Single Particle Characterization of Iron-induced Pore-forming α-Synuclein Oligomers

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Aggregation of α-synuclein is a key event in several neurodegenerative diseases, including Parkinson disease. Recent findings suggest that oligomers represent the principal toxic aggregates during the early phases of disease. Using confocal single-molecule fluorescence techniques, such as scanning for intensely fluorescent targets and atomic force microscopy, we monitored α-synuclein oligomer formation at the single particle level. Organic solvents were used to trigger aggregation, which resulted in small oligomers (“intermediate I”). Under these conditions, Fe3+ at low micromolar concentrations dramatically increased aggregation and induced formation of larger oligomers (“intermediate II”). Both oligomer species were on-pathway to amyloid fibrils and could seed amyloid formation. Notably, only Fe3+-induced oligomers were SDS-resistant and could form ion-permeable pores in a planar lipid bilayer, which were inhibited by the oligomer-specific A11 antibody. Moreover, baicalein and N′-benzylidene-benzohydrazide derivatives inhibited oligomer formation. Baicalein also inhibited α-synuclein-dependent toxicity in neuronal cells. Our results may provide a potential disease mechanism regarding the role of ferric iron and of toxic oligomer species in Parkinson diseases. Moreover, scanning for intensely fluorescent targets allows high throughput screening for aggregation inhibitors and may provide new approaches for drug development and therapy.

All common neurodegenerative diseases are characterized by the formation and deposition of fibrillar aggregates of specific proteins, such as tau protein and Aβ in Alzheimer disease, prion protein in prion diseases, and α-synuclein (α-syn) in Parkinson disease (PD), dementia with Lewy bodies, and multiple system atrophy (1). PD is the most common movement disorder and, after Alzheimer disease, the second most common neurodegenerative brain disorder. It affects about 1% of people beyond 65 years of age (2). Pathologically, PD is characterized by degeneration of dopaminergic neurons in the substantia nigra, which leads to disruption of neuronal systems responsible for motor functions.

Initial evidence for a central role of α-syn in the pathogenesis of PD came from the discovery of point mutations in the α-syn gene in families with familial PD (3, 4). Subsequently, α-syn has been identified as the major component of Lewy bodies and in Lewy neurites, which are characteristic deposits of aggregated protein in PD, dementia with Lewy bodies, and Lewy body variant of Alzheimer disease, and as the major component of the glial cytoplasmic inclusions that characterize multiple system atrophy (5, 6). So far, three different point mutations have been described in the α-syn gene (3, 4, 7). Further evidence for a fundamental role of α-syn in the pathogenesis of PD came from the recent observation that an increased gene dose in PD patients caused by a duplication or triplication of the α-syn gene is sufficient to trigger disease (8).

The transformation of amyloidogenic proteins from the monomeric state into fibrillar aggregates seems to progress via intermediates that have been termed protofibrils, protofilaments, or oligomers (9–11). Although the cause of neurodegeneration in PD is not fully understood, recent findings suggest that small oligomers rather than the fibrillar amyloid deposits of α-syn represent the principal toxic species (12). Overexpression of human α-syn can cause apoptosis and damage of cell organelles and enhance susceptibility to oxidative stress in the absence of detectable fibril formation (13).

It has been shown that certain soluble α-syn oligomers share a common structure with oligomers derived from other amyloidogenic proteins, such as the Aβ, amylin, insulin, prion pro-

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4 The abbreviations used are: α-syn, α-synuclein; PD, Parkinson disease; FCS, fluorescence correlation spectroscopy; FIDA, fluorescence intensity distribution analysis; BSA, bovine serum albumin; Mops, 4-morpholinepropanesulfonic acid; NBB, N′-benzylidene-benzohydrazide; AFM, atomic force microscopy.
tein, and others, implying a common mechanism of pathogenesis (9, 14). One mechanism of oligomer toxicity that has been proposed is the formation of pores that lead to permeabilization of lipid bilayers (12, 15). Additionally, oligomeric forms were found to inhibit the proteasome, leading to decreased protein degradation and increased oxidative stress (16).

In the aggregation process from the monomeric protein to the end stage amyloid fibril, different distinct intermediates like globular oligomers and protofibrils have been proposed (11). In addition, several studies have shown the presence of disease-specific α-syn oligomers in synucleinopathies, such as PD and multiple system atrophy, some of which were suggested to form annular pore-like structures (17–19). So far, it remains unclear which oligomer species exhibit toxic properties.

Epidemiological studies suggest an involvement of heavy metals in the etiology of PD (20, 21). Several lines of evidence indicate that iron ions play an important role in PD pathogenesis. The neurons that are affected most severely in PD are located in the substantia nigra and locus coeruleus. These brain areas are enriched with neuromelanin that sequesters reactive metals, mainly iron. In PD patients, a correlation between increased iron levels and severity of neuropathological changes has been observed (22). Significantly high levels of Fe3+ have been found in Lewy bodies (23). Notably, recent evidence suggests that an increase in iron levels is an early event in patients at risk for developing PD and precedes loss of dopaminergic neurons (24–27). Interestingly, iron chelators showed neuroprotective activity against proteasome inhibitor-induced, MPTP-induced, and 6-hydroxydopamine-induced nigral degeneration (28–30). Additionally, it has been suggested that iron can induce the formation of intracellular α-syn aggregates (31, 32). It has been reported that metal ions, such as iron and aluminum, can accelerate amyloid formation from α-syn and trigger the generation of oligomeric α-syn forms in vitro (33). Taken together, these findings implicate iron ions in the generation of α-syn aggregates and in disease progression in PD. However, the underlying molecular events have not been elucidated so far. Therefore, the aim of our study was to improve the understanding of the molecular events in the pathological processes underlying the formation and toxicity of α-syn oligomers and the role of iron in PD.

