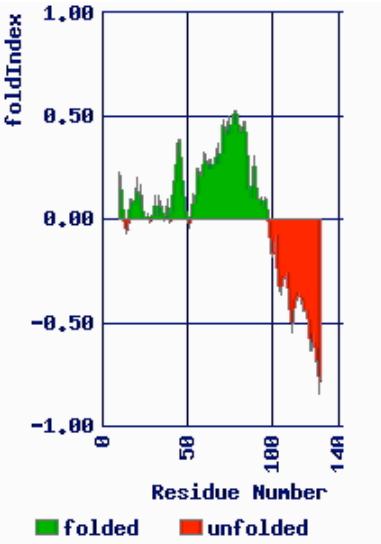
Supplementary figures for:

A soluble alpha-synuclein construct forms a dynamic tetramer

Wang et al.



Summary:

Number Disordered Regions: 1
Longest Disordered Region: 43
Number Disordered Residues: 43
Predicted disorder segment: [98]-[140] length: 43 score: -0.41 ± 0.19

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  1 MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV GSKTKEGVVH
 51 GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL
101 GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA
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(Predicted disordered segment)

Fig. S1. Prediction from FoldIndex©, a program to determine which regions of a protein's sequence are likely to be structured and which are intrinsically disordered (13), on the structure of α-synuclein. It shows that the N-terminal 70% of the protein (residues 1-97) is predicted to be ordered and the C-terminal region (the last 43 residues) is predicted to be disordered. This is in almost perfect agreement with the NMR structure of the α-synuclein oligomer presented here. We thank Joel Sussman for calling our attention to this method.

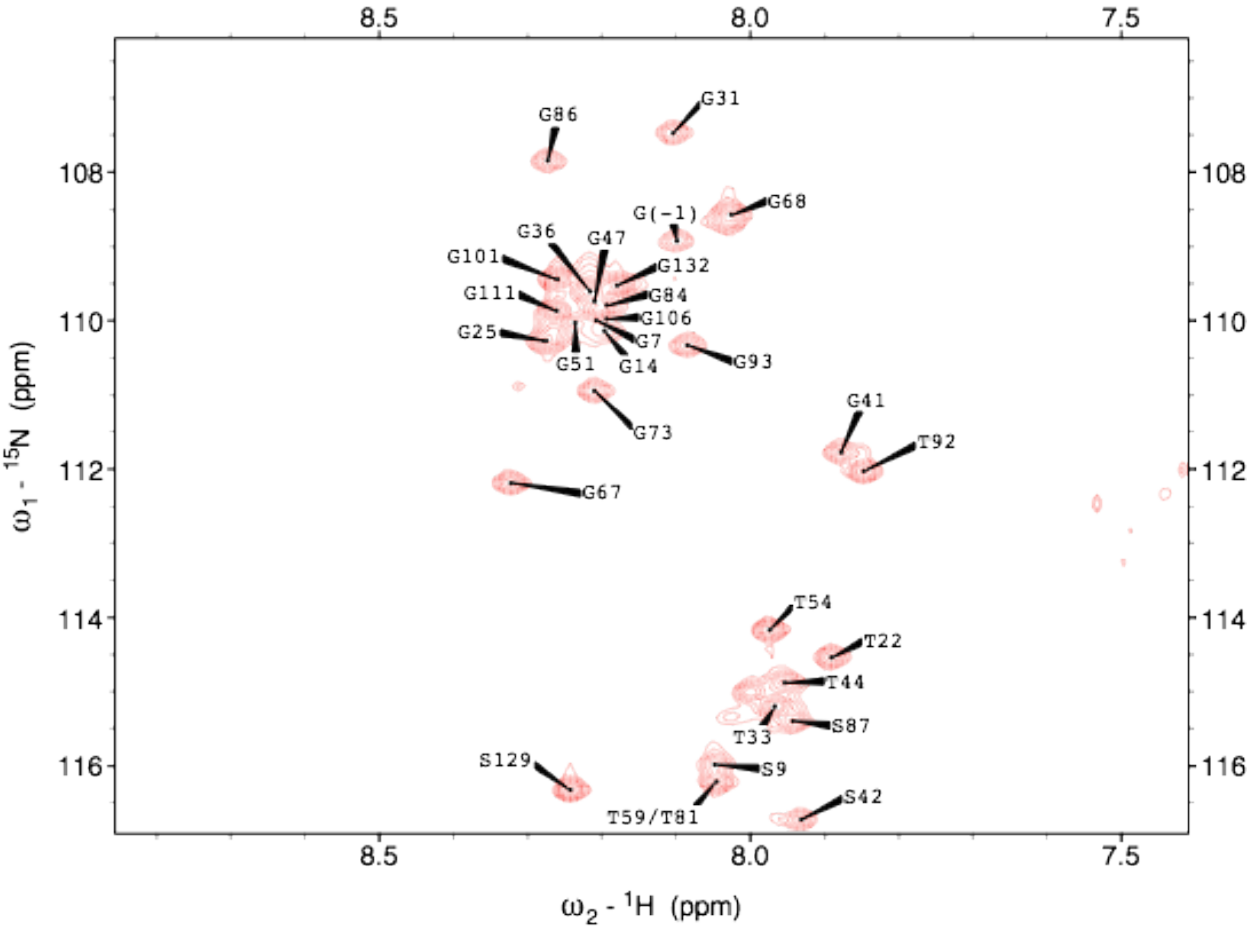

Fig. S2. Glycine/threonine region of 800 MHz ^{1}H,^{15}N TROSY-HSQC spectrum of αSyn with assignments. Spectrum was obtained at 800 MHz (^{1}H), 298 K, 0.5mM in 100mM Tris HCl pH 7.4, 100mM NaCl, 0.1% β-octyl-glucoside, 10% glycerol, 10% D_2O. Resonance assigned as G(-1) refers to the glycine in the N-terminal extension resulting from the GST tag.

