyunsatured fatty acids (PUFAs) has been found to result in a significant increase in the extent of α-synuclein gene expression in rat brain (*21*, *22*). In addition, α-synuclein inhibits phospholipase D activity both in vitro and in vivo, indicating a role in lipid-mediated signal transduction (*23*, *24*). An interaction between α-synuclein and lipids may also play a role in a pathophysiological context, since lipid membranes, artificial liposomes, and arachidonic acid (AA) have been found to promote the multimerization and oligomerization of α-synuclein (*25*−*28*). In addition, in a yeast model, genes involved in lipid metabolism were identified as enhancers of α-synuclein toxicity (*29*). Lewy bodies have also been reported to contain an as yet uncharacterized lipid component (*30*).

Docosahexaenoic acid (DHA, C22:6n-3) is an essential omega-3 PUFA that is abundant in brain (*31*). DHA and AA are the main fatty acids in gray matter phospholipids, where they account for 6% of the dry matter of the cerebral cortex. Both are dynamically released following phospholipid hydrolysis (*32*). DHA levels have been shown to be elevated in brain areas with α-synuclein inclusions from patients with PD and DLB (*33*), as well as in cerebral cortex in incidental Lewy body disease, prior to α-synuclein deposition (*34*). Despite this, little is known about the effects of DHA on α-synuclein. There is only one report suggesting that α-synuclein can oligomerize in response to DHA, as judged by SDS−PAGE (*28*). Here we have examined the interactions between α-synuclein and DHA using biophysical, biochemical, and morphological techniques. We report that DHA rapidly triggers an α-helical conformation in both recombinant and native α-synuclein. After a longer exposure to DHA, α-synuclein assembles into amyloid-like fibrils. Interestingly, α-synuclein strongly affects the micellar properties of DHA.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of Recombinant Human α-Synuclein.* Wild-type human α-synuclein was expressed and purified as described previously (*35*), but with minor modifications. Briefly, following bacterial lysis using a

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[1] Abbreviations: AA, arachidonic acid; αS, α-synuclein; CD, circular dichroism; CMC, critical micelle concentration; DHA, docosahexaenoic acid; DLB, dementia with Lewy bodies; $EC_{50}$, half-maximal effective concentration; PAGE, polyacrylamide gel electrophoresis; PD, Parkinson's disease; RFU, relative fluorescence units; $R_h$, apparent hydrodynamic radius; FA, fatty acid; PUFA, polyunsaturated fatty acid; SDS, sodium dodecyl sulfate; ThT, thioflavin T.

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French press, the protein was eluted with a 0 to 500 mM NaCl gradient using a 5 mL HiTrap QFF column (GE Healthcare) and further purified using a Superdex 200 Hiload 16/60 gel filtration column (GE Healthcare). Peak fractions were applied to a Mono Q column (GE Healthcare), and pure α-synuclein was eluted using a 0 to 500 mM NaCl gradient.

*Intrinsic Tyrosine Fluorescence.* Emission fluorescence spectra of α-synuclein solutions at 0.25 mg/mL in buffer A [100 mM NaCl and 20 mM HEPES (pH 7.2)] were obtained using a Fluoromax-2 spectrophotometer (Jobin Yvon-Spex) at 20 °C in the presence and absence of 200 μM fatty acids. The measurements were performed by using an excitation wavelength of 280 nm (1 nm band-pass) and recording the emission spectra from 290 to 400 nm (1 nm band-pass). All spectra were corrected for their corresponding protein-free sample.

*Circular Dichroism.* Far-UV CD spectra of 0.06 mg/mL α-synuclein in 5 mM PBS in the presence and absence of fatty acids were recorded using a Jasco J-810 spectropolarimeter (Jasco Corp.) at 20 °C in the spectral range from 190 to 260 nm with 0.2 nm resolution. Spectra were recorded as averages of five scans and at a scanning speed of 50 nm/min. The response time was 2.0 s with a bandwidth of 2.0 nm. Quartz cuvettes with an optical path of 1 mm were used. The spectra were obtained in triplicate and corrected for the corresponding protein-free sample.