To this aim, we used three independent single particle-based methods to analyze aggregation pathways and oligomer formation of α-syn. Biophysical and structural characterization was performed by confocal single particle fluorescence techniques and atomic force microscopy. Functional characterization included single-pore electrophysiology in a lipid planar bilayer set-up. By this approach, we could characterize two different oligomer species that were both on-pathway to amyloid fibrils ("intermediate I" and "intermediate II") for the first time. Notably, intermediate II oligomers, which could be induced by low micromolar concentrations of ferric iron, could also form ion-permeable pores in a planar lipid bilayer assay. Neither a well-defined multistep pathway with a crucial role of iron that results in pore formation nor a biophysical and electrophysiological characterization of oligomer pores at the single particle level have been described so far. Moreover, we observed consistent effects of aggregation inhibitors at the level of protein aggregation, pore formation, and cellular toxicity that may provide new approaches for drug discovery.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Wild-type α-Synuclein**—Expression and purification was performed as described previously by Nuscher et al. (34). Briefly, pET-5a/α-synuclein wild type plasmid (kind gift of Philipp Kahle, Ludwig-Maximilians-Universität München) was used to transform *Escherichia coli* BL21(DE3) pLys, and expression was induced with isopropyl-β-d-thiogalactopyranoside for 4 h. Cells were harvested, resuspended in 20 mM Tris and 25 mM NaCl, pH 8.0, and lysed by freezing in liquid nitrogen followed by thawing. After 30 min of boiling, the lysate was centrifuged at 17,600 × g or 15 min at 4 °C. Supernatant was filtered through a 0.22-μm filter (Millex-GV, Millipore Corp., Bedford, MA), loaded into a HiTrap Q HP column (5 ml), and eluted with a 25–500 mM NaCl salt gradient. The pooled α-syn peak was desalted by Superdex 200 HR10/30 gel filtration with 20 mM Tris, 25 mM NaCl, pH 8.0, running buffer. The pooled α-syn peak was concentrated using Vivaspin columns (molecular weight cut-off 5000 (Vivascience, Stonehouse, UK)) and equilibrated with water. The protein concentration was determined with a BCA protein quantification kit (Pierce). Samples were aliquoted and lyophilized.

**Separation of Monomeric and Oligomeric α-Synuclein**—Size exclusion chromatography was performed as previously described (10). Briefly, a solution of purified recombinant α-syn (1–5 mg/ml) in 10 mM PBS, prepared from lyophilized protein, was filtered through a 0.22-μm filter. The filtrate was eluted from a Superdex 200 gel filtration column (Amershambiosciences) in PBS at a flow rate of 0.5 ml/min. The eluate was monitored at 215–280 nm.

**Fluorescent Labeling of α-Synuclein**—Protein labeling was performed with amino-reactive fluorescent dyes Alexa Fluor-488-O-succinimidylester and Alexa Fluor-647-O-succinimidylyester (Molecular Probes, Inc., Eugene, OR), respectively. Unbound fluorophores were separated by two filtration steps in PD10 columns (Sephadex G25; Amershambiosciences, Munich, Germany) equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Quality control of labeled α-syn was performed by mass spectrometry and by FCS measurements on an Insight Reader (Evotec-Technologies). The typical labeling ratio achieved was ~1–2 dye molecules/α-syn molecule. In order to remove preformed aggregates, the stock solution of labeled α-syn was subjected to size exclusion chromatography (see above).

**Confocal Single Particle Analysis**—FCS, fluorescence intensity distribution analysis (FIDA), and SIFT measurements were carried out on an Insight Reader (Evotec-Technologies) with dual color excitation at 488 and 633 nm, using a 40 × 1.2 numerical aperture microscope objective (Olympus, Japan) and a pinhole diameter of 70 μm at FIDA setting. Excitation power was 200 microwatts at 488 nm and 300 microwatts at 633 nm. Measurement time was 10 s. Scanning parameters were set to 100-μm scan path length, 50-Hz beam scanner frequency, and 2000-μm positioning table movement. This is equivalent to...
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~10-mm/s scanning speed. All measurements were performed at room temperature.

The fluorescence data were analyzed by autocorrelation analysis using the FCSPP evaluation software version 2.0 (Evo- tec-Technologies). Two-color cross-correlation amplitudes G(0) and FIDA data were evaluated using the same software. For FIDA (35) and SIFT analysis (36), fluorescence from the two different fluorophores was recorded simultaneously with two single-photon detectors. Photons were summed over time intervals of constant length (bins) using a bin length of 40 μs. The frequency of specific combinations of “green” and “red” photon counts was recorded in a two-dimensional intensity distribution histogram, as described previously (36). Evaluation of SIFT data in two-dimensional intensity distribution histograms was performed by summing up the numbers of high intensity bins as described using a two-dimensional SIFT software module (Evo- tec-Technologies). For threshold setting, nonaggre- gated reference samples were used.

Aggregation Assay—A 5-fold stock solution of fluorescently labeled α-syn was prepared by mixing α-syn labeled with Alexa- 488 and α-syn labeled with Alexa-647. The concentrations of α-syn-Alexa-488 and α-syn-Alexa-647 were adjusted to ~10 molecules/focal volume and 15 molecules/focal volume, respectively. Quality control SIFT measurements were used to confirm that the stock solution was free of α-syn aggregates. Experiments were started by diluting the stock solution in 50 mM sodium phosphate buffer, pH 7.0, or in 50 mM Tris-HCl buffer, pH 7.0, respectively, containing ethanol concentrations of 0–50% and a final concentration of labeled α-syn of 10–20 nM in a total assay volume of 20 μl. FeCl₃ (Merck), FeCl₂, AlCl₃, CuCl₂, MnCl₂, and CaCl₂ (Sigma) were used at final concentra- tions of 0.1–10 μM. In some experiments 1% DMSO (Sigma) was used instead of ethanol to induce α-syn aggregation. In some experiments, sodium ascorbate (Sigma), desferoxamine (Sigma), Nonidet P-40 (Igepal; Sigma), bovine serum albumin (BSA; Sigma), or SDS (Roth) were added to the reaction mixture. All experiments were performed in 96-well plates with a cover slide bottom (Evotec-Technologies). To reduce evaporation, plates were sealed with adhesive film. Typically, aggregation was monitored for at least 5 h in 2–4 independent samples for each experimental group.

Electrophysiology with Planar Lipid Bilayers—Planar lipid bilayers were produced by the painting technique. A solution of 25% sodium KCl, 20 mM Mops/Tris, pH 7.0, in both chambers (equivalent to symmetrical conditions), the solutions were changed by perfusion to asymmetrical conditions 250/20 mM KCl, 10 mM Tris/HCl, pH 7.0, cis/trans. An osmotic gradient of a channel-permeant solute is, in addition to the abso- lute necessity of the channel in the vesicle being in the open state (37), a prerequisite for fusion of membrane vesicles with the bilayer. To promote attachment of the membrane vesicles to the bilayer, CaCl₂ was added to the cis chamber to a concentra- tion of 10 mM. Membrane vesicles were then added to the cis chamber directly below the bilayer, affecting a slow flow of membrane vesicles along the bilayer surface. After fusion, the electrolytes were changed to the final composition.

