

α -Synuclein occurs physiologically as a helically folded tetramer that resists aggregation

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Parkinson's disease is the second most common neurodegenerative disorder^{1,2}. Growing evidence indicates a causative role of misfolded forms of the protein α -synuclein in the pathogenesis of Parkinson's disease^{3,4}. Intraneuronal aggregates of α -synuclein occur in Lewy bodies and Lewy neurites⁵, the cytopathological hallmarks of Parkinson's disease and related disorders called synucleinopathies⁴. α -Synuclein has long been defined as a 'natively unfolded' monomer of about 14 kDa (ref. 6) that is believed to acquire α -helical secondary structure only upon binding to lipid vesicles⁷. This concept derives from the widespread use of recombinant bacterial expression protocols for *in vitro* studies, and of overexpression, sample heating and/or denaturing gels for cell culture and tissue studies. In contrast, we report that endogenous α -synuclein isolated and analysed under non-denaturing conditions from neuronal and non-neuronal cell lines, brain tissue and living human cells occurs in large part as a folded tetramer of about 58 kDa. Several methods, including analytical ultracentrifugation, scanning transmission electron microscopy and *in vitro* cell crosslinking confirmed the occurrence of the tetramer. Native, cell-derived α -synuclein showed α -helical structure without lipid addition and had much greater lipid-binding capacity than the recombinant α -synuclein studied heretofore. Whereas recombinantly expressed monomers readily aggregated into amyloid-like fibrils *in vitro*, native human tetramers underwent little or no amyloid-like aggregation. On the basis of these findings, we propose that destabilization of the helically folded tetramer precedes α -synuclein misfolding and aggregation in Parkinson's disease and other human synucleinopathies, and that small molecules that stabilize the physiological tetramer could reduce α -synuclein pathogenicity.

To identify the native state of α -synuclein in cells while avoiding the potential breakdown of physiological assemblies by detergents, we initially used native gel electrophoresis. α -Synuclein is expressed endogenously in many cell types, so we chose to analyse the dopaminergic human neuroblastoma line, M17D (ref. 8) and the commonly used cell lines HEK293, HeLa and COS-7. Each of these cell lines predominantly contained a non-denatured α -synuclein-immunoreactive species migrating in blue native-polyacrylamide gel electrophoresis (BN-PAGE) at ~45–50 kDa (Fig. 1a, lanes 1–4). Because these initial results suggested an apparently stable oligomeric form under native conditions, we next probed the endogenous state of α -synuclein in normal brain. The frontal cortex of wild-type mice also revealed a ~45–50 kDa form of endogenous α -synuclein as the main species in the buffer-soluble fraction (Fig. 1a, lane 6).

To assess the state of endogenous α -synuclein in living human cells, we examined freshly collected red blood cells (RBCs), which were recently found to have high α -synuclein expression⁹. Human RBCs contained a ~45–50 kDa α -synuclein immunoreactive band on BN-PAGE (Fig. 1a, lane 5). As a second non-denaturing gel system that precludes effects of the Coomassie dye used in BN-PAGE, we performed clear native-PAGE (CN-PAGE)¹⁰. The main α -synuclein species in all samples migrated at ~55–60 kDa, suggesting a tetramer (theoretical mass of monomer = 14,460 Da) (Fig. 1b, lanes 1–6). The

better resolution of CN-PAGE without Coomassie dye also revealed small amounts of apparent monomers running below the 14 kDa molecular weight marker (Fig. 1b, lanes 1–4, 6) and distinguished the small differences in amino acid length of the human and mouse α -synuclein monomers and putative tetramers (Fig. 1b, lane 6). The endogenous ~55–60 kDa species was detected by monoclonal α -synuclein antibodies Syn1, Syn211 and LB509 and polyclonal antibody C20 in both native gel systems.

Because the migration of a protein on BN- or CN-PAGE does not depend solely on its mass but also on its conformation and charge, we used *in vitro* crosslinking to preserve the assembled state of the putative α -synuclein oligomer, followed by denaturing SDS-PAGE. We observed SDS-stable α -synuclein bands migrating at the expected positions of a tetramer (~55 kDa) and non-crosslinked monomer in all