*Analysis of α-Synuclein Assemblies.* α-Synuclein in buffer A was incubated in the presence or absence of fatty acids at 37 °C without agitation. The extent of aggregation was routinely determined by native PAGE on 12% Ready Gels (Bio-Rad). For transmission electron microscopy, 1 μL of protein sample was placed onto glow-discharged carbon-coated 400-mesh grids and stained with 1% potassium phosphotungstate (*36*). Micrographs were recorded on a Philips model EM208S microscope.

*Thioflavin T (ThT) Fluorescence.* Following incubation of α-synuclein in the presence or absence of fatty acids without shaking, the reaction mixtures were diluted 100-fold in buffer A containing 20 μM ThT. ThT fluorescence intensity was determined using a Fluoromax-2 spectrophotometer (Jobin Yvon-Spex) at 20 °C. The emission spectrum from 470 to 560 nm was recorded at an excitation wavelength of 450 nm with an integration time of 1 s and a slit width of 5 nm.

*Purification of Native, Rat Brain α-Synuclein.* Rat brain fractionation was performed as previously described (*37*, *38*). The total brain cytosol was obtained by homogenization of rat brain in 20 mM HEPES (pH 7.2) in a glass−Teflon Potter homogenizer followed by centrifugation of the homogenate for 1 h at 100000*g* to remove membrane material. An anti-synuclein immunoaffinity column was synthesized by attaching monoclonal antibody SYN204 (*39*) to BrCN-Sepharose beads (GE Healthcare, Amersham, U.K.) according to the manufacturer's instructions. Affinity chromatography was performed essentially as described previously (*40*). Brain synucleins in the pH 2.5 eluate were concentrated and further purified on a Superdex 200 size-exclusion column (GE Healthcare, Amersham, U.K.). SDS−PAGE was carried out using 12% Bio-Rad Ready Gels. Western immunoblotting was performed using a rabbit anti-synuclein serum (*35*).

*Photon Correlation Spectroscopy and Turbidity Measurements.* Photon correlation spectroscopy measurements were performed using a multi-tau correlator and a Beckman Coulter N4Plus particle size analyzer with a 10 mW He−Ne laser at a wavelength of 633 nm. Particle sizes were typically obtained at an angle of 90° with a run time of 120 s. Turbidity measurements were performed at 350 nm and 25 °C using a UV-1601 Shimadzu spectrophotometer. First, a baseline was obtained for a 200 μM fatty acid in protein-free buffer A followed by addition of α-synuclein to a final concentration of 50 μg/mL.

## RESULTS

*Docosahexaenoic Acid Rapidly Changes the Conformation of α-Synuclein.* In solution, recombinant α-synuclein behaves as an unfolded protein (*12*). We hypothesized that the proposed involvement of α-synuclein in lipid-related functions may require the protein to adopt a variety of structures depending on the availability of binding partners. First, we used intrinsic protein fluorescence to follow conformational changes in the presence of DHA C22:6, AA C20:4, and two saturated fatty acids (palmitic acid C16:0 and arachidic acid C20:0). Since α-synuclein does not contain tryptophans, its intrinsic fluorescence is due to tyrosine residues. We measured the fluorescence emission within 1 min of fatty acid addition (200 μM). Figure 1A shows that the two PUFAs, particularly DHA, triggered a major reduction in α-synuclein fluorescence, suggesting a conformational change. In contrast, neither palmitic nor arachidic acid changed the fluorescence signal of α-synuclein.