The Ag/AgCl electrodes were connected to the chambers through 2 m KCl-agar bridges. The electrode of the trans compart- ment was directly connected to the head stage of a current amplifier (Axon Gene Clamp 500; Axon Instruments). Mem- brane potentials are referred to the trans compartment. The amplified currents were digitized at a sampling interval of 0.2 ms, filtered with a low pass filter at 5 kHz, and fed into a Digi- data1200 A/D converter (Axon Instruments, Foster City, CA). For analysis, analysis software (single channel investigation program, or SCIP) developed in our laboratory was used in combination with Origin 6.0 (Microcal Software Inc.).

Preparation of α-Synuclein Aggregates for Pore Formation Assay—a α-syn and other chemical components were incubated in the presence of preformed liposomes (Ionomation, Osnabrück, Germany) with a mean diameter of 100 nm at room temperature. After 3 h, samples were treated in an ultrasonic bath for 10 s. Then 1-μl aliquots were added to the cis chamber of the bilayer setup. The cis chamber was stirred by a magnetic stir bar. When no fusion occurred for 5 min, another aliquot was added. Every 30 min, the chamber solutions and the bilayer were rebuilt. After fusion of an α-syn-induced membrane leak, the electrolytes were changed to the final composition (250 mM KCl, 10 mM Mops/Tris, pH 7) in both chambers.

AFM—After incubation, samples were mixed gently to sus- pend any aggregates; 5–6-μl aliquots were placed on freshly cleaved mica (muscovite, Veeco Europe, Dourdan Cedex, France) and incubated for 80–90 s, after which mica was care- fully rinsed twice with 100 μl of filtered, deionized water to remove salt and loosely bound proteins. The micas were dried under dry N₂ gas. Images were obtained with a MultiMode™ SPM (Veeco, Mannheim, Germany) equipped with an E-Scan- ner and operating in the TappingMode, using etched silicon NanoProbes (model FESP, Veeco Europe, Dourdan Cedex, France). Data were corrected with regard to the sample tip size (38). Some typical values were as follows: free oscillation ampli- tude, 0.8–1.8 V; drive frequency, 65–80 kHz; amplitude set point, 300–600 mV; and scan rates 0.5–1.4 Hz. The measuring conditions were 39–42% relative humidity and 21–23 °C.

Thioflavin T Fluorescence Assay—The thioflavin T assay was modified from Munishkina et al. (39). Briefly, α-syn (1 mg/ml) was incubated in 20 mM Tris-HCl and 0.1 M NaCl, pH 7.4, con- taining 20 μM thioflavin T with ethanol and/or FeCl₃, as indi- cated. The sample volume of 150 μl was pipetted into a 96-well plate (black clear bottom; BD Bioscience), and Teflon-coated magnetic stir bars with a diameter of 4 mm were added to each well. The sample plate was covered with adhesive aluminum plate sealer and incubated in an Ascent Fluoroscan plate reader at 37 °C with shaking at 540 rpm. The fluorescence was measured at 15-min intervals with a sampling time of 100 ms in the fluorescent plate reader in bottom read mode with excitation at...
450 nm and emission at 490 nm. Seeding was performed with preformed oligomers added at 10% (v/v). Data were measured in duplicates or triplicates and averaged for evaluations.

Compound Testing—N’-benzylidene-benzohydrazide (NBB) compounds were obtained from ChemBridge Corp. (San Diego, CA). Baicalein was obtained from Fluka. Stock solutions (10 mM) were prepared in DMSO.

Compound screening was done at a concentration of 10 μM in a total assay volume of 20 μl. Aggregation was induced by the addition of 1% DMSO to a mixture of α-syn monomers labeled with Alexa-488 or Alexa-647 at a final protein concentration of ~10 nM in 50 mM sodium phosphate at pH 7.0. Compounds were added together with DMSO.

Generation of Inducible PC12 Cell Lines for Expression of E46K Mutant α-Synuclein—Tet-off PC12 cells (Clontech), stably expressing tTA, were co-transfected with 4 μg of E46K mutant α-syn construct with 0.2 μg of pTK-Hyg (Clontech) plasmid using Lipofectamine Plus (Invitrogen). The cells were selected in Dulbecco’s modified Eagle’s medium (Invitrogen) with 5% fetal bovine serum, 10% horse serum, 100 μg/ml G418, 200 μg/ml hygromycin, 200 ng/ml doxycycline, 100 units/ml penicillin, and 100 units/ml streptomycin. After 3–4 weeks of selection at 37 °C in a humidified 5% CO2 incubator, G418/ hygromycin-resistant colonies were isolated and screened for transgene expression by Western blot analysis using an anti-α-syn antibody (1:500; BD Pharmingen). We chose line 34, which was highly expressing E46K α-syn, and consistent toxicity for this study. Cells were maintained in the presence of doxycycline (200 ng/ml). Expression of the mutant transgene and induction of differentiation were initiated by withdrawing doxycycline and adding 50 ng/ml nerve growth factor to the medium at the same time. Nerve growth factor and doxycycline were replenished every second day after differentiation.

Lactate Dehydrogenase Assay—PC12 cells expressing E46K α-syn were treated with baicalein for 6 days. A cytotoxicity detection kit (Roche Applied Science) was used for measurement of lactate dehydrogenase released into the medium by dead cells. 25 μl of culture medium was transferred to 96-well plates for the lactate dehydrogenase assay. The lactate dehydrogenase working solution was made fresh according to the manufacturer’s instructions. 25 μl of lactate dehydrogenase working solution was added into the 25 μl of culture medium and incubated for 30 min at room temperature. Absorbance was read at 492 nm with a plate reader ( Molecular Devices Spectra Max 340 PC).

RESULTS

Single Particle Detection and Characterization of α-Synuclein Oligomers—As shown by Munishkina et al. (39), organic solvents (e.g. ethanol at a concentration of 5–25%) can induce the generation of a partially folded intermediate and accelerate amyloid formation, whereas higher concentrations of ethanol resulted in the formation of a different oligomer species unable to form amyloid. Thus, this approach can be used as a model system to study aggregation pathways of α-syn in vitro. However, high concentrations of α-syn have been used in these studies. Therefore, we asked whether this effect can also be seen at low protein concentrations in the nanomolar range, since we reasoned that oligomeric α-syn aggregation intermediates might be kinetically trapped under these conditions, since amyloid formation is known to be highly inefficient at low α-syn concentrations. Moreover, nanomolar protein concentrations allow efficient oligomer characterization by confocal single particle fluorescence techniques. In recent years, FCS, FIDA, and SIFT have been recognized as methods that allow highly sensitive analysis of protein aggregation in neurodegenerative diseases, such as prion diseases, synucleinopathies, and poly(Q) diseases at the molecular level (36, 40–42). FCS is based on the analysis of fluctuations in fluorescence caused by the diffusion of fluorescently labeled molecules at nanomolar to picomolar concentrations through an open detection volume of ~1 fl that is created by a focused laser beam. From these fluctuations, the concentration and the diffusion time can be calculated. In addition, the molecular brightness of fluorescent particles can be obtained (35). When molecules labeled with two different fluorophores form complexes, the amount of complex formation can be easily monitored by cross-correlation analysis in a dual color set-up. Moreover, aggregate formation can be analyzed with high sensitivity by SIFT analysis (36).