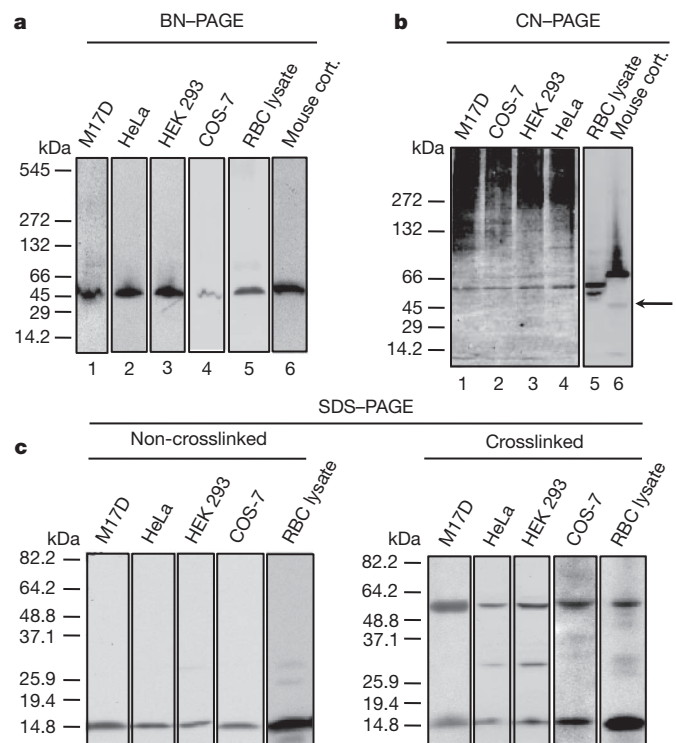


Figure 1 | Western blot analysis of lysates of M17D, HeLa, HEK293 and COS-7 cells, mouse cortex and human RBCs probed for endogenous α -synuclein. **a**, BN-PAGE. **b**, CN-PAGE. The band just below the main ~55–60 kDa RBC species (lane 6) may represent an alternatively spliced form of α -synuclein. Arrow marks a possible dimeric species. **c**, Left, SDS-PAGE/western blot (antibody C20) analysis of cell lysates without crosslinking. Right, proteins were crosslinked in intact living cells with membrane permeable disuccinimidyl suberate (DSS) (M17D, HeLa, HEK 293, COS-7) or in RBC lysate with water soluble bis(sulphosuccinimidyl) suberate (BS³) and then run on SDS-PAGE.

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cells, plus some putative dimer in the HeLa, HEK and red blood cells (right panel of Fig. 1c). This *in vivo* crosslinking supports the existence of native tetramers in cells. We performed two-dimensional gel analysis after the *in vivo* crosslinking; that is, isoelectric focusing (IEF) to separate proteins by charge in a pH gradient followed by denaturing SDS-PAGE. The higher-migrating α -synuclein species in the cross-linked RBC lysates had the same pK_a as monomers, within the limits of IEF resolution (Supplementary Fig. 1), consistent with their being homo-oligomers.

Next, we developed a non-denaturing method to purify native α -synuclein from soluble RBC lysates (see Methods and Nature Protocol Exchange (<http://www.nature.com/protocolexchange/protocols/2136>)). This allowed us to estimate the mass of native α -synuclein based on distinct measurement principles that are not affected by protein conformation, unlike gel electrophoresis. Scanning transmission electron microscopy (STEM) is useful for measuring the masses of purified, non-covalently bonded complexes that may not resist ionization during mass spectrometry^{11,12}. STEM images of α -synuclein purified under non-denaturing conditions from human RBCs (Supplementary Fig. 2) yielded a homogenous distribution of roughly spherical particles measuring ~ 3.0 – 3.5 nm in diameter (Fig. 2a). Unbiased automatic sampling of 1,000 particles gave a size distribution pattern with a peak at ~ 55 kDa (Fig. 2b). Importantly, we next applied sedimentation equilibrium analytical ultracentrifugation (SE-AUC), a technique commonly used to establish the oligomeric state of native proteins independent of their conformation. SE-AUC analysis of purified, native RBC α -synuclein performed at three different concentrations and at different rotor speeds yielded an average molecular weight of 57.8 kDa (4.78 Svedbergs), strongly supporting a tetrameric assembly state (Fig. 2c).

Numerous studies have reported conformational changes in α -synuclein, with a focus on the natively unfolded recombinant monomer undergoing a random coil to α -helix transition upon *in vitro* interaction with small lipid vesicles⁷. This change is believed to be relevant to the poorly defined physiological function of α -synuclein in cells and could potentially decrease the likelihood of its aggregation into β -sheet-rich neurotoxic assemblies¹³. Unexpectedly, we found