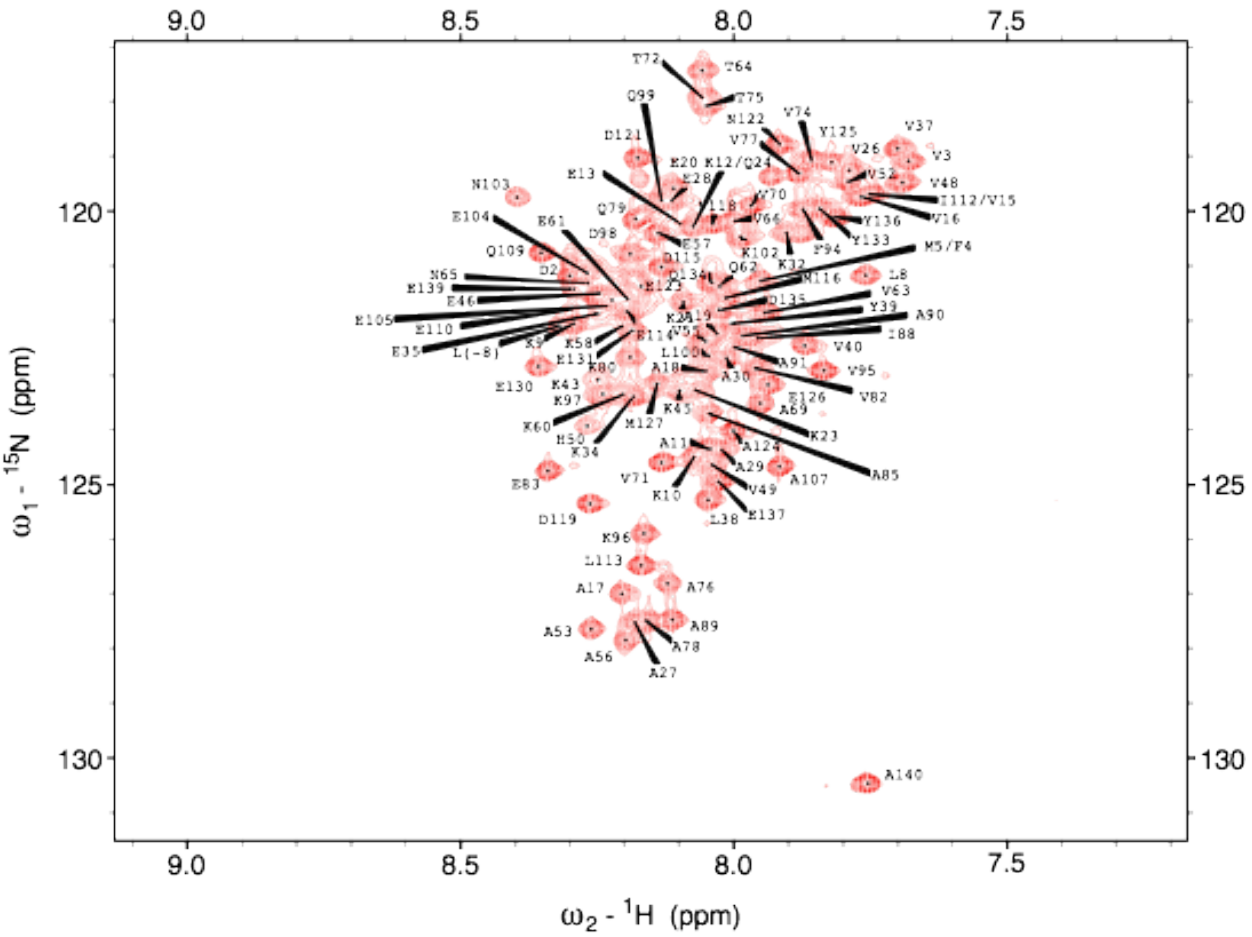

Fig. S3. Downfield (^{15}N) region of 800 MHz ^{1}H,^{15}N TROSY-HSQC spectrum of αSyn with assignments. Spectrum was obtained at 800 MHz (^{1}H), 298 K, 0.5mM in 100mM Tris HCl pH 7.4, 100mM NaCl, 0.1% β-octyl-glucoside, 10% glycerol, 10% D_2O

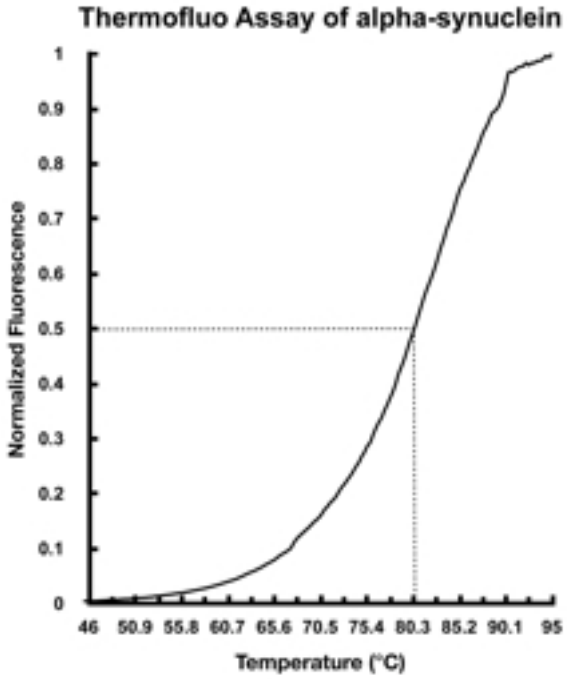

Fig. S4. Thermofluo assay denaturation curve of recombinant oligomeric alpha-synuclein.