We used α-syn labeled with fluorescent dyes to monitor the aggregations process with a single particle approach that has also been used to detect huntingtin and prion protein oligomers (40, 42, 43). After a 4-h incubation of 10–20 nM α-syn at room temperature, virtually no oligomeric forms were observed. In contrast, when ethanol was added at concentrations of 5–20%, small oligomers could be detected by FCS, cross-correlation analysis, and FIDA (Fig. 1). Diffusion times obtained for these oligomers by cross-correlation analysis indicated that the oligomers consisted on average of ~20 monomers. This was confirmed by the detection of particles with a corresponding increase in particle brightness by FIDA analysis (supplemental Table S1). When the sample was diluted in ethanol-free buffer, the process was reversible (data not shown). In contrast to oligomers formed at ethanol concentrations >30%, aggregation at intermediate concentrations of ethanol could be blocked by the addition of detergents, such as Nonidet P-40, and by BSA (Fig. 1D), which is in line with the findings of Munishkina et al. (39) that different oligomer species are formed depending on the concentration of ethanol. It has been described that BSA can bind the acidic C-terminal domain of α-syn (44). Therefore, we tested if BSA could block oligomer formation. Indeed, 0.01% BSA was sufficient to block oligomerization completely (Fig. 1D and supplemental Fig. S1). At the low concentration of α-syn used in our assay, no formation of amyloid-like large aggregates was detectable by highly sensitive SIFT analysis (41). This indicates that aggregation may be kinetically trapped at the level of small oligomers under these conditions.

Effect of Metal Ions on Synuclein Oligomer Formation—In contrast to other groups that used a combination of high protein and high metal ion concentrations (33), we studied the influence of Fe3+ concentrations close to physiological serum levels (5–20 μM) on the aggregation process with nanomolar α-syn concentrations. First, we screened various metal ions (Fe3+, Al3+, Cu2+, Mn2+, and Ca2+) in our assay. Of the metal ions tested, only Fe3+ and Al3+ resulted in an increase in aggregation (Fig. 2A and supplemental Fig. S2).
Notably, Fe$^{3+}$ (and to a lesser extent Al$^{3+}$) resulted in the formation of significantly larger oligomers that were detectable as a high intensity signal in scanned measurements by SIFT analysis (Fig. 2 and supplemental Fig. S2). Diffusion time analysis is not useful for these large aggregates, since large particles diffuse very slowly and are detected only inefficiently when no scanning device is used. Brightness analysis by FIDA indicated the formation of >100-mers (supplemental Table S1). To distinguish these aggregates from the small oligomers described above, we termed the small ones “intermediate I” and the large ones “intermediate II.”

Importantly, in these experiments, Fe$^{3+}$ only caused an effect on α-syn aggregation when added in the presence of intermediate concentrations of ethanol (Fig. 2, B and C). Fe$^{3+}$ affected aggregation of α-syn neither in the absence of ethanol nor at concentrations of ethanol >30%. Moreover, the addition of either Nonidet P-40 or BSA, which specifically inhibited the formation of the intermediate I at intermediate concentrations of ethanol, also inhibited the effect of Fe$^{3+}$. This indicates that the effect of Fe$^{3+}$ seems to depend on the presence of the intermediate species, which suggests a synergistic effect of Fe$^{3+}$ and ethanol on α-syn aggregation. To exclude the possibility that the observed effect of iron was related to the sodium phosphate buffer used in these experiments, we also analyzed aggregation in Tris buffer and obtained similar results (supplemental Fig. S3).

**AFM Analysis of Intermediates I and II**—In order to exclude an influence of the fluorescence label on the aggregation process we observed with confocal single particle fluorescence techniques, we chose AFM as a fluorescence-independent single particle detection method suited for low particle concentrations. The α-syn aggregates formed in the presence or absence of ethanol and/or Fe$^{3+}$ were imaged by tapping mode atomic force microscopy. AFM has been shown to be a powerful method for the characterization of protein aggregates (38). AFM has been used for the identification of the different morphological structures formed during α-syn aggregation (10, 11, 45).

AFM images of dissolved nonlabeled α-syn, incubated for 4 h at room temperature, show globular features with a height between 0.9 and 1.9 nm. Based on their size distribution in the AFM images and their comparison with images of other similar peptides with a similar molecular weight, these globular structures appear to be mostly monomers. Compared with freshly prepared protein samples, α-syn retained its globular structure over a period of several h (4 h) with no significant change in the size distribution and without noteworthy aggregation. The particle distribution was quite homogeneous. Assuming a cylindrical shape of the particle, the average calculated volume is around 200 nm$^3$ (Table S2). About 80% of the particles were smaller than 300 nm$^3$, and just about 5% had a volume between 400 and 500 nm$^3$.

The presence of 20% ethanol induced an increase of the particle size (Fig. 3). The average particle size was estimated to be ~34 nm, and the height was estimated to be 2.1 nm, with an average volume of 2500 nm$^3$ (Table S2). 75% of the particles were smaller than 3000 nm$^3$. The combination of 20% ethanol and 20 µM FeCl$_3$ showed an average particle size of 70 nm and 6.4 nm in height. The calculated volume of these particles was around 33,000 nm$^3$ (Table S2). Since the spatial resolution of our AFM measurements is not sufficient to infer a detailed structural arrangement of potential subunits within these aggregates, we chose a simple cylindrical model for the purpose of volume calculation.