We next analyzed the effect of DHA on α-synuclein by far-UV circular dichroism (CD). The recombinant protein exhibited an unordered conformation in the absence of fatty acids or in the presence of saturated palmitic and arachidic acids, with a local minimum at 198 nm (Figure 1B). Remarkably, both DHA and AA triggered, within 10 min, a large conformational change in α-synuclein, resulting in α-helical structure which was apparent by two local minima at 222 and 208 nm. Analyses of the obtained spectra using a CDsstr algorithm (*41*) and DichroWeb (*42*) showed that the helical content of α-synuclein rose to 60% upon addition of either PUFA. Titration experiments revealed that DHA and AA caused the conformational change in α-synuclein in the micromolar range, the $EC_{50}$ values for the two being 50 and 75 μM, respectively (Figure 1C). Addition of DHA at concentrations higher than 100 μM did not result in any further increase in helicity.

*α-Synuclein Affects the Micellar Properties of Polyunsaturated Fatty Acids.* Since we detected a conformational transition in α-synuclein at concentrations where DHA and AA may form micelles, we investigated micellar behavior in detail. First, we employed dynamic light scattering, based on the principle of photon correlation spectroscopy, to estimate micellar size (*43*, *44*). The initial concentration at which DHA formed micelles was 40 μM (Figure 2A, top panel). However, addition of α-synuclein led to a noticeable shift, increasing the critical micelle concentration to approximately 80 μM. Furthermore, at higher DHA concentrations, the presence of α-synuclein was sufficient to reduce the micellar size of DHA. We obtained similar results for AA with a shift of the critical micelle concentration from 60 to 100 μM (Figure 2A, bottom panel), indicating that α-synuclein binds PUFAs and disperses their micelles, suggesting a possible protective function in a cellular environment.

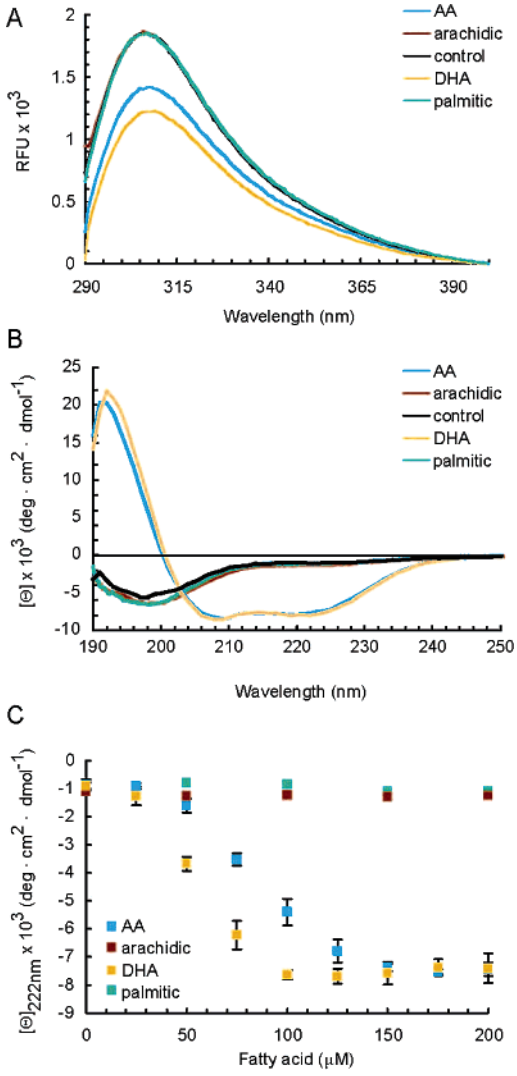


FIGURE 1: Docosahexaenoic acid and arachidonic acid convert unstructured α-synuclein into an α-helical protein. (A) DHA and AA induce a significant decrease in tyrosine intrinsic fluorescence, suggesting a conformational change in α-synuclein. Saturated palmitic and arachidic acids do not affect the α-synuclein intrinsic fluorescence. All fatty acids are at 200 $\mu$M. (B) DHA and AA trigger conversion of α-synuclein from a predominantly random coil to an α-helical conformation, as evidenced by CD spectroscopy. Saturated palmitic acid and arachidic acid do not affect α-synuclein structural properties. All fatty acids are at 200 $\mu$M, i.e., exceeding their critical micelle concentrations. (C) Titration experiment showing that the half-maximal effective concentrations of DHA and AA for inducing α-synuclein α-helical conformation are 50 and 75 $\mu$M, respectively. Palmitic acid and arachidic acid were used as negative controls in this CD experiment.