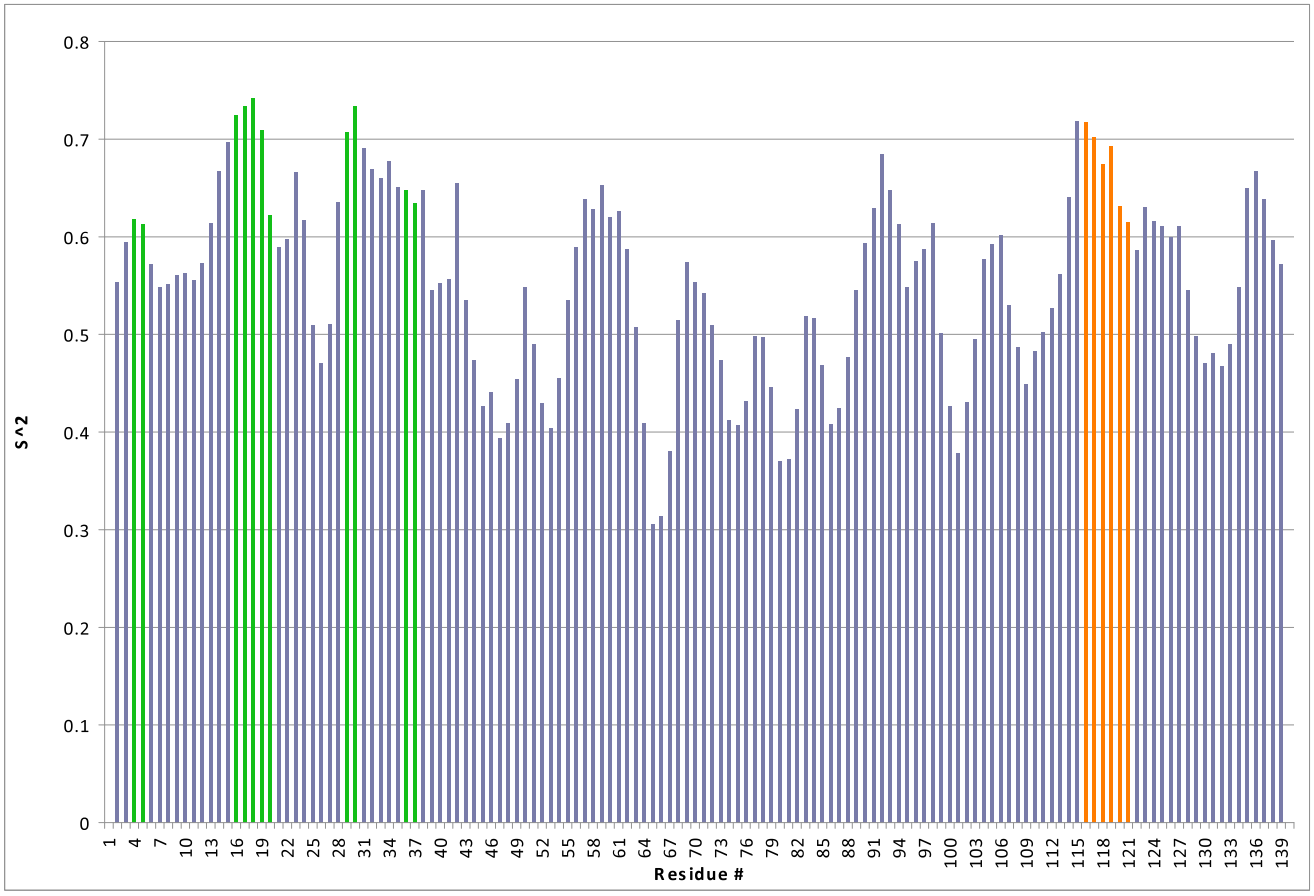

Figure S5. Order parameters (S^2) derived from TALOS+ analysis of chemical shifts for αSyn tetramer described here (BMRB entry 17665). Residues shown in green are predicted by TALOS+ to be helical, those in orange are predicted to be extended. No prediction was made for other residues.

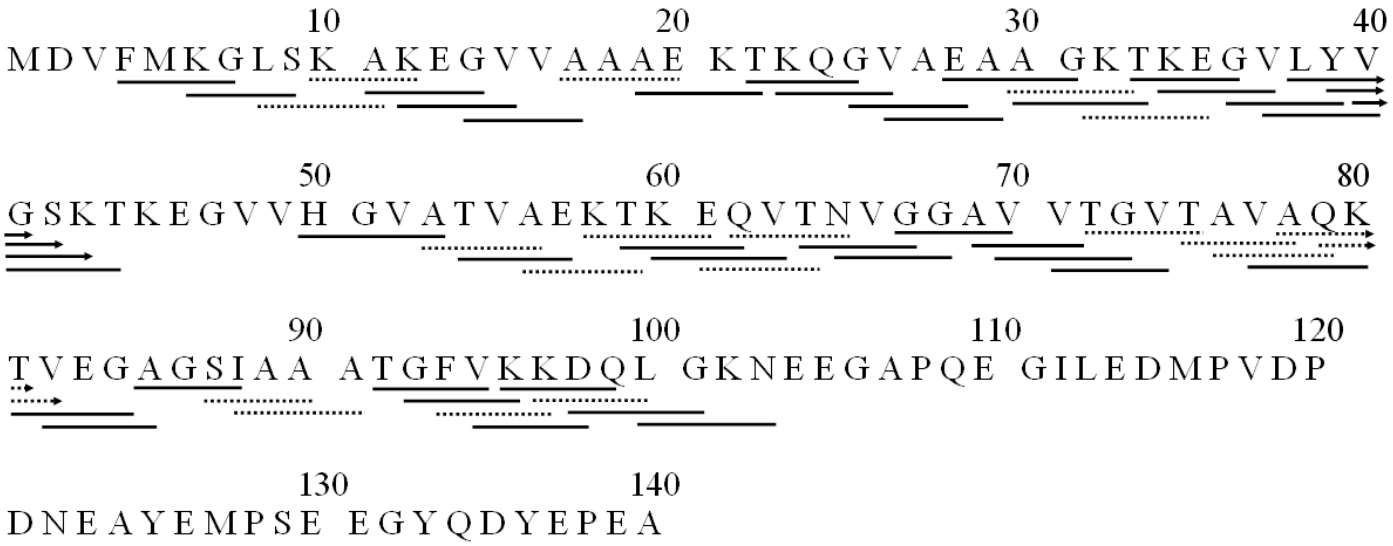

Figure S6. Regions of αSyn fractionally occupying helical structures as defined by *i, i+3* Ha-HN NOEs. Observed NOEs are indicated by solid lines. Ambiguous connectivities due to spectral overlap are indicated by dotted lines.

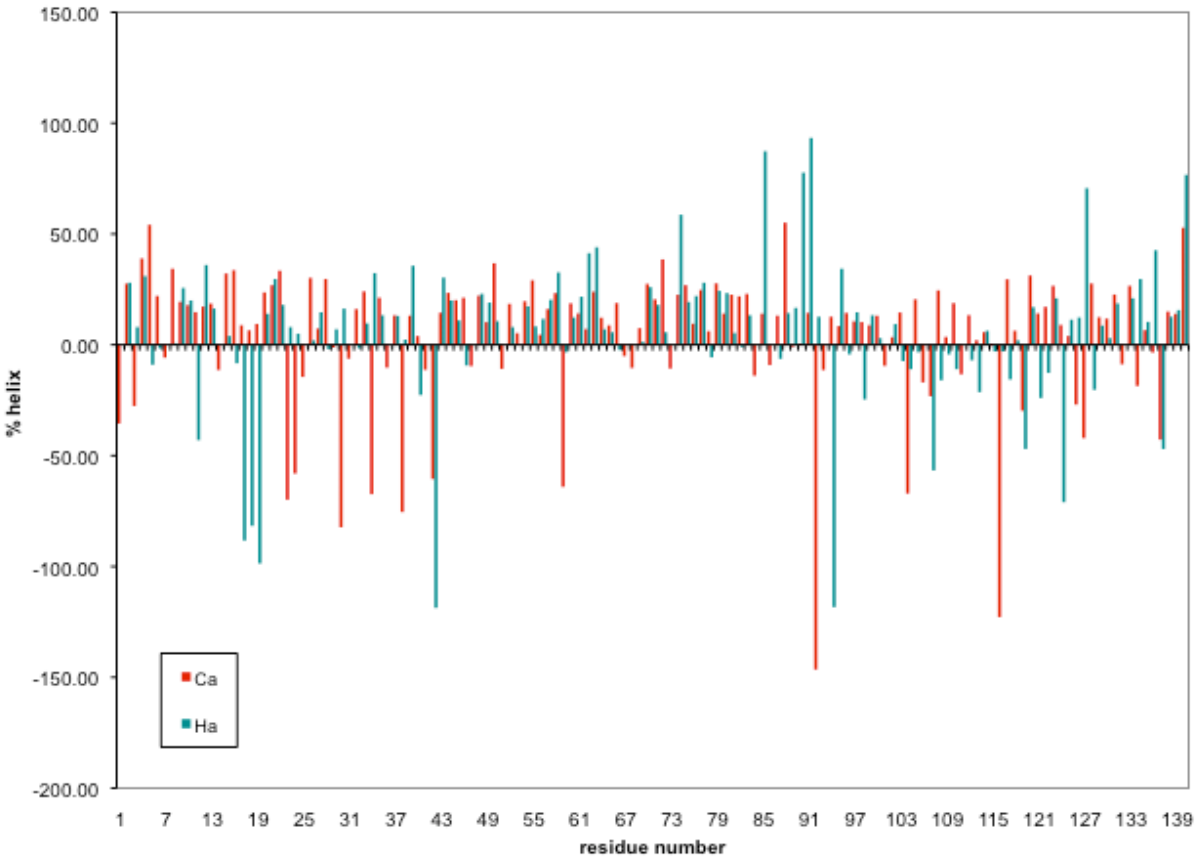

Figure S7. Percent helical character expected for αSyn construct based on $\Delta\delta^{13}C\alpha$ and $\Delta\delta^{1}H\alpha$ versus calculated random coil shifts as described in text.

