Soluble, Prefibrillar α-Synuclein Oligomers Promote Complex I-dependent, Ca\(^{2+}\)-induced Mitochondrial Dysfunction*

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Eric S. Luth‡, Irina G. Stavrovskaya§, Tim Bartels†, Bruce S. Kristal†, and Dennis J. Selkoe†‡
From the‡Center for Neurologic Diseases, Department of Neurology, and §Department of Neurosurgery, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

Background: Mitochondrial dysfunction and aggregation of α-synuclein both contribute to Parkinson disease.
Results: Prefibrillar α-synuclein oligomers reduce the Ca\(^{2+}\) retention time of isolated mitochondria respiring with complex I but not II substrates.
Conclusion: Oligomeric α-synuclein promotes mitochondrial dysfunction in a Ca\(^{2+}\)- and respiratory substrate-dependent manner.
Significance: The Ca\(^{2+}\)-dependence of α-synuclein’s effects may contribute to selective neuronal vulnerability in Parkinson disease.

α-Synuclein (αSyn) aggregation and mitochondrial dysfunction both contribute to the pathogenesis of Parkinson disease (PD). Although recent studies have suggested that mitochondrial association of αSyn may disrupt mitochondrial function, it is unclear what aggregation state of αSyn is most damaging to mitochondria and what conditions promote or inhibit the effect of toxic αSyn species. Because the neuronal populations most vulnerable in PD are characterized by large cytosolic Ca\(^{2+}\) oscillations that burden mitochondria, we examined mitochondrial Ca\(^{2+}\) stress in an in vitro system comprising isolated mitochondria and purified recombinant human αSyn in various aggregation states. Using fluormetry to simultaneously measure four mitochondrial parameters, we observed that soluble, prefibrillar αSyn oligomers, but not monomeric or fibrillar αSyn, decreased the retention time of exogenously added Ca\(^{2+}\), promoted Ca\(^{2+}\)-induced mitochondrial swelling and depolarization, and accelerated cytochrome c release. Inhibition of the permeability transition pore rescued these αSyn-induced changes in mitochondrial parameters. Interestingly, the mitotoxic effects of αSyn were specifically dependent upon both electron flow through complex I and mitochondrial uptake of exogenous Ca\(^{2+}\). Our results suggest that soluble prefibrillar αSyn oligomers recapitulate several mitochondrial phenotypes previously observed in animal and cell models of PD: complex I dysfunction, altered membrane potential, disrupted Ca\(^{2+}\) homeostasis, and enhanced cytochrome c release. These data reveal how the association of oligomeric αSyn with mitochondria can be detrimental to the function of cells with high Ca\(^{2+}\)-handling requirements.

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† To whom correspondence should be addressed: Center for Neurologic Diseases, Harvard Institutes of Medicine, 77 Ave. Louis Pasteur, Boston, MA 02115. Tel.: 617-525-5200; Fax: 617-525-5252; E-mail: dselkoe@rics.bwh.harvard.edu.
‡ The abbreviations used are: PD, Parkinson disease; αSyn, α-synuclein; ETC, electron transport chain; ThT, thioflavin T; TMRM, tetramethylrhodamine methyl ester; CRT, Ca\(^{2+}\) retention time; mPTP, mitochondrial permeability transition pore; ΔΨ\(_{\text{m}}\), mitochondrial membrane potential; CsA, cyclosporin A; ROS, reactive oxygen species; CRC, Ca\(^{2+}\) retention capacity; normally soluble cytoplasmic protein α-synuclein (αSyn) into insoluble amyloid fibrils that accumulate as cytoplasmic aggregates termed Lewy bodies and Lewy neurites (1). Duplication or triplication of the wild-type αSyn locus causes gene dose-dependent early onset PD (2, 3), and five pathogenic missense mutations have been reported to cause autosomal dominant PD (4). Moreover, 3’-UTR variants and other single nucleotide polymorphisms in the SNCA gene that lead to increased αSyn expression are found in a subset of cases of sporadic PD (5). In addition, mutations in glucocerebrosidase (GBA1), the most common genetic risk factor for PD, can elevate αSyn protein levels, perhaps via reduced lysosomal degradation (6). Thus, impaired proteostasis of αSyn is probably a key step in the pathogenesis of PD and related human synucleinopathies.

A growing body of evidence suggests that αSyn normally exists in an equilibrium between partially helical tetramers and unfolded monomers (7–10). Helical, oligomeric αSyn is aggregation-resistant in vitro (7), whereas the unfolded monomer readily aggregates into insoluble, β-sheet-rich amyloid-type fibrils through soluble oligomeric intermediates (11). Recent studies indicate that these abnormal soluble oligomers, in contrast to the fibrillar end products, are neurotoxic in vitro and in disease models (12–15). The pathological mechanisms of these toxic oligomeric intermediates and the basis for the selective vulnerability of certain brain regions to their effects are as yet undetermined.

Many observations suggest that mitochondrial dysfunction is associated with PD. Toxins targeting complex I of the electron transport chain (ETC) can cause parkinsonism in humans and animal models (16–18), and postmortem brain tissue of PD patients shows deficits in mitochondrial complex I activity (19–21). Markers of mitochondrial oxidative stress, including oxidized complex I subunits (22) and mitochondrial DNA mutations (23), are also elevated in PD patients, although it is unclear whether these are a cause or consequence of ETC dysfunction. The neuronal populations most impaired in PD, including the substantia nigra pars compacta, locus ceruleus, and dorsal motor nucleus of the vagus, share an unusual phys-

ANOVA, analysis of variance; AFU, arbitrary fluorescence units; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
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...iological phenotype; they consist primarily of broad-spike pacemaking neurons with high transmembrane Ca²⁺ currents and low Ca²⁺ buffering capacities (24). This combination places a great metabolic burden on mitochondria to continually reestablish the resting cytosolic Ca²⁺ concentration. Mitochondria-targeted, redox-sensitive GFP reveals a more oxidative mitochondrial environment in these neurons relative to those of regions less affected in PD (25, 26). The basal level of oxidative stress in the mitochondria of vulnerable neurons may put them at risk of dysfunction caused by an additional stressor.

alpha-Syn may associate abnormally with mitochondria in PD patients and animal models (27–33). An apparent partial subcellular redistribution of alpha-Syn from the cytoplasm to the inner and outer mitochondrial membranes (27, 34, 35) is correlated with mitochondrial dysfunction, including increased oxidative stress, reduced mitochondrial membrane potential (ΔΨm), altered Ca²⁺ homeostasis, and cytochrome c release (27, 29–31, 33, 36, 37). Few studies have directly investigated the effect of different forms of alpha-Syn on mitochondrial function. One reported that incubation of isolated mitochondria with aggregated alpha-Syn can increase markers of oxidative stress, but the aggregation state (fibrillar or oligomeric) was not thoroughly characterized (36). No previous studies have tested the role of mitochondrial Ca²⁺ stress or specific respiratory substrates in the ability of diverse alpha-Syn species to induce mitochondrial dysfunction. It therefore remains unclear what form of alpha-Syn is most damaging to mitochondrial function and which conditions promote or inhibit the effect of toxic alpha-Syn species.

