Properties of native brain α-synuclein

ARISING FROM T. Bartels, J. G. Choi & D. J. Selkoe *Nature* **477,** 107–110 (2011)

α-Synuclein is an abundant presynaptic protein that binds to negatively charged phospholipids[1,2], functions as a SNARE-complex chaperone[3] and contributes to Parkinson's disease pathogenesis[4,5]. Recombinant α-synuclein in solution is largely unfolded and devoid of tertiary structure[6–11], but Bartels *et al.*[12] have proposed that native α-synuclein purified from human erythrocytes forms a stably folded, soluble tetramer that resists aggregation. By contrast, we show here that native α-synuclein purified from mouse brain consists of a largely unstructured monomer, exhibits no stable tetramer formation, and is prone to aggregation. The native state of α-synuclein is important for understanding its pathological effects as a stably folded protein would be much less prone to aggregation than a conformationally labile protein. There is a Reply to this Brief Communication Arising by Bartels, T. & Selkoe, D. J. *Nature* **498,** http://dx.doi.org/10.1038/nature12126 (2013).

We examined native α-synuclein from brain, the most relevant organ for understanding neurodegeneration. Separation of mouse brain homogenates into soluble and membrane fractions revealed that during ultracentrifugation, most α-synuclein partitioned into cytosol fractions similar to complexins, but different from membrane proteins such as cysteine string protein (CSP)-α and SNAP25 (Fig. 1a, b). Using gel filtration, we analysed the size of native α-synuclein in brain cytosol and of recombinant myc-epitope-tagged human α-synuclein, purified without boiling or detergents[3]. Both α-synucleins eluted in a single peak with an apparent molecular mass of ~63 kDa (Fig. 1c–f), close to that predicted for a folded tetramer[12].

These results seem to confirm that α-synuclein forms a stable tetramer in solution. However, dynamic or unstructured states of a protein may increase its hydrodynamic radius and apparent molecular mass