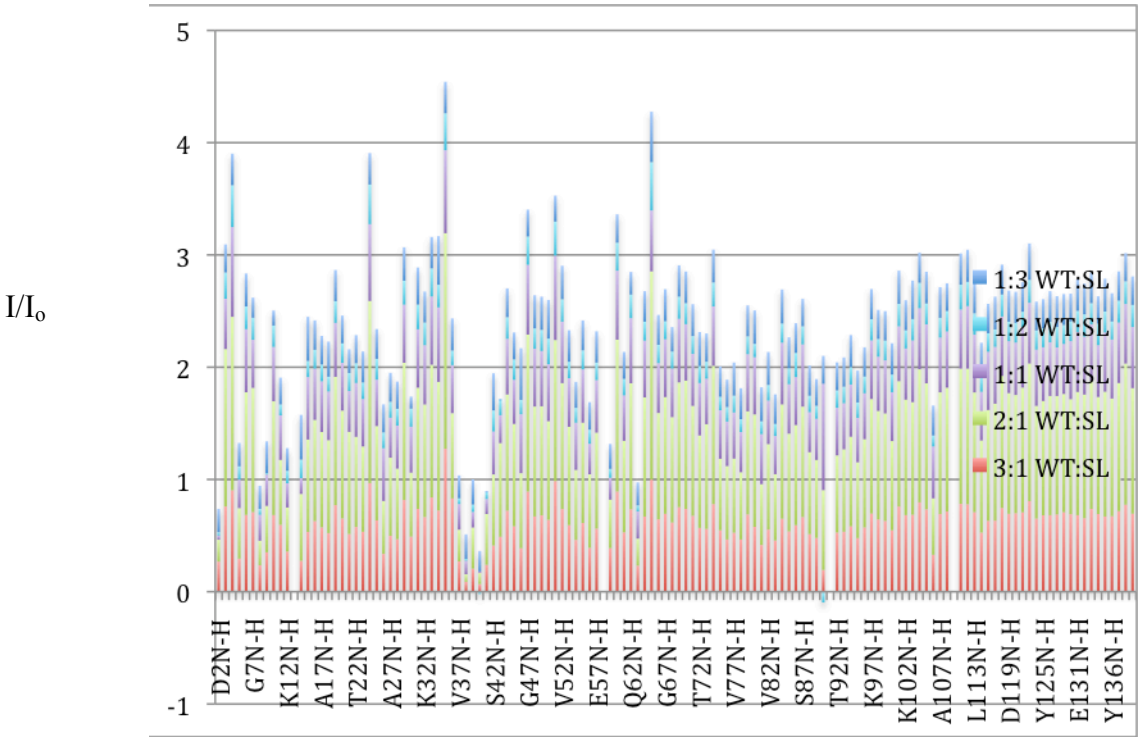

Figure S8. Inter-subunit (i.e., intermolecular) paramagnetic relaxation effects (PRE) on ^{1}H-^{15}N HSQC correlations of WT ^{15}N-labeled αSyn with natural abundance S9C αSyn labeled with MTSL as a function of WT:S9C (SL) ratios. Plotted are the sums of peak intensities divided by that signal intensity for the spectrum of WT αSyn prior to titration (vertical axis, I/I_o) from each titration point (see legend) with the contribution of each titration point indicated by color. Correlations with the overall lowest total intensity are the most affected by PRE. Data for residues 13, 58, 91 and 110 could not be accurately measured and are not shown.

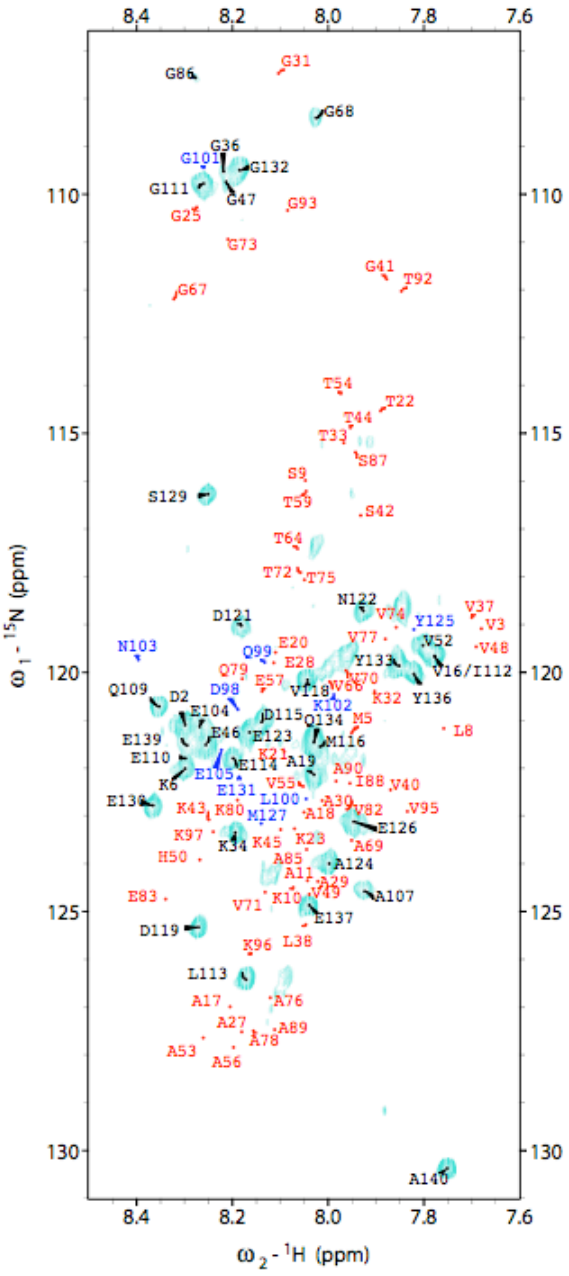

Fig. S9. TROSY-HSQC of ^{15}N labeled cross-linked alpha-synuclein. Peaks of residues affected by cross-linking could not be observed at their original positions due to broadening or change in chemical shift. Affected residues are represented by labels at their original chemical shifts (no peak). Red - perturbed residues in helices 1, 2 or 3 or turns 1or 2 (structured region); blue - perturbed residues in C-terminal tail (unstructured region). Black - unperturbed residues.