We sought to investigate the functional consequences of alpha-Syn on Ca²⁺-challenged mitochondria, with a particular focus on the alpha-Syn aggregation state. We modeled mitochondrial Ca²⁺ stress in an in vitro system comprising isolated mitochondria from liver and brain and pure, recombinant human alpha-Syn at various aggregation states. Soluble, oligomeric alpha-Syn aggregates generated by two independent methods, but not monomeric alpha-Syn or mature fibrils, sensitized mitochondria to Ca²⁺-induced dysfunction. The effects of oligomeric alpha-Syn were observed only when mitochondria were respiring under complex I-dependent conditions and were challenged with exogenous Ca²⁺ addition. Our results demonstrate a specific effect of oligomeric alpha-Syn on complex I-dependent function and reveal a mechanism by which the physical association of alpha-Syn with mitochondria impairs their function.

EXPERIMENTAL PROCEDURES

Purification and Preparation of alpha-Syn Species

Purification—Recombinant human alpha-Syn was purified essentially as described (38). Briefly, E. coli transformed with human wild type alpha-Syn were grown to A600 = 0.5–1, at which time isopropyl 1-thio-β-D-galactopyranoside was added to 1 mM to induce alpha-Syn expression. At A600 = 1.5–1.8, bacteria were pelleted by centrifugation and boiled in anion exchange buffer (20 mM Tris, 25 mM NaCl, pH 8.0). Boiled bacterial lysate was purified sequentially by anion exchange chromatography (using two 5-ml HiTrap Q HP columns, GE Healthcare) and size exclusion chromatography (using a Superdex 200 XK26/00 column, GE Healthcare). Protein not used immediately was lyophilized and stored at 4 °C. Lyophilized protein was reconstituted either in PBS or 10 mM ammonium acetate, depending on the method used to prepare oligomers (see below).

Lag Phase Preparation—0.6 mg/ml recombinant alpha-Syn in 10 mM ammonium acetate was incubated at 37 °C with nutation. The aggregation state was monitored using Thioflavin T (ThT) fluorescence (see below). “Unaged” alpha-Syn was sampled at time 0 (prior to 37 °C incubation or nutation). alpha-Syn aged 3–9 days without ThT fluorescence above background was considered “ThTneg”; alpha-Syn that had plateaued in ThT fluorescence was considered “ThTpos.”

Sonicated Preparation—2 mg/ml recombinant alpha-Syn in PBS was aggregated for 5 days at 37 °C with nutation to form ThTpos fibrils. To generate oligomers, alpha-Syn fibrils were diluted to 1 mg/ml and sonicated at power level 50 for 5 × 10 s using a Sonic Dismembrator model 300 (Fisher). Aliquots of the resultant material were flash-frozen in liquid nitrogen and stored at −80 °C. Fractions of this material were prepared by serial differential centrifugation, first at 16,000 × g for 5 min. The supernatant was transferred to a new tube, and the pellet was resuspended in an equal volume of PBS. The 16,000 × g supernatant was then spun at 100,000 × g for 30 min at 4 °C. The supernatant was transferred to a new tube, and the pellet was resuspended in an equal volume of PBS. The use of a programmable Ultrasonic Liquid Processor (Misonix) equipped with a microtip (settings: amplitude of 20, sonication for 1 s on and 1 s off for a total of 60 s) also produced alpha-Syn oligomers with bioactivity on mitochondria comparable with the bioactivity of oligomers produced via our standard manual sonication technique. For both sonicated and lag phase preparations, concentrations of fibrillar and oligomeric alpha-Syn listed are estimated based on the monomer concentration before aggregation as determined by A280 using a NanoDrop spectrophotometer (Thermo Scientific).

Electron Microscopy

alpha-Syn fibrils, total sonicated alpha-Syn, and 100,000 × g soluble sonicated alpha-Syn were each diluted 1:10 in PBS. 5 µl of these alpha-Syn samples were adsorbed for 1 min to a carbon-coated grid that had been made hydrophilic by a 30-s exposure to a glow discharge. Excess liquid was removed with filter paper (Whatman), and the samples were stained with 0.75% uranyl formate for 30 s. After removing the excess uranyl formate with filter paper, the grids were examined using a TecnaiG2 Spirit BioTWIN transmission electron microscope. Images were acquired with an AMT 2k CCD camera.

Aliquots of mitochondrial suspensions (see below) were spun at 14,000 × g for 5 min to pellet mitochondria. Mitochondrial pellets were fixed in 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) for at least 2 h at room temperature and then washed in 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide (OsO4), 1.5% potassium ferrocyanide (KFeCN6) for 1 h. Samples were then washed three times in H2O and incubated in 1% aqueous uranyl acetate for 1 h followed by two washes in H2O and subsequent dehydration in grades of alcohol (10 min each: 50%, 70%, 90%; twice for 10 min each: 100%). The samples were then put in propylene oxide for
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1 h and infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). The following day, the samples were embedded in TAAB Epon and polymerized at 60 °C for 48 h. Ultrathin sections (~60 nm) were cut on a Reichert Ultracut-S microtome and transferred onto copper grids stained with lead citrate. Sections were examined in a JEOL 1200EX Transmission electron microscope or a TecnaiG2 Spirit BioTWIN, and images were recorded with an AMT 2k CCD camera. Measurements of mitochondrial diameter were obtained using ImageJ (National Institutes of Health).

**Dynamic Light Scattering**

Experiments were performed using a DynaPro (Wyatt Technology) instrument equipped with a 20 °C temperature-controlled microsample. \( \alpha \)Syn samples in PBS were placed in a 1.5-mm path length quartz cuvette, and light scattering was measured in 10-s intervals for 20 cycles. Data were analyzed using Dynamic version 5 software.

**Thioflavin T Assays**

2.5 \( \mu \)M \( \alpha \)Syn at various aggregation states (i.e. unaged/monomer, fibrils, sonicated oligomers and fractions thereof, and lag phase samples) was added in triplicate to 10 \( \mu \)M ThT in 10 mM glycine buffer, pH 9, to a total of 200 \( \mu \)L. Fluorescence at excitation/emission of 447/485 nm was measured in a black 96-well plate using the Synergy H1 Hybrid Reader (BioTek). Background fluorescence of buffer (PBS or 10 mM ammonium acetate) was subtracted from \( \alpha \)Syn-containing samples. For aggregation seeding assays, 100 \( \mu \)L of 7–20 \( \mu \)M monomeric \( \alpha \)Syn in PBS plus 1 mol % of \( \alpha \)Syn seeds at various aggregation states and 10 \( \mu \)M ThT were added in triplicate to a black 96-well plate. Plates were incubated at 37 °C under constant agitation at 300 rpm using a titer plate shaker (Lab Line Instruments). ThT fluorescence at excitation/emission of 447/485 nm was measured periodically as above.

**Mitochondrial Isolation**

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. All the measurements of these parameters were performed simultaneously on a multichannel dye fluorimeter (C&L Instruments, Inc.) as described previously (39, 40). Liver mitochondria were isolated from \( \alpha \)Syn-containing samples. For aggregation seeding assays, 100 \( \mu \)L of 7–20 \( \mu \)M monomeric \( \alpha \)Syn in PBS plus 1 mol % of \( \alpha \)Syn seeds at various aggregation states and 10 \( \mu \)M ThT were added in triplicate to a black 96-well plate. Plates were incubated at 37 °C under constant agitation at 300 rpm using a titer plate shaker (Lab Line Instruments). ThT fluorescence at excitation/emission of 447/485 nm was measured periodically as above.