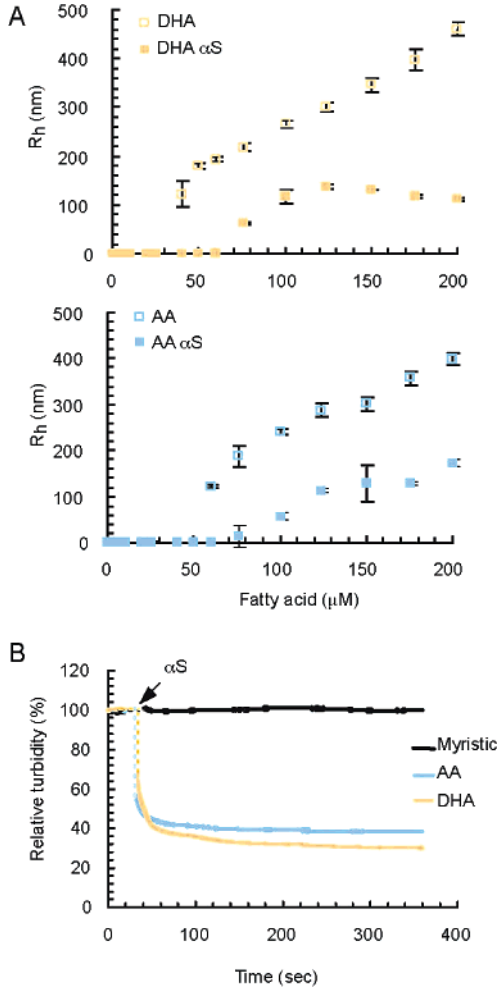


FIGURE 2: α-Synuclein disrupts micelle formation of docosahexaenoic acid and arachidonic acid. (A) The apparent hydrodynamic micelle radius, $R_h$, of DHA (top panel) and AA (bottom panel) was measured by dynamic light scattering. α-Synuclein (50 $\mu$g/mL) approximately doubles the critical micelle concentration for both DHA and AA. At high DHA and AA concentrations, α-synuclein decreases the micellar size. (B) α-Synuclein (50 $\mu$g/mL) clears DHA and AA micellar solutions, whereas a micellar solution of saturated myristic acid remains turbid. All fatty acids are at 200 $\mu$M. The turbidity was measured at 350 nm.

The presence of micelles can be monitored in solution by turbidity measurements (*45*), providing a simple spectrophotometric approach for kinetics analysis. Figure 2B shows that solutions containing 200 $\mu$M DHA or AA were rapidly cleared following addition of α-synuclein. In contrast, α-synuclein did not affect micelles made of saturated myristic acid (C14:0), again highlighting the link between α-synuclein function and essential PUFAs.

*Fibrillation of α-Synuclein following Prolonged Exposure to Docosahexaenoic Acid.* Next, we investigated whether prolonged exposure to DHA would promote the assembly of α-synuclein into amyloid-like fibrils. We incubated α-synuclein for 20 h at 37 °C, without agitation, in the presence or absence of a selection of fatty acids. First, we analyzed α-synuclein aggregation by native gel electrophoresis (Figure 3A). Interestingly, DHA had the strongest aggregating effect on α-synuclein at two different fatty acid concentrations, as evidenced by the appearance of protein material at the bottom of the loading wells. AA also aggregated α-synuclein, whereas saturated fatty acids were without effect. We then tested α-synuclein aggregation using thioflavin T (ThT) fluorescence, a widely used technique for assessing fibril formation (*46*). We detected major changes in ThT fluorescence following incubation of α-synuclein with DHA or AA, each at 100 $\mu$M (Figure 3B). In control experiments, where no fatty acids were present, only a slight enhancement of the ThT fluorescence signal was noticeable after incubation for 4 days at 37 °C. To define the kinetics of α-synuclein aggregation more precisely, we employed dynamic light scattering to follow fibrillation by particle size measurement. The detectable increase in the size of α-synuclein material occurred after incubation for approximately 5 h with 75 $\mu$M DHA, whereas in the case of AA,