that circular dichroism spectra of the human RBC tetramer purified under non-denaturing conditions showed two minima of mean residue ellipticity at 222 and 208 nm (Fig. 3a), characteristic of an α -helically folded protein¹⁴. This result is inconsistent with the common assumption that α -synuclein is natively unfolded. The addition of negatively charged, small unilamellar lipid vesicles (SUVs) did not induce a significant conformational change in the native tetramer by circular dichroism (Fig. 3a), but a random coil to α -helical conversion did occur (as reported) with recombinant monomer that had been expressed in bacteria (Fig. 3b). Incubation of the purified RBC α -synuclein tetramer with Lipidex 1000, a reagent used to strip proteins of bound lipids and fatty acids¹⁵, did not change the conformation of the α -helical α -synuclein tetramer (Supplementary Fig. 3), suggesting that significant lipid association is not required to maintain the folded structure of cellular α -synuclein. To support this possibility, we conducted a quantitative elemental phosphate analysis¹⁶ on the purified native α -synuclein to search for phospholipid. We obtained an average value of 0.25 mol phosphate per mol α -synuclein, making a significant presence of phospholipids on the α -helical α -synuclein purified from normal cells unlikely. Because post-translational modifications also could have an impact on the conformational differences between the native human RBC tetramer and the bacterially expressed, recombinant human monomer, we performed mass spectrometry. The recombinant protein showed a mass peak at 14,462 kDa, very close to the theoretical predicted mass of 14,460 kDa, whereas the purified erythrocyte α -synuclein showed a peak at 14,505 kDa, indicative of only an N- α -acetylation commonly present on human proteins (theoretical predicted mass = 14,502 kDa) (Supplementary Fig. 4).

To validate the above results obtained on RBC α -synuclein using a different human cell type and a different non-denaturing purification method, we isolated α -synuclein from a M17D human neuroblastoma cell line stably overexpressing wild-type human α -synuclein (3D5 cells¹⁷). α -Synuclein from untransfected M17D cell lysates migrated above bacterially expressed α -synuclein of confirmed random coil structure on CN-PAGE (Supplementary Fig. 5A). This higher electrophoretic migration was also true of native (α -helical) but not

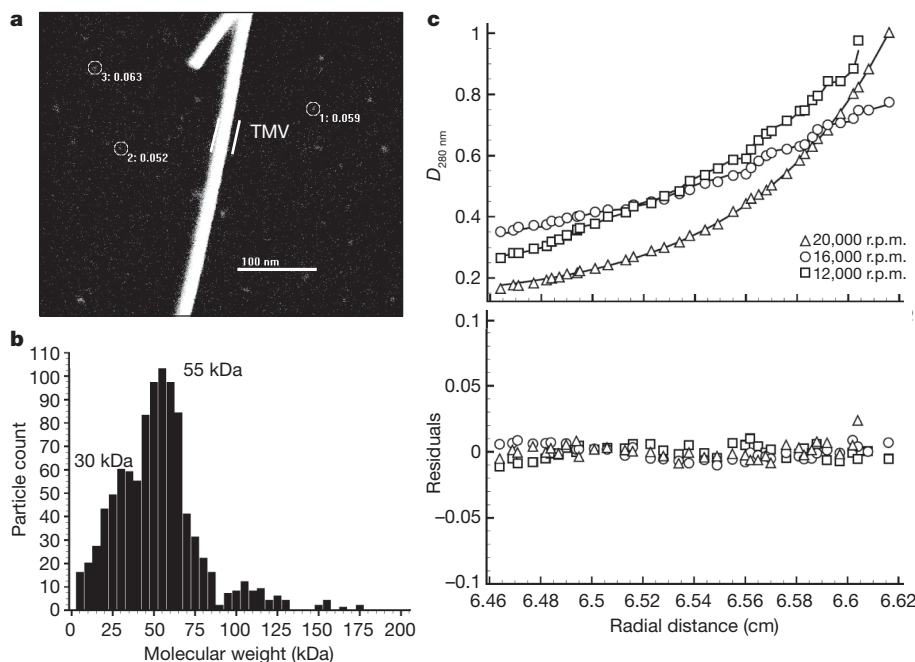


Figure 2 | Sizing analyses of α -synuclein from human RBCs.

a, Representative large-angle dark-field STEM image of purified α -synuclein from human RBCs. A few representative particles are circled. As an internal size standard, tobacco mosaic virus (TMV) helical rod was included during electron microscopy specimen preparation. **b**, Mass histogram (bin size = 5 kDa) of 1,000 automatically selected α -synuclein particles. **c**, SE-AUC of purified,

native RBC α -synuclein. Top panel shows the individual experimental analyses fitting an ideal single-species model to the equilibrium data obtained at 11,612, 20,644 and 32,256g for 1.1 mg ml^{-1} α -synuclein solution. The fitting yielded a molecular weight of 57,753 Da (standard deviation (s.d.) ± 655.199) with a root mean squared deviation of 0.004533. D , attenuation. Bottom panel shows an overlay of the residuals of data and theoretical fit for the three different speeds.