**Mitochondrial Isolation**

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. All procedures for animal use and euthanasia were approved by the institutional animal care and use committee. Liver mitochondria were isolated from 11–13-week-old mice by the standard differential centrifugation method in sucrose-based buffers as described previously (39, 40). Liver mitochondria were incubated in buffer containing 240 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM K\(^+\)-EGTA, and 0.5% fatty acid-free bovine serum albumin (BSA) and centrifuged at 12,000 \( \times \) g for 8 min. The supernatant was then centrifuged again for 3 min at 12,000 \( \times \) g. The pellet from this spin was resuspended in 5 ml of 320 mM sucrose, 10 mM K\(^+\)-HEPES, pH 7.4, 1 mM K\(^+\)-EGTA buffer, layered on Ficoll gradient (7.5%/10%), and centrifuged again for 12 min at 100,000 \( \times \) g. The supernatant was resuspended in 0.05 ml of 240 mM sucrose, 10 mM K\(^+\)-HEPES, pH 7.4.

**Measurement of Mitochondrial Ca\(^{2+} \) Uptake Capacity, Membrane Potential, NAD(P)H Oxidation, and Swelling**

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. All the procedures for animal use and euthanasia were approved by the institutional animal care and use committee. Liver mitochondria were isolated from 11–13-week-old mice by the standard differential centrifugation method in sucrose-based buffers as described previously (39, 40). Liver mitochondria were incubated in buffer containing 240 mM sucrose, 10 mM HEPES, pH 7.2, 1 mM KH\(_2\)PO\(_4\), 3 \( \mu \)M EDTA, and either 5 mM glutamate/malate or 5 mM succinate plus 1 \( \mu \)M rotenone and were used at a concentration of 0.25 mg of mitochondrial protein/ml. Changes to mitochondrial membrane potential (\( \Delta \Psi _{m} \)) were estimated by measuring changes in the fluorescence intensity of tetramethylrhodamine methyl ester (TMRM) (60 nm) (Invitrogen) at excitation and emission wavelengths of 543 and 590 nm, respectively. Mitochondrial Ca\(^{2+} \) flux was measured as change in extramitochondrial Ca\(^{2+} \) concentration, measured by fluorescence of CaGreen-5N (125 nm) (Invitrogen) at excitation and emission wavelengths of 482 and 535 nm, respectively. The redox state of pyridine nucleotides in the mitochondrial suspension was followed by monitoring NAD(P)H autofluorescence at excitation and emission wavelengths of 350 and 450 nm, respectively. Mitochondrial swelling was measured by light scattering at a wavelength of 587 nm. Mitochondria were challenged by single Ca\(^{2+} \) additions of 20–40 nmol Ca\(^{2+} \)/mg mitochondrial protein. Mitochondrial Ca\(^{2+} \) retention time (CRT) was defined as the time between the Ca\(^{2+} \) addition and the plateau in CaGreen-5N fluorescence. To induce maximal swelling, the nonspecific pore-forming agent alamethicin was added to a final concentration of 2.5 \( \mu \)M at the conclusion of each run.

**Measurements of Ca\(^{2+} \) retention capacity (CRC) in isolated brain mitochondria in the presence or absence of \( \alpha \)Syn were achieved by incubating mitochondria in buffer containing 100 mM sucrose, 75 mM KCl, 10 mM HEPES, pH 7.4, 3 \( \mu \)M EDTA, 2 mM KH\(_2\)PO\(_4\), 150 \( \mu \)M ATP, 150 \( \mu \)M MgCl\(_2\). Mitochondrial
parameters were measured as mentioned above for liver mitochondria. Mitochondria were challenged with multiple Ca$^{2+}$ additions of 10 nmol Ca$^{2+}$/mg mitochondrial protein. CRC was defined as the maximum amount of Ca$^{2+}$ that could be completely buffered by mitochondria as measured by the return to baseline fluorescence. Fluorimeter data were analyzed using Origin version 8.0 (OriginLab) software. The specific conditions of each experiment (i.e., substrates, inhibitors, concentration of Ca$^{2+}$ or other mitochondrial permeability transition pore (mPTP) inducers, and αSyn species) are noted throughout. CRT and CRC were normalized to vehicle control.

**Assessment of Mitochondrial Cytochrome c Release**

Mitochondria were incubated as described for fluorescence measurements of mitochondrial parameters. In all cases, glutamate/malate and αSyn were added to the mitochondrial suspension. 20 μM Ca$^{2+}$ or an equivalent volume of water was added 2 min after αSyn, and after an additional 5 min, 200-μl aliquots of the suspension were removed. Aliquots were spun at 8,000 × g for 10 min to pellet mitochondria, and the supernatant was removed. Mitochondrial pellets were washed four times in 50 μl of assay buffer without substrates, after which they were lysed with 50 μl of 1% Nonidet P-40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% (v/v) Nonidet P-40, complete mini-EDTA-free protease inhibitor tablet) for 25 min on ice. Lysed mitochondria were spun for 5 min at 6,000 × g, and the supernatant was collected. The supernatants of the mitochondrial suspension, washes, and lysed mitochondrial pellets were analyzed by SDS-PAGE/Western blot for αSyn. To quantify the total αSyn immunoreactivity of lysed mitochondrial pellets, densitometric analysis was performed using ImageJ (National Institutes of Health).

**SDS-PAGE/Western Blotting**

Samples for Western blotting were electrophoresed on NuPAGE 4–12% BisTris gels (Invitrogen) with MES-SDS running buffer. Gels were then transferred onto 0.45-μm Immobilon-P PVDF membranes (Millipore) for 60 min at 400-mA constant current at 4 °C in transfer buffer consisting of 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, membranes were blocked in 5% nonfat milk in PBS with 0.1% (v/v) Tween 20 (PBS-T) for 30 min at room temperature and then incubated in primary antibody either overnight at 4 °C or for 60 min at room temperature. Membranes were then washed three times for 5 min in PBS-T, incubated with secondary antibody, washed three more times for 5 min in PBS-T, and then developed with ECL Plus or ECL Prime (GE Healthcare) according to the manufacturer’s directions.

**Antibodies**

2F12, an in-house generated mouse monoclonal antibody against αSyn (9), was used at 0.18 μg/ml in PBS-T plus 5% milk. The mouse monoclonal anti-cytochrome c antibody 7H8 (Santa Cruz Biotechnology, Inc.) was used at 200 ng/ml in PBS-T plus 1% milk. Horseradish peroxidase-conjugated mouse secondary antibody (GE Healthcare) was diluted 1:10,000 in PBS-T plus 1% milk.

**Statistical Analysis**

Data are presented as the mean ± S.D. unless otherwise specified. Comparisons across two groups were made using an unpaired t test. When three or more groups were compared, a one-way ANOVA followed by Tukey’s multiple-comparison test were used. Data are considered significant at a p value of ≤0.05.
RESULTS

Prefibrillar Thioflavin T-negative αSyn Promotes Complex I-dependent, Ca\textsuperscript{2+}-mediated Mitochondrial Dysfunction—To assess whether αSyn can directly compromise mitochondrial function, we prepared recombinant human αSyn for application to isolated mitochondria. To this end, we monitored the aggregation state of αSyn solutions incubated at 37 °C under nutation. Assaying αSyn at different time points in this in vitro “aging” process using the amyloid binding dye ThT produced a sigmoidal curve of ThT fluorescence (Fig. 1A). We compared unaged monomer, aged ThT-negative (ThT\textsuperscript{neg}) αSyn sampled during the aggregation lag phase and ThT-positive (ThT\textsuperscript{pos}) aggregates (Fig. 1A, blue, red, and green, respectively) for their ability to alter the function of mitochondria. We hypothesized that ThT\textsuperscript{neg} αSyn from the aggregation lag phase would be bioactive when applied to the isolated mitochondria, because this phase is characterized by the presence of soluble oligomers (45–47) that are

![Diagram](image-url)
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believed to be more toxic than ThT^{pos} fibrils that are abundant at the end stage of aggregation.