Our AFM measurements confirm the results obtained by confocal single particle fluorescence measurements. Ethanol induces an ~10-fold increase in particle size, and ethanol/Fe$^{3+}$ induces an ~100-fold increase in particle size compared with monomeric α-synuclein. To study the potential influence of fluorescence dyes on the aggregation process, we compared labeled and nonlabeled α-syn. Coupling the fluorescence dye...
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A

B

C

FIGURE 2. Effect of Fe\textsuperscript{3+} and other metal ions on α-syn oligomerization. A, aggregation was monitored by cross-correlation amplitude (G(0)) in a mixture of α-syn labeled with Alexa-488 and α-syn labeled with Alexa-647 in samples containing either no ethanol (empty bars) or 20% ethanol (black bars) in the presence of 5 μM FeCl\textsubscript{3}, AlCl\textsubscript{3}, CaCl\textsubscript{2}, CuCl\textsubscript{2}, or MnCl\textsubscript{2}, respectively, and in controls. Data were obtained after ∼4 h of aggregation in triplicate measurements. Shown is the mean ± S.E. The corresponding two-dimensional intensity distribution histograms are provided in the supplemental materials (Fig. S2). B, two-dimensional intensity distribution histograms obtained in an independent experiment show that the synergistic action of ethanol and Fe\textsuperscript{3+} results in the formation of much larger aggregates than found with ethanol. Notably, Fe\textsuperscript{3+} alone does not induce aggregate formation. A detailed quantitative SIFT- and FIDA analysis of aggregate concentration and functionality on membrane conductivity (Fig. 4 and Table 1). Pore formation in the bilayer was reproducibly observed at a success rate of 12–15% (Table 1). These pores were permanently open. The distribution of conductance states obtained (Fig. 4B) indicates the presence of a smallest unit pore with a conductivity of about 50 picoammeters in 250 mM KCl.

The observed reversal potential in 250/20 mM KCl was \( V_{\text{rev}} = -21 ± 2 \text{ mV} \) (Fig. 4A); using the GHK approach, this corresponds to an ion selectivity of \( P_{\text{Cl}^-}/P_{\text{K}^+} \approx 2.75 \). The known Ca\textsuperscript{2+} channel blocker cobalt did not show an effect on the pores formed. Importantly, similar results were obtained with nonlabeled and labeled α-syn.

Intermediate I and II Are On-pathway in Regard to Amyloid Formation—It has previously been shown that preformed aggregates of α-syn can seed the generation of amyloid fibrils from α-syn monomers, arguing for a nucleation-dependent polymerization process (46). To analyze whether buffer conditions that favor formation of intermediate I and II oligomers at nanomolar concentrations of α-syn also accelerate amyloid formation at higher concentrations of α-syn, we used a thioflavin T assay. As expected (39), both 20% ethanol and 20% ethanol plus 100 μM Fe\textsuperscript{3+} accelerated amyloid formation (supplemental Fig. S4, A and B). Consistent with our results obtained in single particle measurements, generation of thioflavin T-positive aggregates was inhibited by 0.1% Nonidet P-40 (supplemental Fig. S4A). Next, we wanted to test whether the intermediate I and II oligomers have seeding properties, which would provide further evidence that they are on-pathway to amyloid structures. The samples used for seeding experiments were generated by overnight incubation of 0.1 mg/ml monomeric α-syn aggregates with 10% ethanol with or without 10 μM ferric chloride. As starting material for the different aggregation approaches monomeric, α-syn (1 mg/ml) containing 10% ethanol with or without 10 μM ferric chloride was used. Nonseeded samples (control reactions) and seeded samples were incubated under these buffer conditions. The seeding was started by adding 10% (v/v) preaggregated α-syn. This resulted in a reduced lag time and an increased thioflavin T fluorescence signal (supplemental Fig. S4C). Therefore, we conclude that both oligomer types can seed amyloid formation and are on-pathway to amyloid fibrils.
In agreement with the observed inhibitory effect of BSA on aggregation, BSA also inhibited pore formation in the bilayer assay (Table 1). To corroborate further that pore formation was α-syn-dependent, we added an α-syn-specific antibody (asy1; a gift from Poul Henning Jensen, University of Aarhus, Denmark) and an anti-Aβ antibody (6E10; Senetec) to the preincubation mix. As expected, only the anti-α-syn antibody was able to prevent pore formation (Table 2). To demonstrate that oligomers were responsible for the pore formation, the recently described oligomer-specific antibody A11 (9) was added to the preincubation mix. This oligomer-specific antibody also inhibited pore formation. Moreover, when applied to the cis chamber after integration of membrane pores into the lipid bilayer, the A11 antibody reduced conductivity by ~30%.

**Intermediates I and II Provide Suitable Target Structures for Drug Development**—In order to further validate intermediates I and II as targets for drug development, we compared the properties of these oligomers with the properties of α-syn oligomers present in vivo. Disease-associated α-syn aggregates in human patients and transgenic mouse models of PD have been reported to be SDS-resistant (48, 49). In our in vitro aggregation assay, 0.2% SDS efficiently blocked the de novo formation of intermediate I and intermediate II, similar to the effect of Nonidet P-40 described above. However, once formed, intermediate II was resistant to dissolution by SDS and Nonidet P-40, respectively, whereas intermediate I was completely dissolved by SDS and Nonidet P-40 (Fig. 5). This finding provides additional evidence that intermediate II oligomers share structural properties with disease-associated aggregates found in vivo. Furthermore, these data corroborate that iron-induced intermediate II oligomers are formed in a multistep pathway from detergent-labile intermediate I oligomers.

It has been reported previously that ion chelators, such as desferoxamine, have neuroprotective effects in animal models...
Interestingly, in post-mortem studies of PD patient brains, it has been shown that iron levels are increased and that the Fe³⁺/H₁₀₀₀¹⁺/Fe²⁺ ratio is shifted toward Fe³⁺ (22, 26, 53).

To test if the effect of iron depends on its oxidation state, freshly prepared Fe²⁺ stock solution was used in some experiments. In contrast to Fe³⁺ (ferric iron), Fe²⁺ (ferrous iron) was unable to induce formation of intermediate II (Fig. 6A). In line with these experiments, sodium ascorbate could strongly inhibit the Fe³⁺-induced formation of intermediate II (Fig. 6B). This effect was correlated with the reduction of ferric iron to ferrous iron (supplemental Fig. S5). Notably, sodium ascorbate had only a minor effect on the formation of Al³⁺-induced large oligomers (Fig. 6B), and H₂O₂ in concentrations of up to 1% did not induce the formation of intermediate II oligomers from intermediate I oligomers (data not shown). This suggests that the formation of intermediate II oligomers is dependent on the availability of trivalent ions and that this process is not due to α-syn oxidation by ferric iron. Based on these findings, we also tested the effect of the Fe³⁺ chelator desferroxamine in our assay. Desferroxamine efficiently blocked the generation of intermediate II oligomers (Fig. 6C).