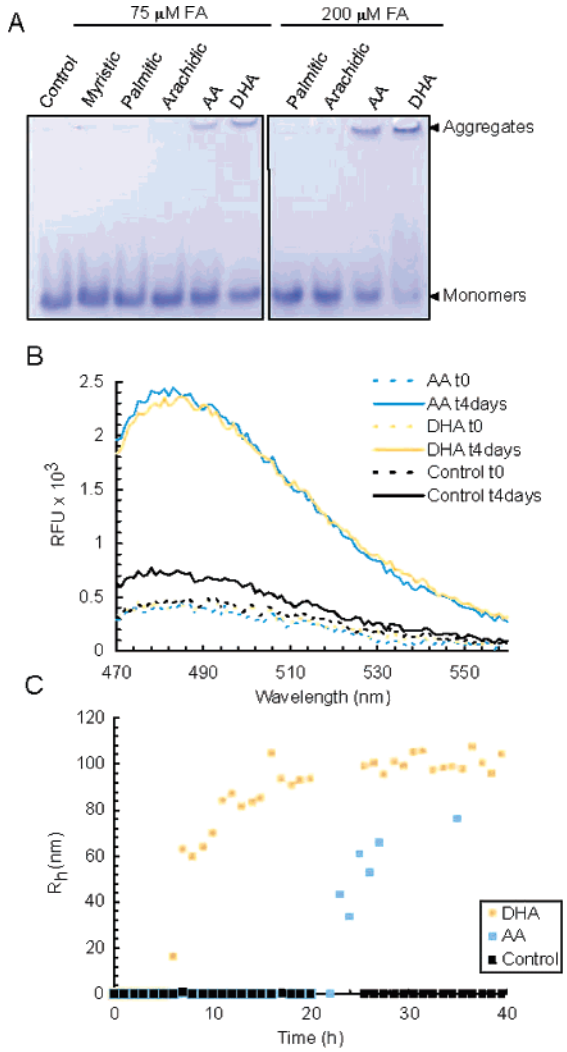


FIGURE 3: Prolonged exposure to docosahexaenoic acid and arachidonic acid leads to α-synuclein aggregation. (A) Native gel electrophoresis was used as a convenient screening method to detect α-synuclein aggregation following a 20 h exposure to the indicated fatty acids (FA). Myristic, palmitic, and arachidic acids are saturated fatty acids C14, C16, and C20, respectively. Aggregation is evidenced by the appearance of protein material at the bottom of the loading wells. Gels were stained with Coomassie Blue. (B) An increase in ThT fluorescence suggests $\beta$-sheet-rich fibril formation by α-synuclein following a 4 day exposure to DHA and AA, both at 200 $\mu$M. (C) Kinetic measurements of α-synuclein aggregation reveal a faster aggregation in the presence of DHA as compared to AA, each at 75 $\mu$M. The particle hydrodynamic radius, $R_h$, was measured by dynamic light scattering.

α-synuclein aggregation required at least 20 h (Figure 3C). Dynamic light scattering confirmed that in the absence of free DHA or AA α-synuclein did not aggregate in the time frame of the experiment. To investigate the morphology of PUFA-triggered α-synuclein aggregates, we analyzed the reactions by transmission electron microscopy. Both DHA and AA caused α-synuclein to form fibrils (Figure 4A), whereas in the control reactions, no fibril material could be seen (Figure 4B).