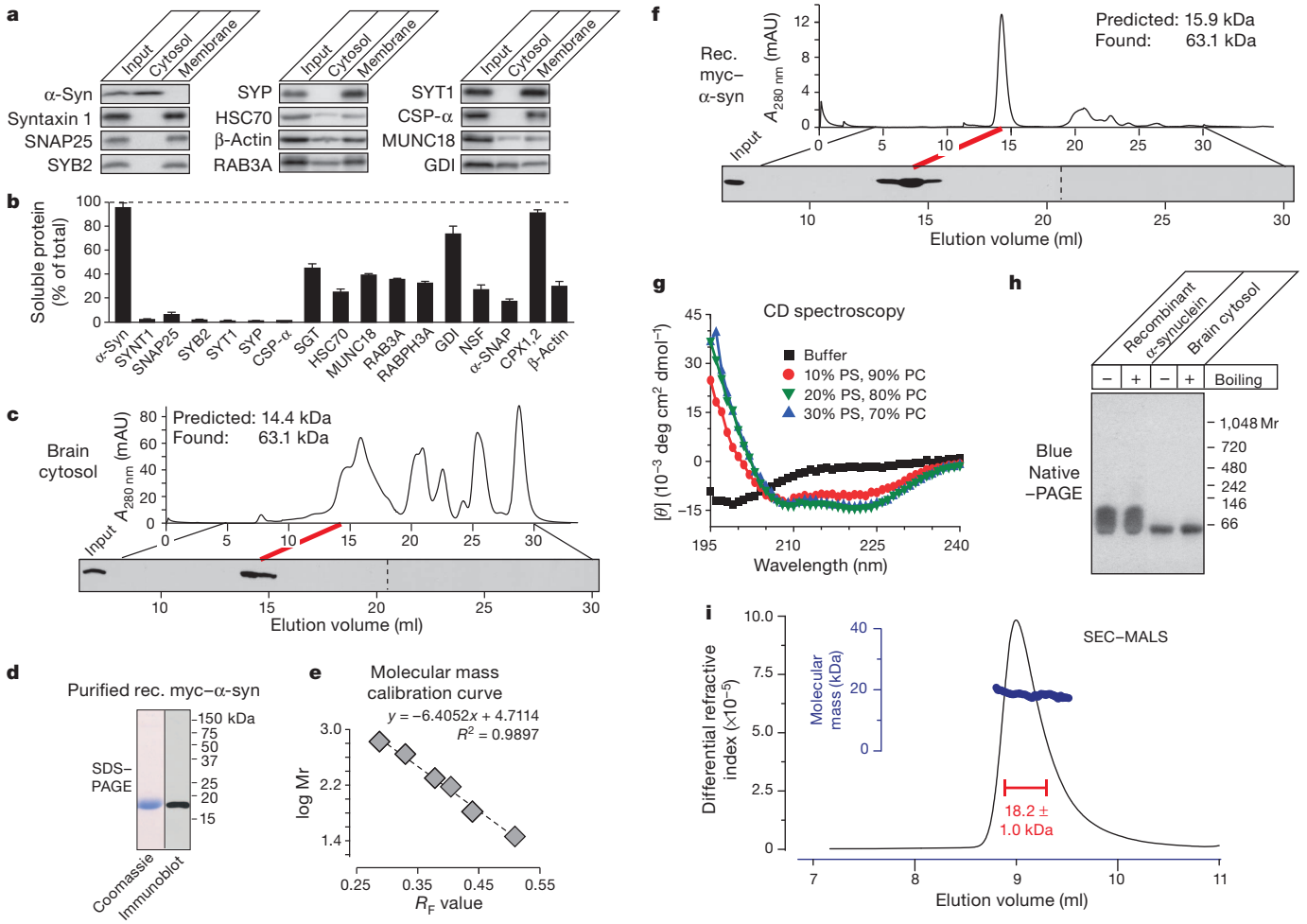

Figure 1 | Recombinant α-synuclein and brain α-synuclein in cytosol are monomeric. **a**, **b**, Immunoblotting analysis of mouse brain homogenate (input), cytosol and membranes (**a**), and quantification of protein levels (**b**; means ± s.e.m.; $n = 3$)[3]. **c**, Native mouse brain α-synuclein (375 μg) elutes as an apparent tetramer during gel filtration on a Superdex 200 column (top), as analysed by α-synuclein immunoblotting (bottom). mAU, milli absorbance unit. **d**, Analysis of purified recombinant myc-epitope-tagged α-synuclein (rec. myc–α-syn) by SDS–PAGE and immunoblotting. **e**, Molecular mass calibration curve for gel filtration (R_F = migration distance of proteins versus total running distance; y axis = logarithm of molecular protein mass (Mr)). **f**, Recombinant myc-tagged human α-synuclein (16 μg) also elutes as an apparent tetramer during gel filtration. **g**, Circular dichroism spectroscopy shows that recombinant α-synuclein (10 μg) is unstructured in solution and becomes α-helical upon liposome binding. PC, phosphatidylcholine; PS, phosphatidylserine. Molar protein-to-lipid ratio, 1:530; θ=molar ellipticity. **h**, Recombinant (0.5 μg) and α-synuclein in brain cytosol (12 μg total protein) run as apparent tetramers on blue native gels without boiling or after boiling for 5 min. **i**, SEC–MALS reveals that recombinant α-synuclein (0.5 mg) is monomeric.

during gel filtration. Indeed, circular dichroism spectroscopy showed that recombinant α-synuclein lacked detectable secondary structure, but became α-helical upon membrane binding (Fig. 1g). Consistent with the gel-filtration analysis, both native and recombinant α-synuclein migrated as a single band of ~65 kDa on blue native gels. Notably, however, both recombinant and native α-synuclein still migrated at that apparent size after boiling, which disrupts secondary and tertiary structures, arguing against a folded multimer (Fig. 1h). Furthermore, size-exclusion chromatography coupled with multi-angle laser-light scattering (SEC–MALS) revealed that recombinant α-synuclein was monomeric (Fig. 1i). As native α-synuclein in brain cytosol and recombinant α-synuclein behave identically in gel filtration and blue native gel-electrophoresis experiments, the SEC–MALS demonstration that recombinant α-synuclein is monomeric suggests that native brain α-synuclein in cytosol is also monomeric.