Mitochondrial stress was modeled by exposing isolated mitochondria to a single bolus of Ca^{2+} to induce eventual membrane permeabilization associated with a collapse of ΔΨ_{m}, release of endogenous and exogenously administered Ca^{2+}, oxidation of pyridine nucleotides, and mitochondrial swelling. We used a fluorimeter-based assay to simultaneously measure these four parameters (ΔΨ_{m}, Ca^{2+} uptake/release, redox state of NAD(P)H, and swelling) in isolated mouse liver mitochondria. Fig. 1B shows a representative record of these four mitochondrial parameters for mitochondria respiring with the complex I substrates glutamate and malate (complex I conditions) and exposed to a single Ca^{2+} addition in the absence (black traces) or the presence of 1 μM monomeric (blue traces) or aged, ThT^{neg} αSyn (red traces). The addition of mitochondria into the incubation buffer, as indicated by the labeled arrow, induced the sharp decline of the TMRM signal (i.e. TMRM concentration in the buffer) due to the accumulation and quenching of the dye by polarized mitochondria. The addition of Ca^{2+} at 180 s induced a transient depolarization (and therefore increase of TMRM signal) as well as a spike in extramitochondrial Ca^{2+} concentration before rapid uptake into mitochondria. Alamethicin, a nonspecific pore-forming agent, was added as a positive control to observe maximal mitochondrial swelling at the end of these measurements (note the rapid reduction in absorbance at 587 nm). Unaged, monomeric αSyn had no effect on mitochondrial CRT, whereas 1 μM ThT^{neg} αSyn significantly reduced mitochondrial CRT by 27% versus control (Fig. 1C). αSyn sampled from the end-stage plateau of aggregation (ThT^{neg}) failed to reduce mitochondrial CRT (Fig. 1D). Taken together, these data suggest that partially aged ThT^{neg} αSyn, but not monomeric or highly aggregated ThT^{pos} αSyn, sensitizes mitochondria to Ca^{2+}-mediated mitochondrial dysfunction.

Because the level of mitochondria-localized αSyn has been inversely correlated with complex I activity in the substantia nigra of PD patients (27), we investigated whether the effect of prefibrillar αSyn was restricted to complex I conditions. Therefore, instead of the complex I substrates glutamate and malate, we added the complex II substrate succinate and complex I inhibitor rotenone (complex II conditions). Interestingly, under complex II conditions, neither ThT^{neg} nor monomeric αSyn significantly affected CRT (Fig. 1C) or the other mitochondrial parameters tested (data not shown). This result suggests that the ability of aged ThT^{neg} αSyn to sensitize mitochondria to Ca^{2+} is specifically dependent on electron flow through complex I and also serves as an important specificity control for our analytical method.

Duration of the Aggregation Lag Phase Is Variable—The data presented in Fig. 1 highlight the fact that obtaining bioactive αSyn is critically dependent on predicting the timing of the aggregation lag phase and “catching” αSyn at the optimal oligomerization state. Although the effects of aged, ThT^{neg} αSyn occurred consistently, the duration of the aggregation lag phase leading to activity varied considerably and was thus difficult to predict a priori. Five examples of αSyn incubated under identical conditions on different days are displayed in Fig. 2. In some cases, the lag phase extended over several days, whereas in others, the samples already contained ThT-binding species at early time points after the start of incubation (Fig. 2, note the broken abscissa). The kinetics of αSyn aggregation are known to be altered by many factors, including pH, temperature, sample volume, degree of agitation, protein concentration, and the presence of even a small amount of oligomeric “seeds.” We therefore sought a more reproducible way of generating bioactive oligomers.

Characterization of αSyn Fibrils before and after Sonication—Sonicated αSyn fibrils have recently been used to seed the aggregation of endogenous αSyn in cells and mice (48, 49).
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![Image](https://example.com/image.png)

**FIGURE 3. Characterization of sonicated and non-sonicated αSyn fibrils.** A and B, representative electron micrographs of αSyn aggregated at 2 mg/ml for 5 days under nutation before (A) and after (B) sonication. Sonicated fibrils contained a heterogeneous mixture of species including spherical oligomers and short fibril fragments. Scale bars, 100 nm. C, hydrodynamic radii (Rh) of fibrils and sonicated fibrils as measured by dynamic light scattering. Error bars, S.E. of three and seven replicates for fibrils and sonicated fibrils, respectively. D, background-subtracted fluorescence values of 2.5 μM (based on starting monomer concentration) fibrillar and sonicated αSyn fibrils in the presence of 10 μM ThT expressed as a percentage of signal obtained for fibrillar αSyn. Sonicated fibrils fluoresced with ~10% of the intensity of fibrils. Error bars, S.D. of three independent experiments. E, an example aggregation time course of 7 μM monomeric αSyn seeded with 1 mol % of fibrillar, sonicated, or additional monomeric αSyn is shown. Sonicated and non-sonicated fibrils accelerated the aggregation of monomeric αSyn compared with additional monomeric αSyn. In this experiment, monomer-seeded αSyn was observed to acquire ThT positivity after 40 h of aging. 100,000 AFU represents the upper limit of detection of our instrument. Error bars, S.D. of 3–4 replicates. Similar data were obtained in three other independent experiments.

Because mature αSyn fibrils can be prepared easily and reproducibly, we asked whether the mechanical disruption of fibrils into small species can produce oligomers that are functionally similar to the prefibrillar oligomers present during the aggregation lag phase (above). We first generated recombinant human αSyn fibrils (see “Experimental Procedures”) and characterized them by several techniques before and after sonication. Electron microscopy of non-sonicated fibrils revealed long, relatively uniform fibers hundreds of nm in length with diameters between 10 and 15 nm (Fig. 3A). In contrast, sonicated fibril samples were more heterogeneous and included short fibril fragments as well as spherical αSyn aggregates (Fig. 3B). Dynamic light scattering analysis revealed the average hydrodynamic radius of non-sonicated fibrils and sonicated fibrils to differ markedly at ~950.5 and ~40.0 nm, respectively (Fig. 3C, note the log scale). We examined the ensemble aggregation state of sonicated αSyn by incubating samples with the β-sheet-binding dye ThT. Sonicated αSyn was only 10% as fluorescent as non-sonicated fibrils (Fig. 3D), illustrating a very substantial loss of β-sheet content. Despite this decrease in β-sheet content, we observed that sonicated αSyn fibrils were able to seed the aggregation of monomeric αSyn (Fig. 3E), confirming that they still contained species that can serve as a template for further aggregation.

**Sonicated αSyn Fibrils Recapitulate the Mitochondrial Effects of Prefibrillar ThTpos αSyn**—We compared sonicated and non-sonicated fibrils for their ability to alter the biochemical function of mitochondria. Under complex I conditions, sonicated αSyn reduced mitochondrial CRT in a dose-dependent manner, with the 1 and 2 μM doses causing significant 14 and 21% reductions, respectively (Fig. 4, A and B). This CRT reduction was again associated with commensurate acceleration in membrane depolarization, oxidation of endogenous pyridine nucleotides, and swelling of the mitochondria (Fig. 4A). In contrast to the sonicated material and in agreement with the ThTpos samples described above (Fig. 1D), non-sonicated αSyn fibrils did not significantly alter CRT (Fig. 4C) or the three other mitochondrial parameters we measured (data not shown). Under complex II conditions, neither 1 nor 2 μM sonicated αSyn led to a reduction in CRT or accelerated depolarization when incubated with mitochondria (Fig. 4D, E). Together, these data confirm that relatively small oligomeric αSyn species, but not ThTpos fibrillar αSyn, promotes Ca$^{2+}$-induced mitochondrial dysfunction specifically under complex I conditions.