To study the native protein, we developed a rapid and reproducible method for isolation of brain α-synuclein. We covalently attached a monoclonal anti-synuclein antibody to BrCN-Sepharose beads and employed immunoaffinity chromatography to purify rat brain α-synuclein. Since synucleins are mainly present in the cytosolic fraction (Figure 5A), we

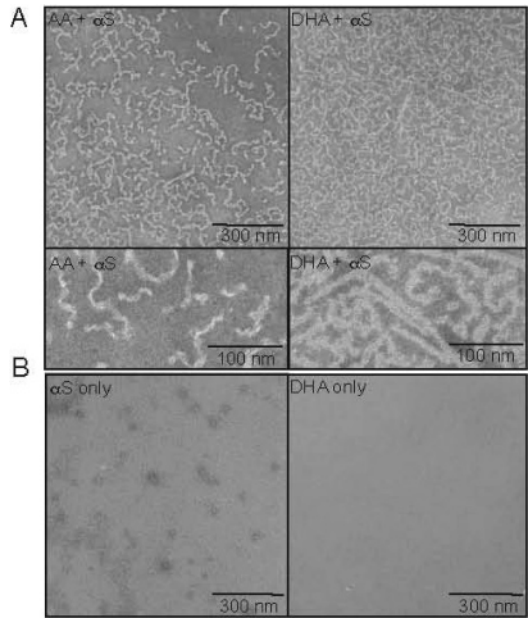


FIGURE 4: Transmission electron microscopy of α-synuclein assemblies. (A) Incubation of 100 $\mu$M α-synuclein in the presence of DHA or AA, each at 100 $\mu$M, leads to formation of α-synuclein fibrils. (B) No fibril material can be detected in α-synuclein only or DHA only samples.

used total brain cytosol as the starting material. The affinity column extracted two synucleins, α and $\beta$, confirmed by both mass spectrometry (data not shown) and Western immunoblotting. The apparent absence of any further protein bands in the eluted fraction (Figure 5B) suggests that the two synucleins are unlikely to interact constitutively with other cytosolic proteins, in agreement with a lipid-related synuclein function. Western immunoblotting of the loading and flow-through material confirmed that extraction of both isoforms was total (Figure 5B). The isolation of synucleins was highly reproducible, allowing us to accumulate the proteins for further experimentation. We first investigated the conformational properties of brain-derived synucleins by far-UV CD. Similar to the recombinant proteins, brain synucleins, in the absence of fatty acids, exhibited spectral properties characteristic of unordered structure (Figure 5C). In the presence of AA and DHA, however, a clear conformational change took place, indicating transition into an α-helical state. Synucleins at a concentration of 25 $\mu$M were then incubated for 20 h in the presence of 100 $\mu$M DHA or AA at 37 °C. Sensitive Sypro Ruby protein staining following native gel electrophoresis revealed that DHA, but not AA, efficiently aggregated brain α-synuclein (Figure 5D).

## DISCUSSION

In this study, we demonstrate that (i) α-synuclein rapidly responds to free DHA and AA by an increase in α-helical content, (ii) α-synuclein can counteract the natural tendency of DHA and AA to form micelles, and (iii) long exposure to free DHA, and to a lesser degree AA, gradually leads to the assembly of α-synuclein into fibrils. Our results suggest that DHA and AA, when liberated through phospholipase action, could lead to fibrillation of α-synuclein away from the phospholipid membranes.