We next tested whether native brain α-synuclein is still monomeric even when purified. We purified α-synuclein from mouse brain without detergents or denaturing conditions (purity >90%; Fig. 2a). Mass spectrometry showed that native brain α-synuclein was substantially larger than predicted (measured mass, 16,408 ± 894 Da ($n = 3$); predicted mass, 14,485 Da). The increased mass is partly due to amino-terminal acetylation of brain α-synuclein[12,13] (Fig. 2b). SEC–MALS revealed that freshly purified native α-synuclein was again predominantly monomeric (Fig. 2c). We also observed a plateau along the left shoulder of the main SEC–MALS peak with a mass of ~58 kDa that contained little detectable α-synuclein (<5% by immunoblotting), and whose observed molecular mass is inconsistent with a putative tetramer. Circular dichroism spectroscopy showed a largely random-coil conformation (34–59%) with α-helical contributions (21–24%; Fig. 2d). Purified α-synuclein aggregated in a time-dependent manner, with a relative increase in overall secondary structure as observed by circular dichroism spectroscopy (Fig. 2d), and the appearance of larger particles as uncovered by dynamic light scattering (Fig. 2e).

Our data show that native brain α-synuclein primarily consists of an unstructured monomer, but readily aggregates in a time-dependent manner. This conclusion was demonstrated both for unpurified α-synuclein as a component of brain cytosol (Fig. 1), and for purified α-synuclein in solution (Fig. 2c). Purified brain α-synuclein — analysed here for the first time — carries significant post-translational modifications (Fig. 2b), which do not, however, seem to alter its folding, as the biophysical properties of recombinant unmodified α-synuclein and native modified α-synuclein were similar (Figs 1 and 2). The differences between our results with brain α-synuclein and those obtained with erythrocyte α-synuclein[12] may be due to erythrocyte-specific post-translational modifications, or to time-dependent multimerization/aggregation of erythrocyte α-synuclein that may have been overlooked. Indeed, the circular dichroism spectrum of erythrocyte α-synuclein[12] is similar to that of purified brain α-synuclein after 75 h incubation (Fig. 2d). Independent of which explanation will account for the differences in results obtained with brain and erythrocyte