In general, the reduction of CRT by sonicated αSyn (1 μM) under complex I conditions was highly reproducible, but in 4 of 23 samples tested, no effect of the αSyn preparation could be detected. In the detailed characterization of the effects of αSyn on mitochondria that follows, we analyzed data from all of...
**FIGURE 4.** Sonicated αSyn fibrils promote Ca\(^{2+}\)-mediated mitochondrial dysfunction in a substrate-dependent manner. **A**, representative traces of basic parameters of isolated liver mitochondria (\(\Delta\Psi_{m}\), extramitochondrial Ca\(^{2+}\) fluorescence, NAD(P)H autofluorescence, and mitochondrial swelling) simultaneously measured by a multichannel fluorimeter and recorded in the presence of a single 20 μM Ca\(^{2+}\) addition, 5 mM glutamate and 5 mM malate as substrates, and either PBS vehicle (black traces) or 1 μM (red traces) or 2 μM (pink traces) sonicated αSyn fibrils. Spikes at 60 s result from the addition of mitochondria; arrows are used to indicate the time of the Ca\(^{2+}\) addition. **B**, the CRT of mitochondria treated with 1 and 2 μM sonicated αSyn fibrils under complex I conditions (glutamate/malate-dependent respiration) were normalized to vehicle-treated mitochondria. Sonicated αSyn dose-dependently reduced CRT under these conditions. Error bars, S.D. from at least nine independent experiments. *, \(p < 0.05\), ANOVA followed by Tukey’s multiple-comparison test. **C**, the relative CRT of isolated mitochondria respiring under complex I conditions and treated with non-sonicated αSyn fibrils was determined. Error bars, S.D. from five independent experiments. **D**, representative traces of \(\Delta\Psi_{m}\) (left) and extramitochondrial Ca\(^{2+}\) fluorescence (right) of mitochondria treated with a single aliquot of Ca\(^{2+}\) in the presence of succinate/rotenone (complex II-dependent respiration) and either vehicle (black traces) or 2 μM sonicated αSyn fibrils (pink traces). **E**, the CRT of mitochondria respiring under complex II conditions (succinate/rotenone-dependent respiration) and treated with 1 and 2 μM sonicated αSyn was compared with vehicle-treated mitochondria under identical conditions. Error bars, S.D. from at least four independent experiments.
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\text{FIGURE 5.} \quad 100,000 \times g \text{ soluble fraction of sonicated } \alpha\text{Syn fibrils contains bioactive oligomers.} \\
A \text{ and } B, \text{ representative electron micrographs of sonicated } \alpha\text{Syn fibrils fractionated into a } 100,000 \times g \text{ supernatant (A) and pellet (B). Large fibril fragments pellet at this speed, whereas smaller, rounded oligomers remain }
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those sonicated preparations that were found to be bioactive (i.e. 19 of 23).

**Bioactivity of Sonicated \(\alpha\text{Syn} is Contained within a 100,000 \times g\) Supernatant—We sought to further define the bioactive component of the sonicated \(\alpha\text{Syn} fibril preparation. Analysis of the CRT reduction capability of the } 16,000 \times g \text{ supernatant revealed that it contained equivalent bioactivity compared with the total sonicated material (data not shown). To determine whether the active component was retained in a high speed supernatant, we fractionated the total sonicated material via ultracentrifugation at } 100,000 \times g. \text{ Electron microscopy of the } 100,000 \times g\)-soluble and -insoluble fractions showed a nearly complete separation of small, rounded particles and longer amyloid fibril fragments into the supernatant and pellet, respectively (Fig. 5, A and B). Further analysis using dynamic light scattering revealed that the } 100,000 \times g \text{ supernatant contained oligomers with a mean hydrodynamic radius of } 20.3 \text{ nm compared with monomeric } \alpha\text{Syn and the } 100,000 \times g \text{ pellet, which had mean hydrodynamic radii of } 3.8 \text{ and } 42.8 \text{ nm, respectively (Fig. 5C). Moreover, the } 100,000 \times g \text{ supernatant was able to seed the aggregation of monomeric } \alpha\text{Syn much faster than did additional monomer (Fig. 5D), suggesting that it contains misfolded species that can promote the conversion of unfolded protein into } \beta\text{-sheet-rich aggregates. Importantly, when applied to mitochondria, the } 100,000 \times g \text{ supernatant significantly reduced CRT to a degree equivalent to the total sonicated sample, whereas the volume-normalized pellet failed to reduce CRT (Fig. 5E). Although the pellet contained considerably less material than the supernatant as determined by total protein assays, subsequent experiments showed that the protein in the pellet had no capability to decrease CRT when protein normalized (data not shown). Together, these data demonstrate that sonication of } \alpha\text{Syn fibrils produces entirely soluble (non-pelletable) oligomers that can disrupt mitochondrial Ca}^{2+} \text{ homeostasis, whereas larger } \alpha\text{Syn species that pellet under these conditions (intact fibrils and larger fragments thereof) have no significant effect on mitochondrial function.}

**Sonicated Fibrils Promote Ca}^{2+}\text{-mediated Mitochondrial Dysfunction and Associated Cytochrome c Release via Permeability Transition Pore Induction—There is mounting evidence that under pathophysiological conditions within a cell, the exposure of mitochondria to elevated cytosolic Ca}^{2+} \text{ can result in mitochondrial damage via a permeability transition mechanism (50–52). The induction of opening of the mPTP is associated with membrane depolarization and release of intramitochondrial Ca}^{2+}. \text{ These changes are followed closely by the rupture of the outer mitochondrial membrane and subsequent release of cytochrome c, which in an intact cell could lead to the downstream initiation of apoptosis (53).}
αSyn Promotes Ca$^{2+}$- and Complex I-dependent Mitotoxicity

To ascertain whether the opening of the mPTP was responsible for the αSyn-induced and Ca$^{2+}$-mediated changes in mitochondrial parameters documented above, we examined the effect of the specific mPTP inhibitor cyclosporin A (CsA). As expected, preincubation of the mitochondria with 1 μM CsA prevented the CRT reduction by sonicated αSyn fibrils (Fig. 6A, left, purple trace) and the associated decrease in the time to complete mitochondrial swelling (Fig. 6A, right, purple trace). We next determined whether the earlier onsets of mitochondrial Ca$^{2+}$ release and swelling caused by incubation with sonicated αSyn oligomers were accompanied by premature cytochrome c release. We removed aliquots of the vehicle- and αSyn-treated mitochondrial suspensions at time points corresponding to ~25, 50, 75, and 100% swelling of vehicle-treated mitochondria. Another aliquot was removed following the addition of alamethicin, which was used to induce maximal swelling and cytochrome c release. Western blots of the supernatants from all of these aliquots indicated that the detection of released cytochrome c correlated with the degree of swelling (change in light scattering) in the mitochondrial suspension as a whole (Fig. 6B). Importantly, CsA preincubation also prevented αSyn-induced cytochrome c release (Fig. 6B), suggesting that, in our system, this swelling occurs as a consequence of mPTP opening rather than via direct membrane permeabilization by αSyn oligomers.