The importance of DHA for brain function is well-known (*47*), and recent evidence suggests its involvement in the

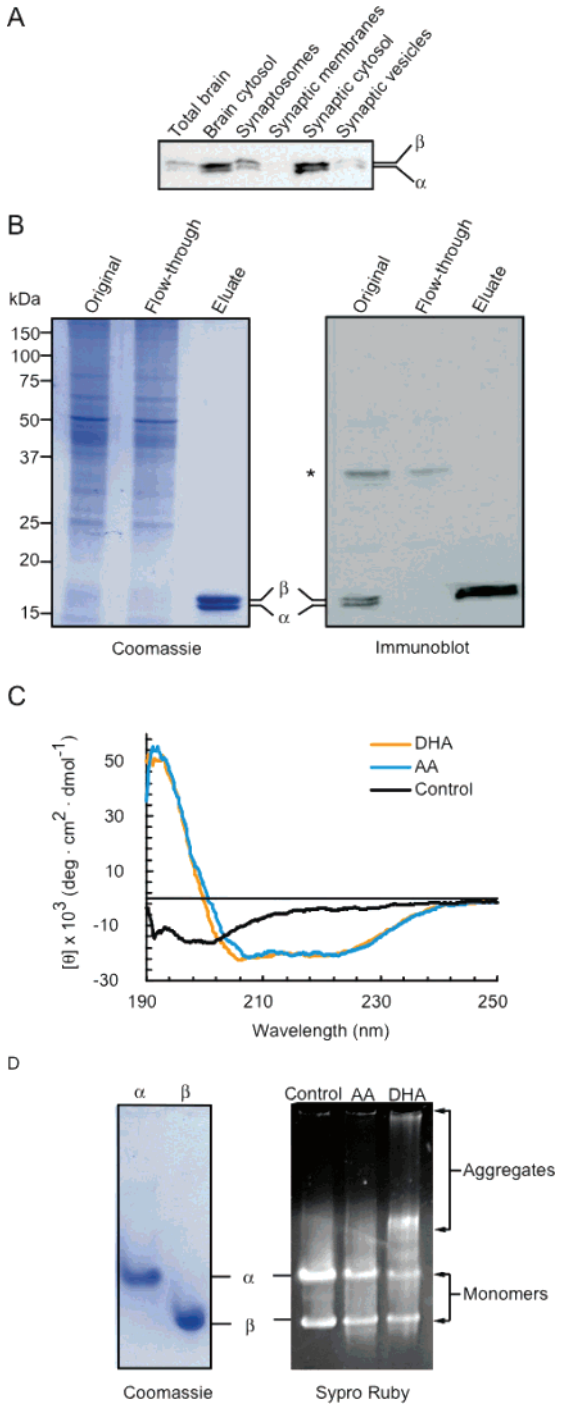


FIGURE 5: Action of docosahexaenoic acid and arachidonic acid on native synucleins. (A) Western immunoblotting of indicated rat brain fractions shows enrichment of α- and *β*-synucleins in cytosolic fractions. A rabbit anti-synuclein antibody was used in this experiment. (B) Immunoaffinity chromatography of brain synucleins using Sepharose-linked monoclonal antibody SYN204. Coomassie-stained gel (left panel). The immunoaffinity column fully extracts both α- and *β*-isoforms as evidenced by immunoblotting of the original load and flow-through material using the rabbit anti-synuclein antibody (right panel). The asterisk indicates a nonspecific band recognized by the rabbit anti-synuclein antibody. (C) Exposure of brain-purified synucleins to DHA or AA, each at 200 *μ*M, triggers the transition from the unstructured to α-helical conformation as measured by CD. (D) DHA causes brain synuclein aggregation. A native gel, stained with Sypro Ruby, shows synuclein aggregation (right panel). Note that AA is less effective. Differential electrophoretic mobility of individual recombinant α- and *β*-synucleins, in the absence of fatty acids, is evidenced in the Coomassie-stained native gel (left panel).