denatured (random coil) purified RBC α -synuclein (Supplementary Fig. 5B). After α -synuclein was purified from the stably transfected 3D5 cell line or from RBCs, the two differentially purified and α -helically folded (by circular dichroism) cellular proteins co-migrated at ~ 55 – 60 kDa on CN-PAGE, as expected (Supplementary Fig. 6). Unbiased, automated STEM measurements of 3,000 particles revealed that the 3D5 neuroblastoma cells contained α -synuclein tetramers of closely similar estimated molecular weight (peak mass ~ 55 kDa) to those of the RBC α -synuclein (Supplementary Fig. 7; compare to Fig. 2b). Circular dichroism spectroscopy revealed the purified 3D5 cell α -synuclein to have two minima of mean residue ellipticity at 222 and 208 nm (Supplementary Fig. 8). To further exclude artefacts arising during purification of cellular α -synuclein such as adventitious association of biomolecules (for example, cellular lipids not removed by Lipidex 1000) that artificially fold the protein, we repeated our experiments with the 3D5 parental line M17D, which has only low levels of endogenous α -synuclein. We added ('spiked') bacterially expressed recombinant human monomer onto the M17D cells before performing cell lysis and the full purification, and then assayed its structural properties. This exposure to cell lysates and the purification procedure led to no induction of helical folding in the recombinant human α -synuclein (Supplementary Fig. 9), whereas simultaneously purified 3D5 cell human α -synuclein did show this conformation, supporting our conclusion that α -helically folded α -synuclein does not arise owing to artificial manipulation of the protein.

Membrane association has been viewed as a principal functional property of α -synuclein *in vitro*⁷ and in living cells¹⁸. We searched for differential binding of recombinant monomeric human α -synuclein versus RBC tetrameric human α -synuclein to a lipid membrane using surface plasmon resonance (SPR). Because recombinant α -synuclein is reported to have preferential affinity for negatively charged lipids, especially phosphatidyl serine⁷, we chose a mixed phosphatidyl choline and phosphatidyl serine (PC/PS) membrane as a model membrane. Exposure of a PC/PS membrane to cell-derived, purified native α -synuclein in a Biacore instrument produced a markedly increased resonance angle shift compared to conventional recombinant monomers at identical concentrations in solution (Fig. 3c), indicating dramatically

increased lipid binding. Fitting a dilution series of α -synuclein tetramer injections to a two-state binding model (Supplementary Fig. 10) gave an apparent dissociation constant of $K_{app} = 56 \pm 61$ nM, which is about two orders of magnitude lower than values obtained for recombinant monomer in analogous SPR studies¹⁹. We next tested the amyloid aggregation propensity of the distinct species in a Thioflavin T (ThT) fluorescence assay. Monomeric and tetrameric α -synuclein showed very different characteristics, with samples of purified cellular α -synuclein incubated under identical conditions showing no evidence of fibril formation in a time (10 days) more than sufficient to form mature, ThT-bound fibrils from equivalent amounts of unfolded recombinant α -synuclein (Fig. 3d). Analysis of protein concentration in the solution after the 10-day incubation showed that the RBC α -synuclein was still present and soluble, making non-amyloid (that is, ThT-negative) aggregation of the tetramers unlikely. Interestingly, melting curves of purified tetrameric α -synuclein showed that heat denaturation (at 95 °C) seemed irreversible under our conditions (Supplementary Fig. 11).

Our experiments provide several independent lines of evidence that endogenous cellular α -synuclein exists in large part as an α -helically folded, ~ 58 kDa tetramer under native conditions. This finding is in contrast to many biophysical and biochemical studies describing α -synuclein as a natively unfolded ~ 14 kDa monomer. In an early study of bacterially expressed recombinant protein purified under non-denaturing conditions or with heat treatment, no conformational differences were observed, and it was concluded that α -synuclein is a natively unfolded monomer⁶. This suggests problems in generating properly folded protein in *Escherichia coli*, although a modified bacterial expression protocol avoiding heating and denaturants has recently been found to yield a helical α -synuclein tetramer closely resembling the species found by us in native human samples (W. Wang *et al.*, personal communication). The reasons for the conformational differences observed in these two bacterial studies are unknown. Using gel filtration on unfolded recombinant α -synuclein also showed an apparent molecular weight of ~ 60 kDa in some earlier studies; the data were interpreted as a decrease in mobility of the extended state of an unfolded protein in the tested matrices⁶. This suggests the possibility