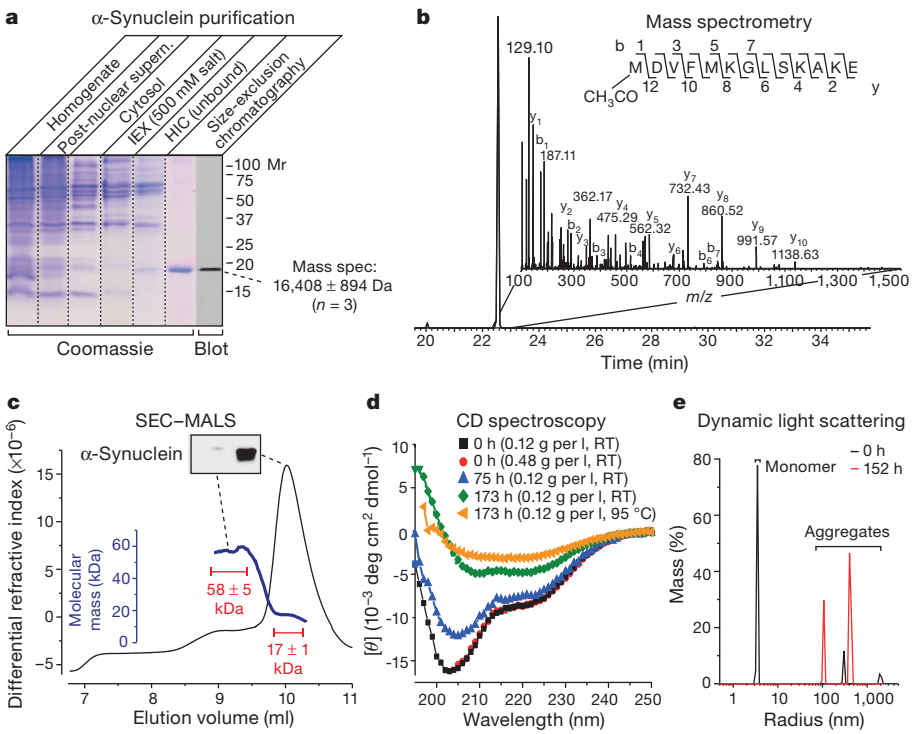

Figure 2 | Purified native brain α-synuclein is predominantly an unstructured monomer that aggregates in a time-dependent manner. **a**, SDS–PAGE analysis of five stages of α-synuclein purification from mouse brain. IEX, anion exchange chromatography; HIC, hydrophobic interaction chromatography. Purified α-synuclein was also analysed by immunoblotting and mass spectrometry as shown. **b**, Mass spectrometry analysis reveals N-terminal acetylation of native α-synuclein. Shown is an extracted ion chromatogram of the N-terminally acetylated α-synuclein peptide. Inset, tandem MS spectrum containing the sequence of the N-terminal peptide and identified b and y ions. **c**, SEC–MALS shows that purified brain α-synuclein (150 μg) is largely monomeric (main peak with a mass of 17 ± 1 kDa), but includes a minor component (plateau along the left shoulder with a mass of 58 ± 5 kDa) that contains little detectable α-synuclein (see immunoblot in boxed region). Calculated masses were extracted from marked areas. **d**, Circular dichroism spectroscopy of freshly purified brain α-synuclein (0.12 g per l = 7.5 μM) shows mainly disordered conformations that progressively acquire structured conformations as a result of time- and temperature-dependent aggregation. RT, room temperature. **e**, Purified brain α-synuclein (0.12 mg ml^{-1}) rapidly aggregates as measured by dynamic light scattering immediately (0 h) or 152 h after purification.

α-synuclein, the conformationally labile state of native brain α-synuclein documented here provides a potential explanation for why α-synuclein is susceptible to pathological aggregation as observed in multiple neurodegenerative disorders[4,5].

Methods

α-Synuclein was purified from mouse brain cytosol (obtained from brain homogenates by ultracentrifugation at 280,000g_{av}) by sequential chromatography on Q sepharose (elution at 0.3–0.5 M NaCl, 20 mM Tris-HCl, pH 7.4), phenyl sepharose (flow-through in 1 M $(NH_4)_2SO_4$) and Superdex-200 10/300GL. SEC–MALS was performed on a WTC-030S5 column (Heleos OptiLab instruments, Wyatt Technology). Circular dichroism spectra were measured in 25% PBS on an Aviv CD Spectrometer and deconvolved (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) with Contin-4 and -7 reference sets. Mass spectrometry was performed on purified α-synuclein or α-synuclein-containing gel pieces digested with Glu-C and Protease Max (Promega, using standard procedures)[14]. All other methods have been described previously[3].

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Bartels & Selkoe reply

REPLYING TO J. Burré *et al.* *Nature* **498,** http://dx.doi.org/10.1038/nature12125 (2013)

In disagreeing with our report that native α-synuclein occurs physiologically as an α-helically folded tetramer in neural and erythroid cells[1], Burré *et al.*[2] conclude instead that 'native brain α-synuclein' consists of a largely unstructured monomer. They make two implications about our paper that are inaccurate: (1) that our findings pertained only to erythrocyte α-synuclein (we reported multiple experiments on neural cells); and (2) that we concluded that cellular α-synuclein is a stable tetramer under all conditions (we did not use the term 'stable', and we observed monomers and some other oligomers in normal cells (e.g., Fig. 1d of ref. 1)). Indeed, we emphasized the need to discover "compounds that … could kinetically stabilize native tetramers and prevent pathogenic α-synuclein aggregation". Although the data in our report suggest that tetramers are the predominant native species, tetramers and other oligomers arise from monomers, so there must be an equilibrium between monomeric and oligomeric forms in cells. Pathogenic events (e.g., mutations) could alter this equilibrium, and some therapeutic compounds could potentially re-establish it, as we explicitly suggested[1].

Most findings in Fig. 1 of Burré *et al.*[2] confirm previous reports (including ours[1]) that recombinant α-synuclein is an unfolded monomer of ~14 kDa but migrates anomalously at ~60 kDa in gel filtration (their Fig. 1f), presumably owing to the large hydrodynamic radius of an extended monomer. We had stated that this made "gel filtration an unreliable indicator [of mass] and therefore [it was] not used here"[1]. That recombinant α-synuclein becomes α-helical upon binding phospholipid vesicles (their Fig. 1g) was also long known[3] and observed by us[1]. The key difference from our work regards their data on the folding and assembly state of native α-synuclein (their Fig. 2). We believe these data are less in disagreement with our conclusions than the authors suggest. First, they show by size-exclusion chromatography coupled with multi-angle laser-light scattering (SEC–MALS) the existence of small amounts of α-synuclein tetramer (58.5 kDa) in their natively purified brain preparation (their Fig. 2c). Then, their Fig. 2d shows circular dichroism spectra of purified brain α-synuclein that display a mixture of unfolded (34–59%) and α-helically folded (21–24%) protein, a clear structural difference from recombinant α-synuclein, which is all unfolded (their Fig. 1g, 'buffer'). Their findings are not entirely incompatible with our paper, as we had stated that helical tetramers were the predominant physiological species but variable amounts of monomers and other oligomers were observed[1].