We then used electron microscopy to confirm the protective effects of CsA on αSyn-induced swelling. Mitochondria at baseline (before the Ca$^{2+}$ addition) appeared dark with electron-dense, compact cristae (Fig. 7A). Electron micrographs of vehicle- and αSyn-treated mitochondrial samples fixed after complete reduction in absorbance (as determined by the fluorimeter) showed similar evidence of swelling (Fig. 7, B and C). We observed a qualitative lightening of the mitochondria caused by a loss of solutes as well as an increase in mitochondrial diameter. After swelling, the average diameters of αSyn- and vehicle-treated mitochondria were significantly larger than samples obtained at baseline but were not different from each other (Fig. 7E). Microscopy confirmed that preincubation of mitochondria with CsA prevented the loss of solutes and membrane swelling associated with permeability transition (Fig. 7D). In the presence of CsA, the average diameter of αSyn-treated mitochondria was not significantly different from mitochondria at baseline, suggesting that, in our system, αSyn-induced changes to mitochondrial diameter are dependent on the mPTP opening rather than direct, gross perturbation of mitochondrial membranes (Fig. 7B).

Activity of Sonicated αSyn Is Dependent on Exogenous Ca$^{2+}$ Uptake—Thus far, we had only investigated whether αSyn can alter mitochondrial function in the presence of exogenously added Ca$^{2+}$. We therefore asked whether the ability of sonicated αSyn to alter mitochondrial function under complex I conditions was dependent on added Ca$^{2+}$. For this purpose, we incubated the isolated mitochondria with sonicated αSyn but without the addition of exogenous Ca$^{2+}$. No changes in all four measured mitochondrial parameters (ΔΨ$m^-$, Ca$^{2+}$ flux, NAD(P)H oxidation state, and swelling) were detected for incubation times up to 1 h (Fig. 8A). To confirm that effects of sonicated αSyn are mediated by exogenous Ca$^{2+}$, we asked whether sonicated αSyn fibrils sensitized mitochondria to a non-Ca$^{2+}$-inducer of mPTP opening, the bifunctional hydrophobic thiol-cross linking agent phenylarsine oxide (54). As in the absence of exogenous Ca$^{2+}$, we observed no sensitizing effect of αSyn in the presence of 1 or 10 μM phenylarsine oxide (PhAsO; Fig. 8B), suggesting that prefibrillar αSyn does not sensitize to all subtoxic stressors.

We then specifically tested whether Ca$^{2+}$ uptake into mitochondria, rather than simply its presence in the mitochondrial suspension, was required to observe αSyn-induced changes to mitochondrial parameters. Preincubation with the specific inhibitor of the mitochondrial Ca$^{2+}$ uniporter Ru360 (10 μM) was sufficient to completely block uptake of exogenous Ca$^{2+}$ (Fig. 9A, compare bottom and top panels) and prevent the mPTP-inducing effects of sonicated αSyn, as exemplified by the swelling traces shown in Fig. 9B. We observed no reduction in absorbance over time, indicating that αSyn did not promote mitochondrial swelling in the presence of Ru360 (compare top and bottom panels of Fig. 9B). To determine whether exogenous Ca$^{2+}$ enhanced the physical association of αSyn with mitochondria, we incubated mitochondria with sonicated αSyn in the presence or absence of 20 μM Ca$^{2+}$, ran the lysate of extensively washed mitochondrial pellets on SDS-PAGE, and blotted for αSyn. We observed no differences in the intensity or...
pattern of αSyn immunoreactivity in the lysed mitochondrial pellet (Fig. 9, C (lanes L) and D). This indicates that, whereas Ca^{2+} uptake is necessary for the downstream functional consequences of the αSyn/mitochondria association (i.e. CRT reduction and sensitization to mPTP opening), the association itself is not regulated by Ca^{2+} under our conditions. These data suggest that the addition of Ca^{2+} and the concomitant metabolic changes that accompany its uptake into mitochondria make the mitochondria vulnerable to the effects of bound αSyn.

Sonicated αSyn Impairs Complex I Activity—We have shown that sonicated αSyn fibril fragments impair complex I-dependent mitochondrial function. One potential explanation for this is that αSyn interferes with proper electron transport at complex I. To test this, we measured the enzymatic activity of complex I directly using the autofluorescence of its substrate NADH. To test this, we measured the enzymatic activity of complex I directly using the autofluorescence of its substrate NADH. This assay is independent of substrate availability or the activity of other respiratory complexes that factor into our CRC of brain mitochondria by ~22% under complex I conditions. Because of the limited ability of brain-derived mitochondria to undergo swelling (56), we quantified the CRC, a widely used measure of a permeability transition-like event (40, 57), rather than CRT. In this assay, additional Ca^{2+} aliquots are needed to promote permeability transition at the population level. Sonicated αSyn reduced the CRC of brain mitochondria by ~22% under complex I conditions (Fig. 11, A and B), thus reproducing our key finding from liver mitochondria. We were able to simultaneously measure ROS production by adding Amplex Red to the assay buffer. However, despite their consistent ability to reduce CRC, sonicated αSyn oligomers did not increase the rate of H_{2}O_{2} production in this system. Catalase is generally less abundant in isolated brain mitochondria. Thus, we chose to examine ROS production by isolated brain mitochondria in the presence of Ca^{2+} plus either vehicle or sonicated αSyn. We first confirmed that sonicated αSyn does indeed reduce Ca^{2+} retention of brain mitochondria under complex I conditions. Based on this ability of sonicated αSyn to reduce complex I activity, we tested whether αSyn-induced reduction in Ca^{2+} retention was associated with increased production of reactive oxygen species (ROS). High activity of catalase in liver mitochondrial preparations (55) makes it difficult to detect H_{2}O_{2} in liver mitochondria. Here, we used a reductionist model system comprising...
isolated liver and brain mitochondria and different, well-characterized forms of pure human H9251 Syn to discover that highly soluble, prefibrillar H9251 Syn oligomers, but not monomers or ThTpos fibrils, impair mitochondrial function. Further, we show that the toxicity of oligomeric H9251 Syn is specifically dependent on both mitochondrial uptake of exogenous Ca\textsuperscript{2+} and electron flow through complex I. The literature is replete with protocols for preparing H9251 Syn oligomers (e.g., by incubation with supraphysiological levels of metal ions, lipids, detergents, or oxidizing agents) that are either not further characterized or vary in their morphological, biochemical, and/or functional characteristics (12, 60–65). In this study, we report highly complementary results using two independent methods for generating soluble prefibrillar H9251 Syn oligomers in the absence of any additives: sonication of fully formed amyloid fibrils (sonicated preparation) and collection of oligomers during the lag phase of initial aggregation (lag phase preparation). Although oligomers obtained with the sonicated preparation are formed by the sonication of fibrils and could therefore be considered “postfibrillar,” they are capable of seeding the aggregation of monomeric protein into ThTpos fibrils. Thus, for simplicity and to distinguish them from physiological, aggregation-resistant helical oligomers (7, 9), we refer herein to oligomers from the sonicated and lag phase preps as “prefibrillar oligomers.” Both of these preparations produce closely similar effects on mitochondrial function, which argues against the likelihood of artifactual effects caused by a specific preparation of oligomers. Under the specific conditions discussed further below, H9251 Syn generated using either of our protocols recapitulated several mitochondrial phenotypes previously reported in animal and cell models of PD, namely complex I-mediated dysfunction, altered ΔΨm, disrupted Ca\textsuperscript{2+} homeostasis, and enhanced cytochrome c release. Our development of simple protocols to generate and enrich for pathologically relevant

FIGURE 8. αSyn does not affect mitochondrial parameters in the absence of exogenous Ca\textsuperscript{2+}. A, comparison of mitochondrial suspensions incubated with vehicle control (black) or 1 μM sonicated αSyn fibrils (red) under complex I conditions in the absence of exogenous Ca\textsuperscript{2+}. The addition of sonicated αSyn resulted in no alteration of steady state ΔΨm, Ca\textsuperscript{2+} flux, oxidation state of pyridine nucleotides, or membrane swelling. Alamethicin (Ala) was added to induce complete swelling after 1 h. Arrows, the addition of mitochondria. Traces are representative of four independent experiments. B and C, representative swelling traces of mitochondria incubated with either vehicle (black) or 2 μM sonicated fibrils (red) under complex I conditions and treated with 1 μM (B) or 10 μM (C) phenylarsine oxide (PhAsO). αSyn incubation did not sensitize mitochondria to undergo mPTP-related swelling in the presence of phenylarsine oxide.
oligomeric αSyn provides an approach for more efficiently studying αSyn cytotoxicity, including further investigations into the mechanisms of mitochondrial dysfunction.