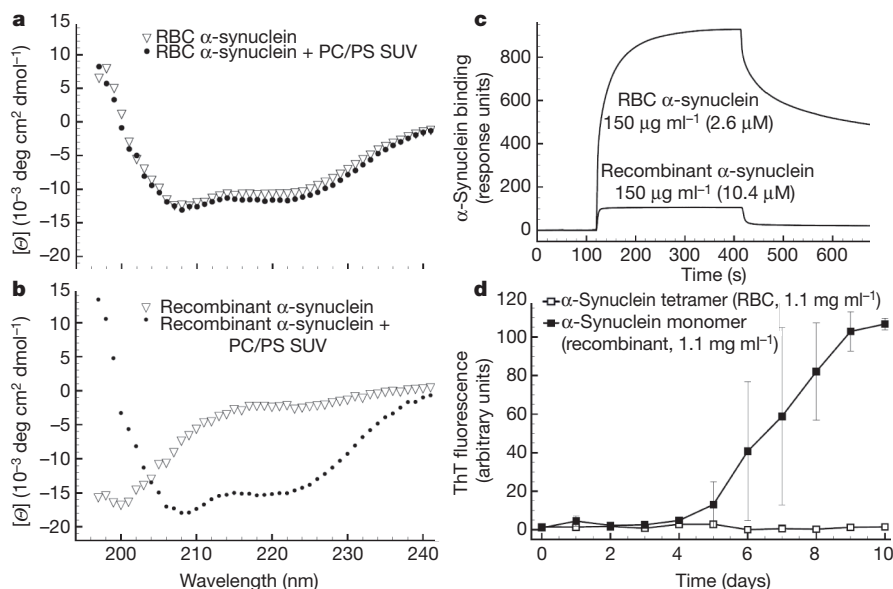


Figure 3 | Comparative analyses of native (cell-derived) and bacterial α -synuclein. **a**, Circular dichroism spectra of native tetrameric α -synuclein (isolated under non-denaturing conditions from human RBCs) before versus after addition of POPC/POPS SUVs (PC/PS 4:1; protein:lipid 1:500). **b**, Circular dichroism spectra of recombinant α -synuclein monomer purified from *E. coli*, alone and with addition of PC/PS SUVs (protein/lipid 1:500). **c**, SPR sensorgram of equal protein concentrations of α -synuclein

recombinant monomer versus endogenous tetramer injected on a L1 chip covered with a PC/PS membrane. **d**, Amyloid-type aggregation kinetics of recombinant α -synuclein monomer versus native RBC tetramer monitored by ThT fluorescence; average values from 3 independent experiments (error bars show s.d.; some s.d. values for RBC-derived α -synuclein are smaller than the symbol size).

of a similar hydrodynamic radius for the unfolded monomer and the more compact, helically folded tetramer, making gel filtration an unreliable indicator (and therefore not used here). Our evidence for a tetrameric molecular mass of endogenous α -synuclein was particularly supported by the analytical ultracentrifugation and the unbiased STEM analysis, both of which sizing methods are not based on conformation. The STEM sizing was performed on intrinsic α -synuclein isolated from two cell types and using two distinct non-denaturing procedures.

Our apparent disagreement with most published findings on the monomeric state of α -synuclein in cells and brain tissue, usually as judged by SDS-PAGE and western blotting, can be explained by the common use of denaturing detergents. Our tetramer aggregation data (Fig. 3d) are consistent with a recent report describing non-neurotoxic, aggregation-resistant α -synuclein oligomers *in vivo*²⁰. Moreover, an oligomeric species of α -synuclein (size undefined) was observed by *in vivo* fluorescence lifetime imaging in an intact cell culture model²¹. Given the close match between our observed molecular weights using SE-AUC (Fig. 2c) and STEM (Fig. 2b and Supplementary Fig. 7) and the theoretical weight of a tetramer, the detection of a tetrameric band on denaturing gels after *in vivo* crosslinking (Fig. 1c), and the IEF evidence post-crosslinking that the endogenous tetramer and dimer bands have pK_a values similar to that of a monomer (Supplementary Fig. 1), we conclude that the predominant physiological species of α -synuclein in cells and brain is a helically folded tetramer, although minor and variable amounts of monomers, dimers and trimers were detected in some cell types. The closely similar properties of α -synuclein observed until now in neural cells and fresh human RBCs recommends the latter as an abundant, available source for future studies of physiological α -synuclein.