Given that even the helically folded tetramer suggested by us[1] (and others[4,5]) contains only about 50% helical structure (as the regions around amino acid 50 form structured loops and the carboxy terminus is conformationally mobile), the fact that their circular dichroism spectrum contains ~24% helical conformation suggests that up to half of their

brain α-synuclein sample is folded and the other half is unfolded. The latter result raises the possibility of either differences in tetramer:monomer equilibria between their (murine brain) and our (human erythrocyte or neuroblastoma) samples or a partial denaturation of the brain sample during purification. Interestingly, room-temperature incubation of their unfolded monomeric/partly folded tetrameric sample led to overall loss of circular dichroism spectral intensity (by ~50%), probably due to aggregation and precipitation of some of the protein out of solution, and a relative increase in helical content of the protein remaining in solution (their Fig. 2d, green). The authors correctly indicate that now their spectrum of purified brain α-synuclein is similar to our spectrum of purified erythrocyte α-synuclein. They say this conversion indicates that "purified a-synuclein aggregated in a time-dependent manner, with a relative increase in secondary structure", but using the term 'aggregation' for this helical change is different from the widely studied pathogenic aggregation of α-synuclein that involves a conversion to a β-sheet-rich structure. It was the latter type of aggregation that we showed native α-synuclein to be resistant to (Fig. 3d of ref. 1). Burré *et. al*[2] only observed loss of α-helical structure after heating brain α-synuclein to 95 °C (their Fig. 2d, orange), a condition that similarly led to denaturation of our purified α-synuclein helical tetramers (Supplementary Fig. 11 of ref. 1) and thus does not disprove our conclusion that native helical α-synuclein does not readily aggregate under physiological conditions.

The loss of overall circular dichroism signal accompanied by an increase in α-helical spectral components that Burré *et al*.[2] show in Fig. 2d could be interpreted in two ways: (1) some sample precipitation occurs, and at the same time the remaining soluble α-synuclein becomes increasingly α-helically folded (such an event could be interpreted as the refolding of a partially denatured protein); or (2) the monomeric, unfolded portion of the mixture (their Fig. 2c) aggregates and precipitates out of solution (their Fig. 2e), whereas the helically folded, apparently tetrameric component (their Fig. 2c) stays unaltered in solution and provides the circular dichroism signal. The latter interpretation would be consistent with our hypothesis that destabilization of helical tetramers into unfolded monomers in cells may precede pathological α-synuclein aggregation[1]. In summary, the difference between their purified brain α-synuclein and our purified erythrocyte and neuroblastoma α-synuclein seems to be the relative abundance of the aggregation-resistant helical material at the time of initial analysis.

Even though the dynamic light scattering data of Burré *et al*.[2] in Fig. 2e imply an increasing amount of aggregates (in agreement with the partial precipitation suggested in their Fig. 2d), no conclusion about the amount of remaining monomers/tetramers in the sample can be drawn from this, given the inability of dynamic light scattering to detect small particles if sufficient amounts of large particles are present in the mixture.

Collectively, the data of Burré *et al*.[2] show the existence of some helically folded, apparently tetrameric (58.5 kDa) protein in purified α-synuclein isolated from normal mouse brain, although in their hands, this constitutes only half (by their Fig. 2d) or a minor portion (by their Fig. 2c) of their total protein immediately after purification and only becomes the major species upon incubation over time (their Fig. 2d). Given that the two studies are therefore debating the relative proportion under native conditions of helically folded tetramers, not their existence *per se*, we believe it is reasonable to pursue attempts to stabilize helically folded native α-synuclein tetramers as an approach to reducing the pathological aggregation of monomers. In light of our findings in Bartels *et al*.[1] and in an extensive α-synuclein crosslinking analysis in intact neurons and other cells[6], the combined recent data support the hypothesis that physiological α-synuclein occurs in cells in an oligomeric (principally tetrameric) state in the cytosol[1,4,5,6] which is in equilibrium with unfolded monomers.

This Reply is written by two out of three of the authors from the original paper[1]. J. G. Choi left the laboratory for Graduate School in 2011.

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