### TABLE 2

| Fusion success rate | N   |
|--------------------|-----|
| α-syn              | 14  | 42  |
| α-syn + A11        | 0   | 42  |
| α-syn-Alexa488 + A11| 0   | 42  |
| α-syn + asy1       | 0   | 42  |
| α-syn + 6E10       | 12  | 42  |

of PD (30, 50–52). Interestingly, in post-mortem studies of PD patient brains, it has been shown that iron levels are increased (22, 26, 53) and that the Fe³⁺/Fe²⁺ ratio is shifted toward Fe³⁺ (54).

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Single Particle Characterization of α-Synuclein Oligomers

In preparation for use of our aggregation assay for compound screening, we employed the organic solvent DMSO instead of ethanol in the experiments described above, since compounds are usually supplied in DMSO. DMSO was able to mimic the effect of intermediate concentrations of ethanol in regard to the induction of intermediate I and II oligomers and in regard to amyloid formation in control experiments (Fig. 6A and supplemental Fig. S4D).

Compounds That Inhibit Synuclein Oligomer Formation Also Block Toxicity in Cell Culture—A major advantage of our single particle-based fluorescence approach is that it lends itself to high throughput screening applications. For example, a SIFT screening assay has recently been employed successfully in the search for new anti-prion compounds (55). In a pilot study, we tested eight NBB derivatives that were identified as anti-prion compounds by us previously (55). Some of these compounds were recently shown to also inhibit poly(Q) aggregation in vitro and in vivo (56). In addition, we used baicalein, which has previously been shown to inhibit fibrillation of α-syn (57). For compound testing, aggregation was induced by DMSO instead of ethanol, since compound stock solutions were supplied in DMSO and since DMSO was shown to mimic the effect of ethanol in control experiments (Figs. 6B and S4D). Baicalein at a concentration of 10 μM efficiently blocked aggregation in our assay system. Similarly, aggregation was inhibited by some of the tested NBB compounds. Interestingly, inhibitory activity was strictly dependent on the presence of two hydroxyl groups at the benzohydrazide ring, indicating a well defined structure-activity relationship (Fig. 7, A and B).

Unfortunately, the NBB compounds showed toxicity at micromolar concentrations in our cell culture model for α-syn-dependent toxicity (see below), so that we were not able to confirm this in vitro activity in cell culture. However, baicalein showed no toxicity up to 10 μM in cell culture and could therefore be used to study a potential effect on α-syn-dependent toxicity. It has been shown previously that inducible expression of α-syn (A53T) in PC12 cells results in endoplasmic reticulum stress and mitochondrial dysfunction, leading finally to cell death. This apoptotic mechanism could be partially blocked by a pancaspase inhibitor (benzyloxy carbonyl-VAD) (58). Interestingly, in our experiments, overexpression of the mutant E46K α-syn resulted in the same reversible toxicity.

DISCUSSION

Protein aggregation and amyloid formation are the key molecular events in a number of human diseases, such as Alzheimer disease, CAG repeat diseases, prion disease, and Parkinson disease (1). Recent research suggests that oligomeric aggregation intermediates rather than mature amyloid fibrils represent the principal toxic aggregate species, which is essential in the pathogenesis of neurodegeneration and a potential therapeutic target in these diseases (12, 14). However, the molecular events that are relevant to the formation of disease-associated toxic oligomer species and the biophysical and func-
Single Particle Characterization of α-Synuclein Oligomers

![Diagram of aggregation pathways of α-syn](image)

The oligomer formation of α-synuclein is characterized by the formation of pores that lead to permeabilization of cellular membranes. Goldberg and colleagues proposed the model of annular protofibrils as toxic species in the aggregation process. In agreement with this model, annular Aβ structures could be isolated from AD brains. On the other hand, several lines of evidence implicate iron ions in the generation of α-synuclein aggregates and in disease progression in Parkinson disease. However, the underlying molecular events have not been elucidated so far. Therefore, the aim of our study was to improve the understanding of the molecular events in the pathological processes underlying the formation and toxicity of α-syn oligomers and the role of iron in Parkinson disease.

We used a combination of three independent single particle-based techniques to analyze aggregation pathways and oligomer formation of α-syn. By confocal single molecule fluorescence techniques in combination with atomic force microscopy and single pore electrophysiology, we could comprehensively characterize for the first time two different oligomer species that were both on-pathway to amyloid fibrils (intermediate I and intermediate II). Notably, intermediate II oligomers, which could be specifically induced by low micromolar concentrations of ferric iron, could form ion-permeable pores in a planar lipid bilayer assay (Fig. 8).

It has been suggested that oligomers formed by different amyloidogenic proteins share specific structural features and similar pathogenetic mechanisms. The recently described oligomer-specific antibody A11 has been shown to bind to specific oligomer species formed from a number of proteins, including α-syn (9), and to label disease-specific protein aggregates in a number of human diseases (9, 60–63). Moreover, A11 was able to inhibit oligomer toxicity in cell culture (9). The finding that A11 inhibited pore formation and reduced conductivity of membrane-inserted pores in our experiments thus provides evidence that the oligomer species we generated in vitro share structural properties with oligomers that have been detected with this antibody in affected patient brains, which supports the view that the pore-forming intermediate II oligomers we characterized in our cell-free studies are also present in affected PD brains. The concept that intermediate II oligomers are formed in a multistep pathway involving iron-induced changes in aggregate structure and that intermediate II oligomers share structural features with α-syn aggregates found in human disease is further affirmed by our experiments that show that intermediate II oligomers are SDS-resistant, whereas intermediate I oligomers are SDS-sensitive, and SDS inhibits de novo formation of intermediate II oligomers. Detergent-resistant α-syn oligomers (19, 49), including annular structures (18), have been isolated from brain tissue of human patients and transgenic animal models. Isolation and electrophysiological characterization of such oligomers from PD brains will be the next step to establish a complete picture how α-syn oligomers trigger neuronal death.

A novel key result of our single particle-based experiments is the crucial role of ferric iron in inducing pore-forming oligomers. These findings shed more light on the underlying toxic mechanism of synucleinopathies and could provide an explanation of why brain areas with higher iron content are more affected in brains of Parkinson’s disease patients (22, 24). It has been shown recently by transcranial sonography that PD patients and even asymptomatic mutation carriers in familial PD show iron-related changes of the substantia nigra (24). Moreover, in post-mortem studies of PD patient brains, it has been shown that the Fe^{3+}/Fe^{2+} ratio is shifted toward Fe^{3+} (54), which corresponds to our molecular findings that show a specific induction of intermediate II oligomers only by ferric iron. Interestingly, mice administered iron during a developmental period equivalent to the first human year of life displayed progressive midbrain neurodegeneration (64). Moreover, in humans, it has been reported that in patients with atypical neurodegeneration related to pathological brain iron accumulation, also α-syn deposition can be detected (27).