The higher lipid-binding capacity of native α -synuclein leads us to speculate that the monomer represents a not fully functional and less abundant species in normal cells. Given the much lower propensity of the native tetramer to aggregate into fibrils (Fig. 3d), it is likely that tetramers undergo destabilization before α -synuclein aggregates into abnormal oligomeric and fibrillar assemblies that can confer cytotoxicity in Parkinson's disease and other α -synucleinopathies. Hypothetically, such a mechanism could be analogous in part to transthyretin amyloidosis, in which a native metastable tetramer circulates in human plasma but can become destabilized (for example, by pathogenic missense mutations) to allow monomers to aggregate aberrantly in tissue²². Our identification of helically folded α -synuclein tetramers encourages the design of compounds that, like those for transthyretin²³, could kinetically stabilize native tetramers and prevent pathogenic α -synuclein aggregation as a novel treatment approach for Parkinson's disease, dementia with Lewy bodies and other synucleinopathies²⁴.

METHODS SUMMARY

Native gel electrophoresis was conducted as described¹⁰. For crosslinking, 1–5 mM disuccinimidyl suberate was added to living cells. RBC lysates were treated analogously but using 1 mM bis(sulphosuccinimidyl) suberate. To purify α -synuclein from fresh or packed frozen RBCs, an initial 25% (NH₄)₂SO₄ cut followed by a 50% (NH₄)₂SO₄ precipitation substantially enriched α -synuclein. The resolubilized 50% pellet was injected onto a hydrophobic interaction column (HiTrap Phenyl HP; GE Healthcare) and eluted in 1 M to 0 M (NH₄)₂SO₄, pH 7. Alternatively, α -synuclein-overexpressing 3D5 neuroblastoma cell lysate after (NH₄)₂SO₄ was injected onto a 5-ml HiTrap Q HP column. A 25–500 mM NaCl (pH 8.0) gradient eluted α -synuclein at ~300 mM NaCl. α -Synuclein from both cell sources underwent a final purification step on a Superdex 75 SEC column. STEM analysis was conducted at the Brookhaven National Laboratory STEM user facility. Sedimentation equilibrium data were acquired on a Beckman XL-I analytical ultracentrifuge at speeds of 11,612, 20,644 and 32,256g (AN-60 Ti rotor) and protein concentrations of 0.6, 1.1 and 1.6 mg ml⁻¹. Circular dichroism spectroscopy for lipid-induced α -synuclein folding was conducted in the presence of 4 mM PC/PS (4:1) SUVs. SPR spectroscopy was conducted as described¹⁹. To quantify amyloid fibril growth, aliquots (10 μ l) of purified α -synuclein were added to a 10 μ M ThT solution in 10 mM glycine buffer, pH 9. ThT fluorescence was measured by exciting at 444 nm and scanning the emission wavelengths from 460–550 nm.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 15 November 2010; accepted 20 June 2011.

Published online 14 August 2011.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements Mass measurements were carried out at the Brookhaven National Laboratory STEM facility, a user facility supported by the US Department of Energy. We are grateful to D. Walker and J. Anderson (Elan Pharmaceuticals) for conducting mass spectrometry of our purified α -synuclein samples and for comments. We thank X. Simon and I. Perovic (Brandeis University) for their assistance with the AUC and phosphate analyses. Supported by NIH grants NS051318 and NS038375 (D.J.S.). We thank our colleagues at the Center for Neurologic Diseases for many discussions.

Author Contributions All experiments were planned by T.B. and D.J.S. and conducted by T.B. and J.G.C. The manuscript was prepared by T.B. and D.J.S.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to D.J.S. (djelskoe@rics.bwh.harvard.edu).

METHODS

Materials. Recombinant human α -synuclein was bought from Anaspec. Recombinant human transthyretin was provided by I. Rappley and J. Kelly (Scripps Research Institute). HEK, COS-7 and HeLa cells were cultured in DMEM with 10% fetal bovine serum, penicillin (100 U ml⁻¹), streptomycin (100 μ g ml⁻¹) and L-glutamine (2 mM). For M17D and 3D5 human neuroblastoma cells, standard DMEM was supplemented with 400 μ g ml⁻¹ G418 and 1 μ g ml⁻¹ puromycin. Frontal cortex was obtained from wild-type mice aged 4–9 months. α -Synuclein immunoblotting used antibodies C20 (1:1,000, Santa Cruz), LB509 (1:400, Santa Cruz), Syn211 (1:200, Santa Cruz) and Syn1 (1:2,000, BD).

Lipid preparation. PC/PS SUVs (30 nm) of 80% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 20% 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] (POPS) (Avanti Polar Lipids) were prepared in 10 mM sodium phosphate, pH 7.4, by sonication.

Crosslinking. Cells were detached and incubated at room temperature (22 °C) for 30 min in DSS crosslinker (1–5 mM), then quenched with 1 M Tris buffer, pH 7.4, for 15 min at room temperature. Human RBC lysates were treated analogously with 1 mM BS³ (Pierce) to covalently crosslink lysine residues.