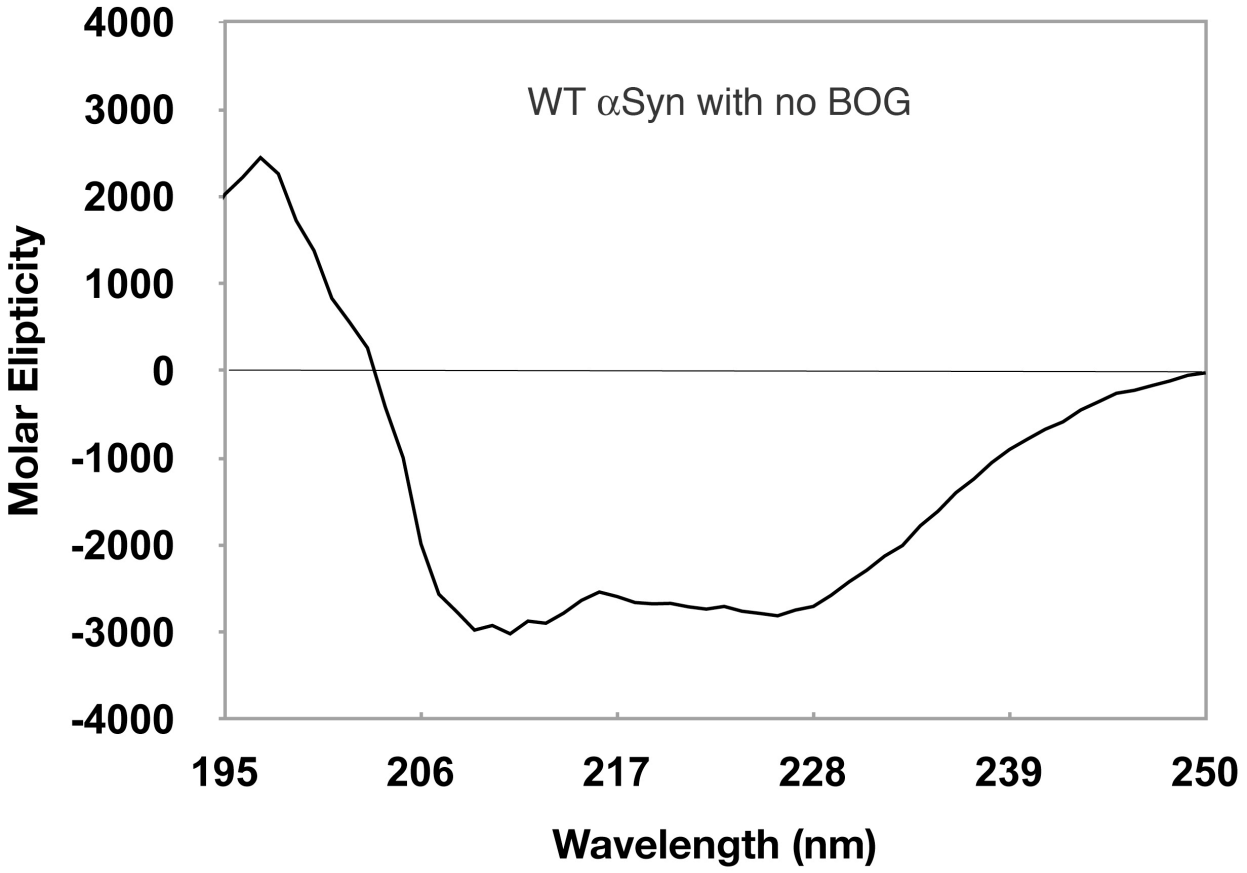

Fig. S10. CD spectrum of αSyn obtained in the absence of glycerol and BOG. Buffer is otherwise the standard buffer reported in the text. Protein concentration is 1.8 mg/mL. Path length 0.2 mm.