In our present work, we combined single particle-based approaches with experiments addressing α-synuclein-dependent toxicity in neuronal cell cultures. We could confirm and extend previous observations in human BE-M17 neuroblastoma cells regarding a synergistic toxic effect of iron and α-syn (32). We used SH-SYSY cells overexpressing α-syn (wild type, A30P, and A53T mutants) and measured depletion of cellular ATP as a marker for early cytotoxicity. The prolonged treatment with high iron levels resulted in a reduction of cellular ATP. Interestingly, the cell clones overexpressing mutant α-syn were significantly more vulnerable to the iron-induced toxicity (supplemental Fig. S6). This effect on the ATP level could be confirmed by the Alamar Blue assay (data not shown). Importantly, neither the stable overexpression of wild type α-syn nor of mutant α-syn resulted in cytotoxicity over time in this cell culture model. Thus, we conclude that a synergistic effect of iron and synuclein in regard to cytotoxicity can be observed also on the cellular level.

Also, we identified several compounds capable of inhibiting oligomer formation in our cell-free system. We show that

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5 C. G. Glabe, unpublished results.
baicalein, which blocks formation of small oligomeric forms in our cell-free system, is able to prevent cell death in a dose-dependent manner (Fig. 7C). This result suggests that α-syn aggregation might be involved in cell death in this model system. It is already known that baicalein can form a Schiff base with a lysine of α-syn, preventing the fibrillation process (57). In that study, baicalein was also able to disaggregate existing fibrils in vitro to high molecular weight oligomer species. Here we used a cellular system where fibril formation has never been observed. Therefore, we exclude a role of fibril deaggregation activity of baicalein in this model. However, the binding of baicalein to the monomeric α-syn as it was proposed by Zhu et al. would also interfere with the α-syn aggregation process in our cell culture model, preventing the generation of toxic oligomers. Taken together with our findings in the cell-free SIFT assay, our finding that baicalein was also able to inhibit α-syn-dependent toxicity in a neuronal cell culture model further argues for a significant role of oligomer forms similar to those obtained and characterized in our in vitro experiments also in physiological conditions in neuronal cells. Thus, our results provide a potential disease mechanism regarding the role of toxic oligomer species in neurodegenerative diseases. Based on our results, we propose that the oligomerization of α-syn is a well defined multistep pathway with a crucial role of ferric iron that can result in pore formation as a final mechanism of toxicity. A detailed biophysical and electrophysiological characterization of oligomer pores at the single particle level (e.g. presence of a smallest unit pore with a conductivity of about 50 picosiemens) has not been described so far. Moreover, the consistent effects of aggregation inhibitors at the level of protein aggregation, pore formation, and cellular toxicity may provide new approaches for drug discovery, since the confocal single particle techniques used in our experiments lend themselves to application in high throughput molecular screening of large compound libraries.

REFERENCES

1. Koo, E. H., Lansbury, P. T., Jr., and Kelly, J. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9899–9990
2. Forman, M. S., Lee, V. M., and Trojanowski, J. Q. (2005) Neuron 47, 479–482
3. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J. T., Schols, L., and Riess, O. (1998) Nat. Genet. 18, 106–108
4. Polymereopoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stroenos, E. S., Chandrasekarappan, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047
5. Goedert, M. (2001) Nat. Rev. Neurosci. 2, 492–501
6. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) Nature 388, 839–840
7. Zarranz, J. J., Alegré, J., Gomez-Esteban, J. C., Leccano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez, T. E., del Ser, T., Munoz, D. G., and de Yebeles, J. G. (2004) Ann. Neurol. 55, 164–173
8. Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Karcher, G., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M. R., Muenter, M., Baptista, M., Miller, D., Blencowe, J., Hardy, J., and Ghwin-Hardy, K. (2003) Science 302, 841
9. Kayed, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cottman, C. W., and Glabe, C. G. (2003) Science 300, 486–489
10. Volles, M. J., Lee, S. J., Rochet, J. C., Stittlerman, M. D., Ding, T. T., Kessler, I. C., and Lansbury, P. T., Jr. (2001) Biochemistry 40, 7812–7819
11. Fink, A. L. (2006) Acc. Chem. Res. 39, 628–634
12. Kayed, R., Sokolov, Y., Edmonds, B., McIntire, T. M., Milton, S. C., Hall, J. E., and Glabe, C. G. (2004) J. Biol. Chem. 279, 46363–46366
13. Gosavi, N., Lee, H. J., Lee, J. S., Patel, S., and Lee, S. J. (2002) J. Biol. Chem. 277, 48984–48992
14. Bacciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) Nature 416, 507–511
15. Volles, M. J., and Lansbury, P. T., Jr. (2002) Biochemistry 41, 4595–4602
16. Tanaka, Y., Engeland, S., Ishigaki, S., Rao, R. K., Wanner, T., Tanzi, R. E., Sawa, A., Dawson, L., Dawson, T. M., and Ross, C. A. (2001) Hum. Mol. Genet. 10, 919–926
17. El Agnaf, O. M., Salem, S. A., Paleologou, K. E., Curran, D. M., Gibson, M. J., Court, J. A., Schlommaecher, M. G., and Allsop, D. (2006) FEBS J. 20, 419–425
18. Pountney, D. L., Lowe, R., Quilty, M., Vickers, J. C., Voelcker, N. H., and Gai, W. P. (2004) J. Neurochem. 90, 502–512
19. Sharon, R., Bar-Joseph, I., Frosch, M. P., Walsh, D. M., Hamilton, J. A., and Selkoe, D. J. (2003) Neuron 37, 583–595
20. Gorell, J. M., Johnson, C. C., Rikoss, G. A., Peterson, E. L., Kortsch, G. X., Brown, G. G., and Richardson, R. J. (1997) Neurology 48, 650–658
21. Zayed, Y., Ducic, S., Campanella, G., Panisset, J. C., Andre, P., Masson, H., and Roy, M. (1999) Can. J. Neurol. Sci. 17, 286–291
22. Gotz, M. E., Double, K., Gerlach, M., Youndm, B. M., and Riederer, P. (2004) Ann. N. Y. Acad. Sci. 1012, 193–208
23. Gaeta, A., and Hider, R. C. (2005) Br. J. Pharmacol. 146, 1041–1059
24. Berg, D. (2007) Neurochem. Res. 32, 1646–1654
25. Michaeli, S., Oz, G., Sorce, D. J., Garwood, M., Ugurbil, K., Majestic, S., and Tuite, P. (2007) Mov. Disord. 22, 334–340
26. Oakley, A. E., Collingswood, J. F., Dobson, J., Love, G., Parrott, H. R., Edwards, J. A., Elstner, M., and Morris, C. M. (2007) Neurology 68, 1820–1825
27. Tofaris, G. K., Reveiz, T., Jacques, T. S., Papacostas, S., and Chataway, J. (2007) Arch. Neurol. 64, 280–282
28. Ben Shachar, D., Behrends, C., Langer, C. A., Boteva, R., Bottcher, U. M., and Schaffar, G. (2004) Biophys. J. 83, 1315–1325
29. Ben Shachar, D., Behrends, C., Langer, C. A., Boteva, R., Bottcher, U. M., and Schaffar, G. (2004) Biophys. J. 83, 1315–1325
30.Goedert, M. (2001) J. Biol. Chem. 276, 44284–44296
31. Nuensch, T., Kupper, F., Mehnert, T., Odoe, S., Haass, C., Kahle, P. J., and Beyer, K. (2004) J. Biol. Chem. 279, 21966–21975
32. Kask, P., Pal, A., Ullmann, D., and Gall, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13756–13761
33. Biernacki, K., Giese, A., Schulz-Schaeffer, W., Zerr, I., Poser, S., Eigen, M., and Kretzschmar, H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5468–5473
34. Woodbury, D. J., and Hall, J. E. (1988) Biophys. J. 54, 345–349
35. Colton, R. J. (1997) Procedures in Scanning Probe Microscopy, John Wiley & Sons, Inc., New York
36. Munishkina, L. A., Phelan, C., Uversky, V. N., and Fink, A. L. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6438–6443
Schaffar, G., Rao, B. V., Giese, A., Kretzschmar, H., Siegers, K., and Hartl, F. U. (2006) *Mol. Cell* **23**, 887–897.