Collectively, our data indicate that highly soluble prefibrillar oligomers, rather than larger, 100,000 g-pelletable assemblies or fibril fragments, can disrupt proper mitochondrial function. ThT assays suggest that our bioactive material is not β-sheet-rich; however, we cannot exclude the possibility that a small percentage of β-sheet-containing oligomers that do not avidly bind ThT contribute to our observed effects, especially in light of a propensity of the active material to act as a template for the formation of β-sheet-containing amyloid fibrils. Our results provide a potential subcellular target of prefibrillar αSyn oligomers recently reported to be cytotoxic in cellular and animal models.

FIGURE 9. Mitochondrial Ca²⁺ cycling is necessary for αSyn-induced mPTP induction but not binding. A, extramitochondrial Ca²⁺ (as determined by Ca-Green 5N fluorescence) of mitochondrial suspensions incubated with vehicle control (black) or sonicated αSyn fibrils (red) and challenged with 20 μM Ca²⁺ in the absence (top) or presence of 10 μM Ru360, an inhibitor of the mitochondrial Ca²⁺ uniporter (bottom). Note that Ru360 prevents uptake of exogenous Ca²⁺. Traces are representative of four independent experiments. B, measurements of mitochondrial swelling (as determined by the decrease in absorbance at 587 nm) obtained from incubations of the same αSyn and mitochondrial preparations shown in A. 20 μM Ca²⁺ was added to mitochondrial suspensions in the absence (top) or presence of 10 μM Ru360 (bottom). In this example, the initial absorbance of the αSyn-treated mitochondrial suspension is somewhat lower than that of the vehicle-treated suspension (due to small differences in the total mitochondrial protein present); however, in neither case is there a reduction in absorbance over time, indicating that there is no Ca²⁺-induced swelling in the presence of Ru360. Traces are representative of four independent experiments. C, SDS-PAGE/Western blot of the supernatant (S), washed pellets (W₁–W₄), and lysed pellets (L) of mitochondrial suspensions after brief incubation with sonicated αSyn in the presence or absence of 20 μM Ca²⁺. Membranes were probed for αSyn (top) and cytochrome c (bottom). The majority of the incubated αSyn remains in the supernatant, but a fraction resists four washes in assay buffer and is specifically associated with the mitochondrial pellet. As expected, cytochrome c is only detectable in the lysed mitochondrial pellets, suggesting that mitochondria remain intact after these washes. Blots are representative of four independent experiments. D, quantification of total αSyn immunoreactivity of lysed mitochondrial pellets after incubation with sonicated αSyn in the presence or absence of Ca²⁺ and after extensive washing. Total lane immunoreactivity was normalized to samples in which 20 μM Ca²⁺ was added to the αSyn/mitochondria suspension. Error bars, S.D. of four independent experiments.

FIGURE 10. Sonicated αSyn can inhibit complex I activity. A, mechanically disrupted mitochondria were incubated with vehicle (black), monomeric αSyn (blue), sonicated αSyn fibrils (red), or intact fibrils (green), and the oxidation of supplied NADH was monitored by the absorbance at 340 nm before and after the addition of rotenone (indicated by the dashed line). The post-rotenone absorbance slope was subtracted from the pre-rotenone slope to obtain the rotenone-sensitive activity. B, rotenone-sensitive complex I activity in mitochondria incubated with various αSyn forms was normalized to vehicle-treated mitochondria. Error bars, S.D. of three independent experiments. *, p < 0.05 when compared with all other groups, ANOVA followed by Tukey’s multiple comparisons test.
models. Karpinar et al. (14) reported greater oligomerization propensity but reduced fibrilization and β-sheet content of recombinant αSyn engineered to contain various proline substitutions. Overexpression of these mutants in rat primary neurons, Caenorhabditis elegans, and Drosophila was associated with neurotoxicity that was inversely proportional to the β-sheet content of in vitro oligomers of the same αSyn proteins (14), but mitochondrial dysfunction was not specifically addressed. Recent work by Winner et al. (13) is also in line with our findings; αSyn mutants that preferentially form β-sheet-poor oligomers rather than β-sheet-rich fibrils in vitro were associated with more neurotoxicity in lentivirus-infected human mesencephalic cells and rat brain. Moreover, interaction of these oligomers with cellular membranes was suggested as a potential mediator of toxicity (13), but again mitotoxicity was not examined. Our sonicated preparation was modeled in part after the sonicated preformed fibrils used by Lee and colleagues (48, 49), who demonstrated the internalization of extraneuronally applied sonicated fibrils and their ability to promote the aggregation of endogenous αSyn. To date, the colocalization, if any, of the seeded protein with mitochondria and the possible functional consequences for these organelles have not been assessed, but in light of our findings, they should be.

Prefibrillar αSyn represents a heterogeneous pool of transient oligomeric species on their way to highly ordered, β-sheet aggregates analogous to those comprising the fibrils in Lewy bodies and Lewy neurites (11, 60). Thus, our results are in agreement with the concept of Lewy pathology as a relatively protective mechanism, at least temporarily (66, 67). In the context of PD, our data support the pursuit of therapeutic strategies designed to stabilize native, aggregation-resistant α-helical tetramers and related oligomers in neurons (7, 9). Alternatively, one could attempt to accelerate the fibrilization process to decrease levels of potentially toxic oligomers, although it is likely that these species are in equilibrium, and the fibrils could later release smaller oligomers that could promote aggregation of monomeric protein via a secondary nucleation mechanism (47, 68).

The mitochondria of neurons of the substantia nigra pars compacta and other brain regions vulnerable to PD are subjected to high Ca²⁺ concentrations due to regular influx through pacemaking L-type channels and low expression of...
αSyn Promotes Ca\textsuperscript{2+} - and Complex I-dependent Mitotoxicity