regulation of α-synuclein levels (*21*, *48*). Of the two most abundant PUFAs, liberated DHA may be present for a longer period in the cytosol while AA is rapidly modified by multiple enzymes into prostaglandins, thromboxanes, and leukotrienes (*49*). Importantly, mobilization of free DHA and AA has been reported during oxidative stress, which is well-known to take place in PD (*32*, *34*, *50*). Furthermore, an increase in the level of DHA has been observed in the early stages of PD neuropathology (*34*). Although a number of studies have suggested a lipid-related function for α-synuclein, the exact meaning of the α-synuclein−lipid interplay remains obscure. The increase in α-helical content in the presence of AA and DHA is indicative of their direct interaction with α-synuclein, in line with microcalorimetry binding data of Golovko et al. (*20*). Our results show, for the first time, that α-synuclein acts to disperse micelles made of the omega-3 and omega-6 polyunsaturated fatty acids DHA and AA. The micelle disruption can be explained by α-synuclein-mediated sequestration of monomeric fatty acids. Such a protective function may be useful during increased phospholipase activity in metabolic stress and/or injury or upon lipid uptake in the brain. The abundance of DHA and AA in brain phospholipids suggests that, upon local phospholipase-induced release, their concentrations can rapidly become micromolar, reaching micellar threshold levels (*21*, *49*). For example, the transient intracellular concentration of AA in pancreatic cells was shown to approach 75 *μ*M and may be even higher in specialized neuronal subcompartments (*51*−*53*).

Our results for the effect of α-synuclein on micellar properties contrast with those obtained by Necula et al. (*54*), who reported that α-synuclein promotes micelle formation in the case of AA. However, the authors relied on a single fluorometric method to assess micelle formation and did not account for the fluorescence appearing upon interaction of their probe, *N*-phenyl-1-naphthylamine, with α-synuclein (data not shown). In contrast, we employed a direct measurement of micellar size by photon correlation spectroscopy and verified our conclusions using another direct approach, determination of solution turbidity. The critical micelle concentration for AA determined in our study (60 *μ*M) is very close to that reported by two independent groups (*55*, *56*). Necula et al. (*54*) also utilized glutaraldehyde cross-linking to detect α-synuclein fibrillation. Here we showed, for the first time, that both DHA and AA can trigger formation of α-synuclein fibrils without any chemical cross-linking.

It is possible that while α-synuclein acts to disperse DHA micelles, upon prolonged exposure α-synuclein may eventually aggregate. Whereas unfolded α-synuclein did not assemble into amyloid-like fibrils following week-long incubations (data not shown), it did so readily (within hours) after addition of DHA. Upon interaction with the micellar PUFA, α-synuclein quickly acquired an α-helical conformation. Upon further incubation with DHA or AA, α-synuclein forms characteristic amyloid-like fibrils, indicating that acquisition of α-helical properties may be an intermediate on the pathway to fibrillation. Previously, an α-helical conformation of α-synuclein was detected in the presence of negatively charged liposomes or organic solvents, and it was suggested that such a partially folded membrane-bound form may act as a "seed" for aggregation (*26*, *57*). Our results

show that brain-enriched DHA forces α-synuclein to adopt an α-helical conformation in the absence of phospholipid bilayers. Intriguingly, solutions that promote helical structure of A$\beta$ and islet amyloid polypeptide also accelerate their fibrillogenesis (*58*−*61*). Furthermore, free fatty acids were shown to induce fibril formation of both A$\beta$ and tau (*62*), although with a specificity lower than that with α-synuclein. The relatively fast conversion, in the presence of DHA, of monomeric α-synuclein into insoluble assemblies could now be utilized for rapid screening to identify drugs that block α-synuclein fibrillation.

Several techniques employed here will facilitate future studies. Native gel electrophoresis provides a quick readout of α-synuclein aggregation and is more sensitive than the previously used SDS−PAGE/Western immunoblotting method. Measuring tyrosine fluorescence offers a useful way of screening compounds that may affect α-synuclein structure, while photon correlation spectroscopy and turbidity measurements provide two independent methods for studying the effects of α-synuclein on micelles. Finally, the rapid, reproducible one-step isolation of brain synucleins provides a source of native protein for future studies of native α-synuclein function and its post-translational modifications.

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