43. Levin, J., Bertsch, U., Kretzschmar, H., and Giese, A. (2005) *Biochem. Biophys. Res. Commun.* **329**, 1200–1207.

44. El Agnaf, O. M., Paleologou, K. E., Greer, B., Abogrein, A. M., King, J. E., Salem, S. A., Fullwood, N. J., Benson, F. E., Hewitt, R., Ford, K. J., Martin, F. L., Harriott, P., Cookson, M. R., and Allsop, D. (2004) *FASEB J.* **18**, 1315–1317.

45. Lashuel, H. A., Petre, B. M., Wall, J., Simon, M., Nowak, R. J., Walz, T., and Lansbury, P. T., Jr. (2002) *J. Mol. Biol.* **322**, 1089–1102.

46. Wood, S. J., Wypych, J., Steavenson, S., Louis, J. C., Citron, M., and Biere, A. L. (1999) *J. Biol. Chem.* **274**, 19509–19512.

47. Meuser, D., Splitt, H., Wagner, R., and Schrempf, H. (1999) *FEBS Lett.* **462**, 447–452.

48. Lee, M. K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A. S., Dawson, T. M., Copeland, N. G., Jenkins, N. A., and Price, D. L. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 8968–8973.

49. Kahle, P. J., Neumann, M., Ozmen, L., Muller, V., Odoy, S., Okamoto, N., Jacobsen, H., Iwatsubo, T., Trojanowski, J. Q., Takahashi, H., Wakabayashi, K., Bogdanovic, N., Riederer, P., Kretzschmar, H. A., and Haass, C. (2001) *Ann. J. Pathol.* **159**, 2215–2225.

50. Youdim, M. B., Stephenson, G., and Ben Shachar, D. (2004) *Ann. N. Y. Acad. Sci.* **1012**, 306–325.

51. Shachar, D. B., Kahana, N., Kampel, V., Warshawsky, A., and Youdim, M. B. (2004) *Neuropharmacology* **46**, 254–263.

52. Obata, T. (2006) *Eur. J. Pharmacol.* **539**, 34–38.

53. Dexter, D. T., Wells, F. R., Lees, A. J., Agid, F., Agid, Y., Jenner, P., and Marsden, C. D. (1989) *J. Neurochem.* **52**, 1830–1836.

54. Riederer, P., Sofic, E., Rausch, W. D., Schmidt, B., Reynolds, G. P., Jellinger, K., and Youdim, M. B. (1989) *J. Neurochem.* **52**, 515–520.

55. Bertsch, U., Winklhofer, K. F., Hirschberger, T., Bieschke, J., Weber, P., Hartl, F. U., Tavan, P., Tatzelt, J., Kretzschmar, H. A., and Giese, A. (2005) *J. Virol.* **79**, 7785–7791.

56. Schiffer, N. W., Broadley, S. A., Hirschberger, T., Tavan, P., Kretzschmar, H. A., Giese, A., Haass, C., Hartl, F. U., and Schmid, B. (2007) *J. Biol. Chem.* **282**, 9195–9203.

57. Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F., and Fink, A. L. (2004) *J. Biol. Chem.* **279**, 26846–26857.

58. Smith, W. W., Jiang, H., Pei, Z., Tanaka, Y., Morita, H., Sawa, A., Dawson, V. L., Dawson, T. M., and Ross, C. A. (2005) *Hum. Mol. Genet.* **14**, 3801–3811.

59. Goldberg, M. S., and Lansbury, P. T., Jr. (2000) *Nat. Cell Biol.* **2**, E115–E119.

60. Glabe, C. G., and Kayed, R. (2006) *Neurology* **66**, S74–S78.

61. Kokubo, H., Kayed, R., Glabe, C. G., and Yamaguchi, H. (2005) *Brain Res.* **1031**, 222–228.

62. Luibl, V., Isas, J. M., Kayed, R., Glabe, C. G., Langen, R., and Chen, J. (2006) *J. Clin. Invest.* **116**, 378–385.

63. Sanbe, A., Osinska, H., Saffitz, J. E., Glabe, C. G., Kayed, R., Maloyan, A., and Robbins, J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 10132–10136.

64. Kaur, D., Peng, J., Chinta, S. J., Rajagopalan, S., Di Monte, D. A., Cherny, R. A., and Andersen, J. K. (2007) *Neurobiol. Aging* **28**, 907–913.

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