BN-PAGE and CN-PAGE. For BN-PAGE, samples were run on 4–16% Bis-Tris BN-PAGE gels (Invitrogen) at 200 V and room temperature. The cathode buffer was 50 mM tricine, 15 mM Bis-Tris, 0.02% Brilliant Blue G (Serva), pH 7.0; the anode buffer was 50 mM Bis-Tris pH 7.0. CN-PAGE was conducted identically to BN-PAGE, but Coomassie blue was omitted from the sample and the cathode buffer. Electroblotting of protein on PVDF membranes (0.45 μ m pore size) was conducted at 400 mA for 2 h. For molecular weight estimation three different molecular weight marker were loaded on each gel (Sigma Non-denaturing, 108K6408, Invitrogen Native Mark, LC0725 GE Healthcare HMW Native Marker Kit, 17-0445-01).

IEF two-dimensional PAGE. We used the IPGphor IEF system (GE Healthcare). Lysates were heated at 65 °C overnight and brought to 200 μ l with sample rehydration buffer (7 M urea, 2 M thiourea, 2% Chapso, 0.5% IPG buffer (GE Healthcare, bromophenol blue) and applied on an 11 cm 1D Ready-strip (Bio-Rad) with a pH gradient of 4–7. Sample was rehydrated for 16 h followed by IEF at 500 V for 30 min, then 1,000 V for another 30 min, and then 8,000 V for 3.5 h. The 1D strip was then applied to a precast NuPAGE ZOOM 4–12% Bis-Tris gel (Invitrogen) and run at 200 V.

Purification of α -synuclein from human RBCs. Freshly collected and washed RBCs were resuspended in a threefold volume of ACK lysing buffer (Lonza). (NH₄)₂SO₄ to a final concentration of 25% was added and incubated at 4 °C for 30 min. The lysate was centrifuged (20,000g, 20 min), and the supernatant brought up to 50% (NH₄)₂SO₄. The pellet was washed several time in 55% (NH₄)₂SO₄ to remove excess haemoglobin. The sample was centrifuged at 20,000g for 20 min and the pellet resolubilized in a 50-fold volume of 50 mM phosphate buffer, pH 7.0, 1 M (NH₄)₂SO₄. Five millilitres of the resultant solution were injected onto a 5-ml HiTrap phenyl hydrophobic interaction column (GE Healthcare) equilibrated with 50 mM phosphate buffer, pH 7.0, 1 M (NH₄)₂SO₄. α -Synuclein was eluted with a 1 M to 0 M (NH₄)₂SO₄ gradient in 50 mM phosphate buffer, pH 7.0 (α -synuclein eluted at ~0.75 M (NH₄)₂SO₄). For anion exchange purification of RBC α -synuclein, we used the protocol used for neuroblastoma cells (see later), but the first run of RBC lysate sometimes showed low binding of α -synuclein and contamination by plasma transthyretin. In these cases we discarded the first eluate and used the flow-through for a second run, which showed significantly higher binding and subsequent purity. As a third alternative to HIC and AX, an XK 16/100 column packed with activated thiolpropyl Sepharose 6B gel media was used (Supplementary Fig. 5B) (binding buffer, PBS; flow rate, 0.2 ml min⁻¹). In this case, the flow-through contained α -synuclein and was processed further. The column was regenerated by eluting bound protein with 5 column volumes of binding buffer with 25 mM dithiothreitol, and reactivated with 1.5 mM dipyrindyl sulphide in 50 mM borate buffer pH 8.0. The final solution was concentrated (Amicon Ultra centrifugal filter units, MWCO 10,000, Millipore) and further purified via gel filtration.

Purification of α -synuclein from human neuroblastoma cells. 3D5 cells (α -synuclein stables) and their parental M17D cells were scraped from the plates, washed in PBS and lysed by sonication. A (NH₄)₂SO₄ precipitation was conducted as described earlier, the 50% (NH₄)₂SO₄ pellet was taken up in 20 mM Tris buffer, pH 8.0, 25 mM NaCl. The sample was injected onto a 5-ml HiTrap Q HP anion exchange column, equilibrated with 20 mM Tris buffer, pH 8.0, 25 mM NaCl.

α -Synuclein was eluted from the column with a 25–500 mM NaCl gradient in 20 mM Tris buffer, pH 8.0. α -Synuclein was eluted at ~300 mM NaCl. The column was regenerated with 1 M NaCl in 20 mM Tris buffer, pH 8.0. The final solution was concentrated (Amicon Ultra centrifugal filter units, MWCO 10,000, Millipore) and further purified via gel filtration. For the addition ('spiking') of exogenous recombinant α -synuclein monomers, bacterially expressed α -synuclein was added to the scraped M17D cell pellet and the purification scheme conducted as just described.