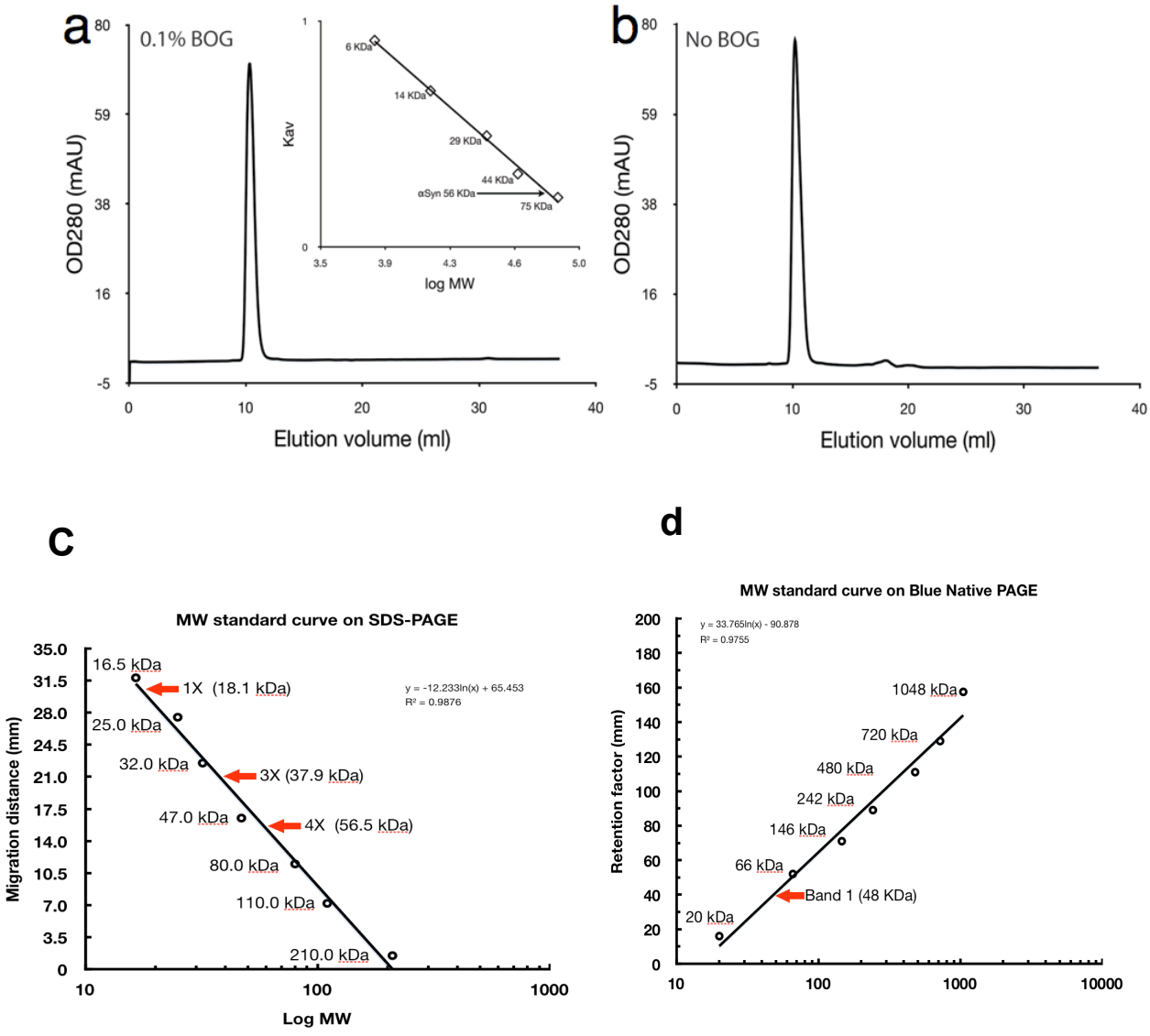

Fig. S11. Size-exclusion chromatography and SDS-PAGE of α-synuclein. (a) Elution profile of α-synuclein from superdex75 column with 0.1%BOG in buffer. Insect shows the column calibration used for calculation Mw from elution volumn. (b) Elution from same column without BOG in buffer. The presence of detergent has no effect on the oligomerization state of the protein. (c) Analysis of SDS-PAGE gel of purified, cross-linked recombinant α-Syn (see Figure 2b of main text). Bands of molecular mass consistent with monomeric, trimeric and tetrameric α-Syn are present. No dimeric species is observed. (d) Analysis of Blue Native PAGE gel of purified recombinant α-Syn (see Figure 2b of main text). The predominant band has a mass estimated at ~ 48 KDa.

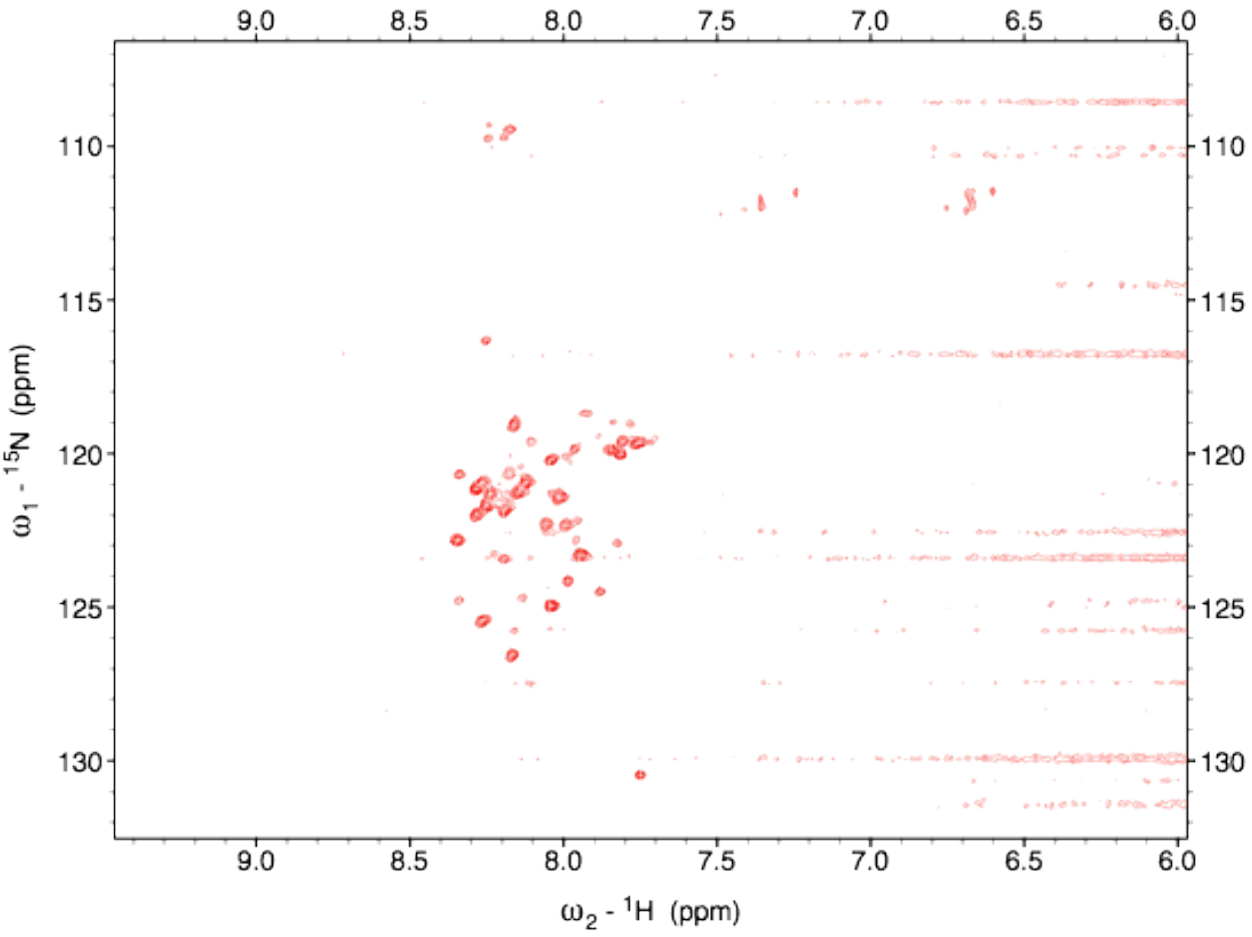

Fig. S12. 800 MHz $^{1}H,^{15}N$ TROSY-HSQC spectrum of αSyn after boiling and repurification from size exclusion column. Spectrum was obtained at 800 MHz (^{1}H), 298 K, <100 micromolar concentration, in 100mM Tris HCl pH 7.4, 100mM NaCl, 0.1% β-octyl-glucoside, 10% glycerol.