cytosolic Ca\textsuperscript{2+}-buffering proteins (25, 26, 69). We therefore studied the effects of αSyn on mitochondrial function in the context of elevated Ca\textsuperscript{2+} levels. We observed that bioactive prefibrillar αSyn disrupted basic mitochondrial parameters (ΔΨ\textsubscript{m}, Ca\textsuperscript{2+} uptake/release, redox state of pyridine nucleotides, and swelling) only in the presence of exogenously added Ca\textsuperscript{2+}. Because Ru360 abolished this effect, we can conclude that the downstream effects of mitochondria-associated αSyn were dependent on the uptake of Ca\textsuperscript{2+} into the mitochondrial matrix. Previous reports suggest that overexpressing αSyn in cells or animals leads to alterations in mitochondrial morphology or function in the absence of overt Ca\textsuperscript{2+} stress (27, 31, 36, 37, 58, 59, 70, 71). These results could be explained by one or more of the following possibilities. First, toxic αSyn species could affect the function of other organelles, such as the endoplasmic reticulum (71), or cellular pathways leading to intracellular Ca\textsuperscript{2+} dyshomeostasis. Second, when overexpressed in cell lines or animals (versus briefly incubated with mitochondria in vitro as here), there may be greater opportunity for αSyn to exert its effects, so that mild alterations of mitochondrial function (which could be exacerbated by our acute Ca\textsuperscript{2+} challenge) can accumulate chronically. In this context, physiological Ca\textsuperscript{2+} transients in neurons could be a sufficient stress in the presence of prefibrillar, oligomeric αSyn. Furthermore, endogenous toxins, such as the monoamine oxidase-generated product of dopamine, 3,4-dihydroxyphenylacetaldehyde, have also been shown to facilitate mitochondrial permeability transition (72) and may act synergistically with pathological αSyn in dopaminergic neurons. Third, the ability of Ca\textsuperscript{2+} to exacerbate the mitochondrial effects of αSyn may not be due to Ca\textsuperscript{2+} uptake per se but rather the associated increase in ETC activity. Mitochondrial Ca\textsuperscript{2+} influx comes at the expense of ΔΨ\textsubscript{m}, which must be reestablished in order to generate ATP and buffer further Ca\textsuperscript{2+} loads. One possibility is that proper ETC function may be disrupted by αSyn (see below), and the increased ETC activity needed to restore ΔΨ\textsubscript{m} in response to the uptake of excess Ca\textsuperscript{2+} could increase the generation of ROS, thereby accelerating mPTP opening. However, we did not observe an elevation in ROS production in our preliminary experiments. Mitochondria targeted by prefibrillar αSyn may be less able to reestablish ΔΨ\textsubscript{m} and would therefore be more susceptible to permeability transition; reduced ΔΨ\textsubscript{m} is a known sensitizing factor for mPTP opening (73, 74). Our direct measurements of complex I activity, obtained under conditions of rapid electron flux, are supportive of this hypothesis. They suggest that prefibrillar αSyn can reduce the maximal enzymatic activity and impair mitochondrial function in the absence of Ca\textsuperscript{2+} if ETC activity is otherwise increased.

The protective effects of CsA on our αSyn-induced changes in mitochondrial parameters demonstrated that the addition of prefibrillar αSyn sensitized mitochondria to mPTP activation. The acceleration of cytochrome c release in αSyn-treated mitochondria was likewise rescued by preincubation with CsA. This result suggests that cytochrome c release was also regulated by the mPTP in our system rather than by channels formed via direct permeabilization of the outer membrane by oligomeric αSyn, as has been suggested (75). Although the role of mitochondrial permeability transition in PD-related cell death requires further study, two recent reports support the in vivo relevance of our data. Findings from Martin et al. (32) indicate that the genetic ablation of cyclophilin D, the mPTP modulator and target of CsA, delayed the onset of mitochondrial abnormalities and neuronal apoptosis in Thy1-αSyn A53T transgenic mice. Also, Büttner et al. (33) showed that overexpression of αSyn in yeast led to cell death and a redistribution of the pro-apoptotic nuclease EndoG from mitochondria to the nucleus, and these effects could both be rescued by genetic modulation of the mPTP components. αSyn-induced, EndoG-dependent cytotoxicity was also observed in C. elegans, flies, and human neuroblastoma cells. Moreover, nuclear translocalization of EndoG was preferentially detected in the brain sections of PD patients compared with age-matched controls (33). Others have recently proposed that WT αSyn binds to mitochondria-associated endoplasmic reticulum membranes rather than mitochondria themselves (76). However, the authors did see a redistribution of αSyn to “pure” mitochondria under pathological conditions, when the oligomer-promoting mutants A53T and A30P were overexpressed in both HeLa cells and mouse brain. Overexpression of these mutants was also associated with altered mitochondrial morphology (76).

Ca\textsuperscript{2+}-induced mPTP opening can be broadly split into three main phases that could be accelerated by αSyn based on our new work: the “initiation phase,” during which Ca\textsuperscript{2+} is taken up into the matrix through the mitochondrial Ca\textsuperscript{2+} uniporter; the “lag phase,” during which mPTP components are recruited and assembled; and the “propagation phase,” during which mPTP induction spreads through the population (39). Inducers can affect one or more phases, and even highly related compounds can show differential effects (39, 77). Our data showing the lack of direct effect of αSyn on ΔΨ\textsubscript{m} and the uninjured initial uptake of Ca\textsuperscript{2+} both suggest that the first phase is not significantly affected by αSyn. The relatively long, stable lag phase even in the presence of prefibrillar αSyn is also consistent with a minimal effect (if any) in the initial phase. There is also no apparent shift in the slope of the final propagation phase, suggesting no significant effect on that aspect of mPTP induction. This suggests that αSyn primarily acts during the lag phase. Accordingly, the lag phase is significantly shortened in our αSyn-treated mitochondria.

Alteration of the lag phase by prefibrillar oligomers is also consistent with our observing a clear cut respiratory substrate-dependent effect. Our results show that prefibrillar αSyn oligomers sensitize mitochondria to Ca\textsuperscript{2+}-induced permeability transition under complex I but not complex II conditions. In light of our findings and existing literature (27, 35, 58), the most likely explanation for these findings is that αSyn acts to inhibit complex I. It has been reported that the brain mitochondria of A53T transgenic mice show reduced complex I activity when compared with wild type (78). The degree of reduction did not correlate with age-dependent αSyn deposition in the mice or with the apparent level of SDS-resistant oligomers (78). It is possible that (a) the results were confounded by the loss of the cells in which complex I dysfunction was exacerbated by age, (b) only a small population of the total brain cells (e.g. nigral dopaminergic neurons) showed an age-dependent reduction in complex I activity, or (c) a “ceiling effect” occurred (i.e. the
initial accumulation of αSyn oligomers in the brain already produced a maximal reduction of complex I function. Prefibrillar αSyn oligomers could act to inhibit complex I function either directly or indirectly via perturbations of mitochondrial membrane lipids. The well demonstrated tendency of αSyn to bind acidic phospholipids, especially cardiolipin (79), is critical for proper electron transfer by complexes I and III (80, 81), suggests that membrane association of pathological αSyn oligomers could destabilize complex I activity. Flux through complex I would be expected to be more sensitive to this interaction/inhibition because it is more rate-limiting (82). In the context of energization with a complex I substrate, even mild inhibition could be detrimental to mitochondria depolarized by Ca\(^{2+}\) uptake. Restoration and maintenance of the ΔΨ\(_{m}\), a primary defense against mPTP opening, would require increased electron flow into a compromised complex I.

Flux into and/or through an impaired complex I could also be expected to lead to initiation of a feed-forward cycle of increasing ROS generation and worsening complex I function. We did not detect elevated ROS production by prefibrillar αSyn in our system, but it is conceivable that extensive optimization of assay conditions could allow for more sensitive measurement of small changes in ROS generation.

In summary, we demonstrate for the first time a direct link between a particular form of αSyn assembly and mitochondrial dysfunction in a system that models the Ca\(^{2+}\) phenotype of cells affected in PD. Our data suggest that, in the context of PD, alterations in αSyn proteostasis that destabilize physiological α-helical oligomers or otherwise shift the equilibrium toward aggregation-prone monomeric αSyn may drive the generation of mitotoxic prefibrillar oligomers that contribute to complex I-dependent dysfunction and the resultant degeneration of vulnerable neuronal populations.

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