Gel filtration. Aliquots (250 μ l) were injected onto either a Superdex 75 (10/300 GL), Superdex 200 (10/300 GL) or a Superose 12 (10/300 GL) column (GE Healthcare) at 4 °C and eluted with 50 mM ammonium acetate, pH 8.5. For size estimation, a gel filtration standard (Bio-Rad, cat. no. 151-1901) was run on each column, and the calibration curve was obtained by semi-logarithmic plotting of molecular weight versus elution volume divided by void volume.

STEM. STEM was carried out at the Brookhaven National Laboratory STEM user facility with 100 μ l of sample at a concentration of 300 μ g ml⁻¹ in 50 mM ammonium acetate, pH 7.4, and diluted to find the appropriate concentration for a homogenous particle distribution. Tobacco mosaic virus (TMV) rods were included during specimen preparation as an internal sizing standard.

Circular dichroism spectroscopy. Circular dichroism spectra were obtained using an Aviv Biomedical spectrometer (model 410) in the presence or absence of 4 mM PC/PS SUVs. The spectral contributions of buffer and SUVs were subtracted. Data are reported as mean residue ellipticities measured at 20 °C and a pathlength of 0.1 mm.

Lipidex 1000 treatment. 10% (w/v) Lipidex 1000 (Perkin Elmer) was washed with 50% methanol-ultra pure water and added to a 100 μ M solution of purified α -synuclein from RBCs. The samples were stirred overnight at 37 °C, and α -synuclein was purified from that mixture via size exclusion chromatography.

SPR. All lipid binding experiments were performed at 20 °C on a BIACORE 3000 apparatus using the L1 sensor chip (Biacore AB). The running buffer was 10 mM sodium phosphate, pH 7.4. SUVs were applied to the sensor chip surface at a flow rate of 10 μ l min⁻¹ in the presence of 0.1 mM NaCl. Injections were done at a flow rate of 10 μ l min⁻¹ with 50 μ l sample volume. Apparent K_d values were calculated from equilibrium data of several dilution series, collected at 300–320 s.

ThT binding. To detect amyloid fibril growth, a discontinuous assay was used. Aliquots (10 μ l) were removed from each purified α -synuclein sample (lyophilized from 50 mM ammonium acetate, pH 7.4, and agitated at 37 °C at a concentration of 75 μ M in 20 mM Bis-Tris propane, 100 mM LiCl, pH 7.4) and added to 2 ml of a 10 μ M ThT solution in 10 mM glycine buffer, pH 9. Fluorescence was directly quantified on a Varian Eclipse fluorescence spectrophotometer at 20 °C by exciting at 444 nm and scanning the emission wavelengths from 460–550 nm with slit widths set at 5 nm (PMT at 750 V).

Quantitative phosphate analysis. Samples (2 \times 15 μ l and 2 \times 30 μ l of 1 mg ml⁻¹ α -synuclein in 50 mM ammonium acetate, pH 7.4) were placed at the bottom of glass test tubes, 225 μ l of 8.9 N H₂SO₄ (in deionized water) was added, and the mixture was heated for 25 min at 200–215 °C. Next, 75 μ l H₂O₂ was added to all tubes at room temperature. After heating for 30 min at 200–215 °C, 1.95 ml deionized water and then 0.25 ml 2.5% ammonium molybdate(VI) tetrahydrate solution (in deionized water) were added at room temperature. After addition of 0.25 ml 10% ascorbic acid solution (in deionized water), the tubes were heated for 7 min at 100 °C, and samples were allowed to cool to room temperature. Absorbance at 820 nm was measured, and phosphate concentration calculated using a calibration curve obtained from 7 phosphate standard solutions ranging from 0–50 nmol phosphate (Sigma-Aldrich).

SE-AUC. AUC experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge. Sedimentation equilibrium experiments were carried out at purified α -synuclein protein concentrations of 1.6, 1.1 and 0.6 mg ml⁻¹ in 50 mM ammonium acetate, pH 8.5. The experiments were performed at 20 °C at 11,612, 20,644 and 32,256g (AN-60 Ti rotor), and data were collected at 278 nm. The software SEDPHAT (version 6.5) was used to calculate the *M* and *s* of the species present in equilibrium in the samples. For molecular weight analysis, we used the model 'Species Analysis' available in the SEDPHAT program with RI noise baseline correction. Analysis was performed for each protein concentration separately, and the molecular weight determined from the average obtained for the analyses of the 3 protein concentrations. The average errors and standard deviations were calculated using Monte-Carlo simulation, with 1,000 iterations and a confidence level of 0.68.