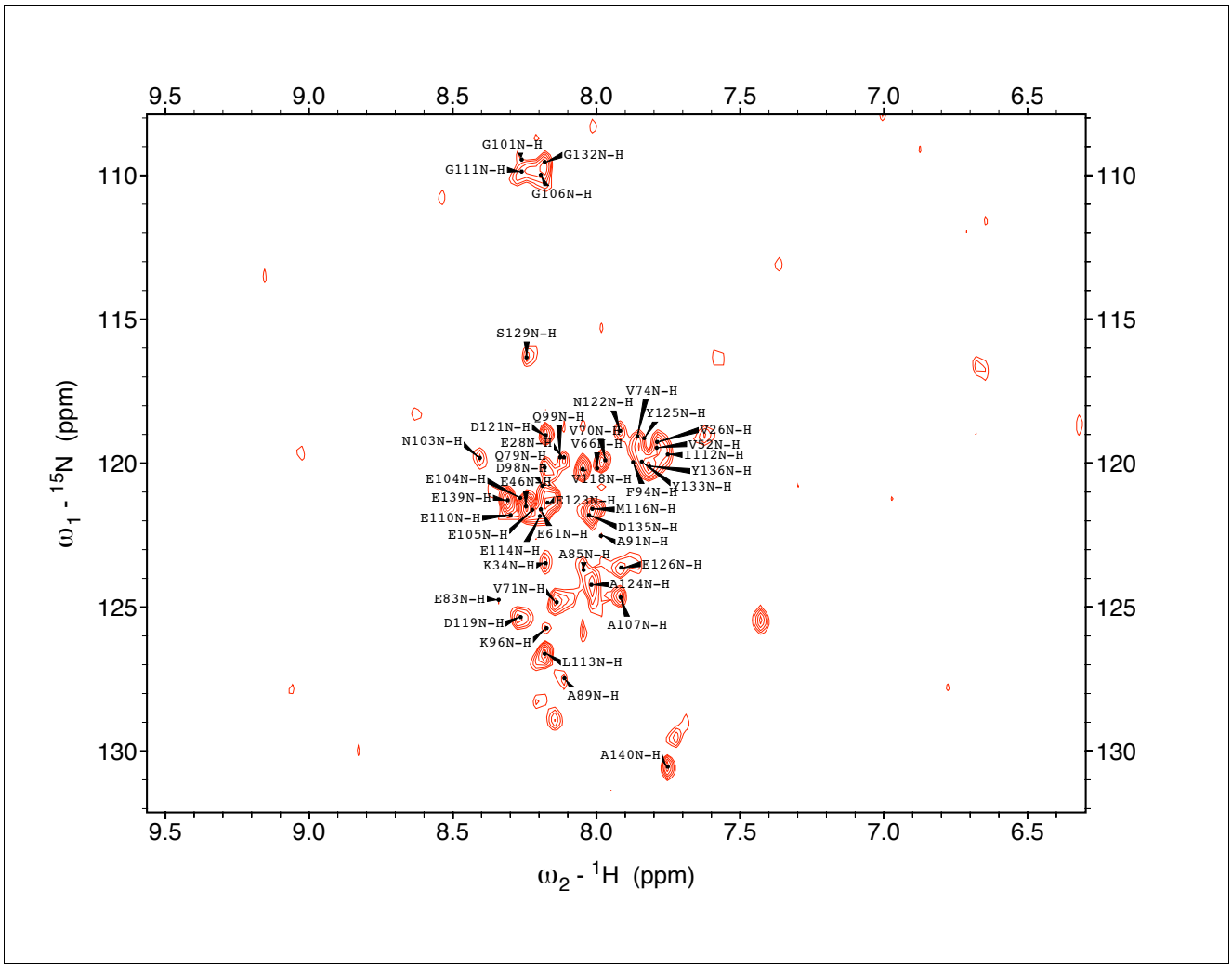

Figure S13. $^{1}H,^{15}N$ HSQC spectrum (800.13 MHz ^{1}H) of a dilute (50 μM) sample of WT αSyn construct in standard buffer (298 K, 0.5mM in 100mM Tris HCl pH 7.4, 100mM NaCl, 0.1% β-octyl-glucoside, 10% glycerol, 10% D_2O). Sample is unboiled, subjected only to normal purification protocol.

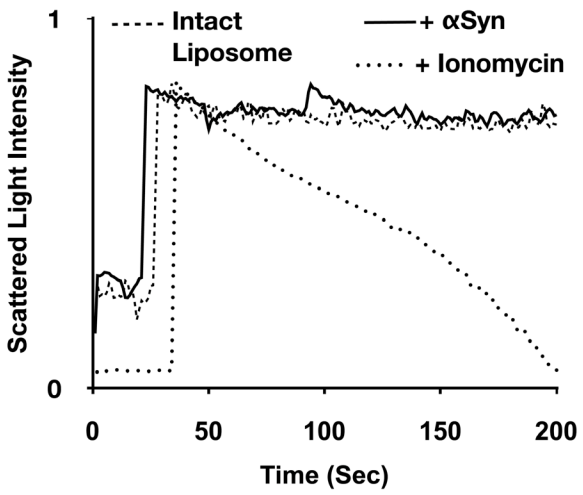

Fig. S14. αSyn has no effect on liposome membrane permeability upon binding. Scattered light intensity increases upon adding high salt (KCl, NaCl, or $CaCl_2$) causing liposomes to collapse due to osmotic pressure. Leaky liposome (+ ionomycin) returns to original shape as ions equilibrate, but intact liposomes are unchanged.

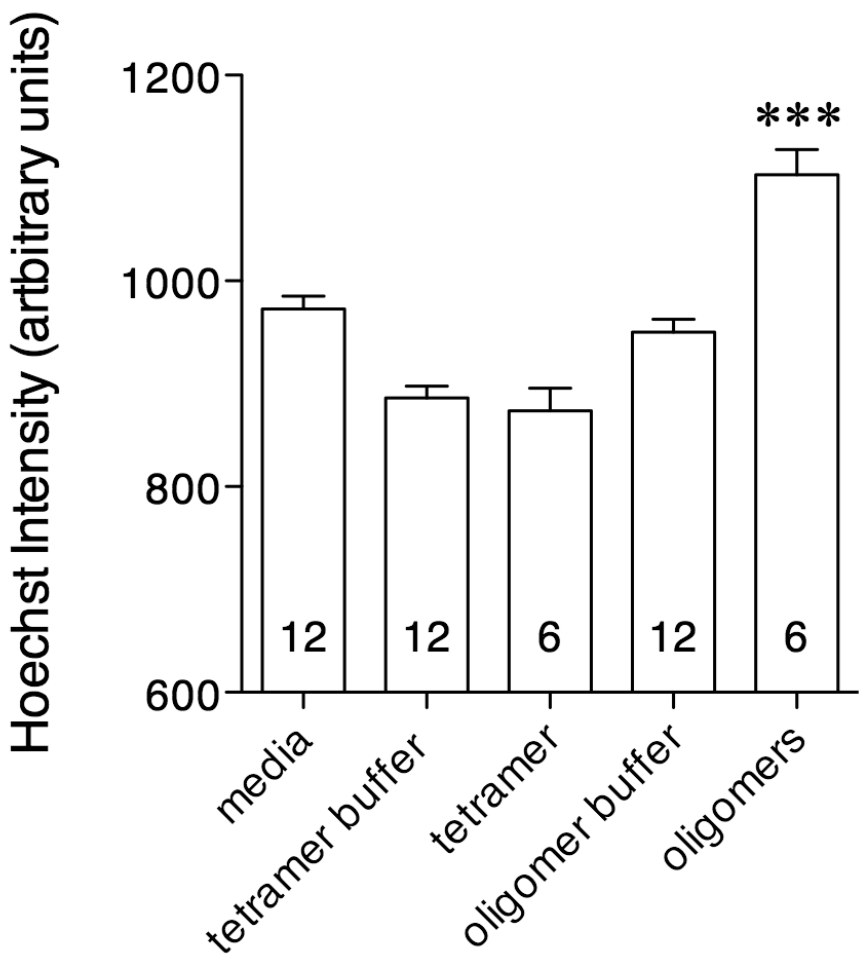

Fig. S15. Cell viability was estimated by measuring average intensity of the nuclear dye Hoechst 33342, which increases because of nuclear shrinkage in dying cells, in M17 human neuroblastoma cell lines. A Cellomics HT automated microscopy system was used to measure intensity in n=100 cells per well with N=6-12 wells per condition as indicated with the numbers on each bar, which show average staining intensity per well with SEM between wells. Conditions included media alone (serum and antibiotic free OptiMEM I), 7µM (in monomer equivalents) tetrameric or oligomeric synuclein (A1 toxic oligomer described in Danzer KM et al. J. Neurosci. 22;27(34):9220. 2007) or appropriate buffer controls at similar dilution factors as indicated. Statistical significance was assessed using a one-way ANOVA with Tukey's post-hoc test; ***, $p<0.0001$ compared to media alone.

Supporting materials for

A soluble alpha-synuclein construct forms a dynamic tetramer

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Construction of protein expression vector. The full-length αSyn open reading frame was amplified by PCR with a forward primer containing a SamI restriction site (5′- AGGTTACCCGGGAATGGATGTATTCATGAAAGGACTTTC-3′), a reverse primer containing an Xho1 restriction site (5′-AGGCTCGAGTTAGGCTTCAGGTTGTAGTCTTG-3′), and pRS-GDP-wt-asyn as template following standard protocol. The amplified insert was cloned into the corresponding sites in a pGEX-6P-1 plasmid (GE Biosciences).

PFG diffusion measurements. Experiments were performed on ^{15}N-labeled wild-type in perdeuterated 100mM HEPES pH 7.0, 100mM NaCl, 0.1% BOG, 10% glycerol, before and after boiling at 96°C for 30 minutes. The diffusion coefficients were calculated form ten consecutive spin-echo experiments implementing varying gradient field strength from 2% to 95%. Gradient field strength was calibrated at 33.7 G/cm, and the sine-shaped gradient pulse length and the diffusion time were set to 3.0 ms and 150 ms, respectively. All spectra were phased and processed identically. Three protein peaks with the least buffer peak contamination at 6.74 ppm, 2.17 ppm and 2.04 ppm were manually picked to be fit into intensity decay curves using the equation:

$$I = I_o \exp\left[-(\gamma G\delta)^2 D(\Delta - \delta/3)\right]$$

Where I_o is the unattenuated signal amplitude, I is the diffusion-attenuated amplitude, γ is the gyromagnetic ratio of the observed nucleus (^{1}H), G is the gradient amplitude in gauss/cm, δ is the duration of the gradient pulse and Δ is the diffusion delay time. Two isolated buffer peaks at 3.78 ppm and 2.88 ppm were also fit and compared between non-boiled and boiled sample as an internal standard. Protein diffusion coefficients were calculated as averages of individually calculated diffusion coefficients for each of three chosen peaks.

Liposome assay. 4 ml of 10 mg/ml of E. coli lipid extract (Avanti), which consists of 67% phosphatidylethanolamine (PE), 23.2% phosphatidylglycerol (PG), and 9.8% cardiolipin in chloroform, was dried under nitrogen while the lipid-containing vial was maintained at room temperature. Residual chloroform was removed by washing with pentane and drying. 100 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% BOG was added to make a solution of 10 mg/ml lipid. The mixture was sonicated for 30 minutes to make liposomes. For assaying, 20 µl of liposome was diluted to 2 ml with and without 60 µg of αSyn or ionomycin. The diluted liposomes were placed into a Hitachi F-2500 FL spectrophotometer where diffracted light (500 nm) was constantly monitored at 90^o from incident beam, at room temperature and constant stirring. After the baseline stabilized, 60 µl of 5 M KCl, NaCl, or $CaCl_2$ was added (time zero) and diffracted light was continuously monitored for 200 seconds.

Cytotoxicity assay. Human neuroblastoma M17 cells stably expressing αSyn (1) were grown in OPTI-MEM I supplemented with 10% FBS, 500µg/ml G418. Cells were seeded overnight at 15 x 103 cells per well in 96-well plates (Greiner). After 12 h, cells were treated with 7µM as monomer equivalents of αSyn tetramer or

oligomer, prepared as descibed by Danzer et al (A1 oligomers) (2), or the same volume of the corresponding buffer and medium controls. After 2h of treatment, both tetramer and oligomer were diluted 1:2 in culture medium without serum for an additional 22 hours of treatment at 37C. After treatment, cells were fixed with 4% paraformaldehyde and 1µg/ml Hoechst 33342 (Invitrogen) in PBS. After washing, the cells were kept in PBS in the dark until future analysis. Plates were analyzed using a Thermo Scientific Cellomics Array Scan VTI, using Compartment Analysis protocol. Intensity of the nuclear Hoechst staining was used as a measure of toxicity. We measured intensity of n=100 cells per well with N=6-12 wells per condition with results confirmed in 2-3 independent experiments.

Spin labeling experiments. For spin-labeling experiments, samples of uniformly ^{15}N-labeled wild-type αSyn and S9C mutant αSyn with no isotopic labels were prepared. The S9C mutation was introduced into the above-described construct using four-primer methodology (3). All samples were purified as described above and the final concentration for NMR experiments was adjusted to ~0.5mM in NMR buffer (100 mM Tris HCl pH 7.0, 100mM NaCl, 0.1% β-octyl-glucoside, 10% glycerol, 10% D_2O). S9C mutant sample purifications were closely monitored by SDS-PAGE, as cysteine mutant had a different mobility on the size-exclusion column comparing to the wild-type due to the formation of disulfide cross-links. The spin-label, MTSL (Anatrace) was introduced into the S9C αS by mixing the protein and the label dissolved in acetonitrile in 1:10 molar ratio, respectively, and then incubating for 1.5 h in the dark at room temperature. The concentrations were adjusted so that only 10-15 microliters of the MTSL solution are needed for each mL of ~0.1 mM protein. Residual spin-label was removed by 5 cycles of centrifugation filtration (Amicon, Millipore), concentrating from 15 mL to 1 mL in each cycle. For the titration ^{15}N-^{1}H HSQC-TROSY experiment ^{15}N-labeled wild-type αS, and spin-labeled S9C mutant with no isotopic labels were mixed in 4:1, 3:1, 1:1, 1:3 and 1:4 molar ratios, thus creating five titration points, not including the zero point. ^{15}N-^{1}H HSQC-TROSY experiments were recorded on ^{15}N-labeled wild-type αS and ^{15}N-labeled S9C mutant before and after the addition of the spin label, and no significant changes in chemical shifts were observed, showing that neither the introduction of the mutation or the spin label disrupted the time-average behavior of the molecule.

1. Bisaglia M*, et al.* (2010) alpha-Synuclein overexpression increases dopamine toxicity in BE(2)-M17 cells. *BMC Neurosci.* 11.
2. Danzer KM*, et al.* (2007) Different species of alpha-synuclein oligomers induce calcium influx and seeding. *Journal of Neuroscience* 27(34):9220-9232.
3. Pochapsky TC, Kostic M, Jain N, & Pejchal R (2001) Redox-Dependent Conformational Selection in a Cys(4)Fe(2)S(2) Ferredoxin. *Biochemistry* 40(19):5602-5614.