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Parkinson-causing α -synuclein missense mutations shift native tetramers to monomers as a mechanism for disease initiation

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β -Sheet-rich α -synuclein (α S) aggregates characterize Parkinson's disease (PD). α S was long believed to be a natively unfolded monomer, but recent work suggests it also occurs in α -helix-rich tetramers. Crosslinking traps principally tetrameric α S in intact normal neurons, but not after cell lysis, suggesting a dynamic equilibrium. Here we show that freshly biopsied normal human brain contains abundant α S tetramers. The PD-causing mutation A53T decreases tetramers in mouse brain. Neurons derived from an A53T patient have decreased tetramers. Neurons expressing E46K do also, and adding 1-2 E46K-like mutations into the canonical α S repeat motifs (KTKEGV) further reduces tetramers, decreases α S solubility and induces neurotoxicity and round inclusions. The other three fPD missense mutations likewise decrease tetramer:monomer ratios. The destabilization of physiological tetramers by PD-causing missense mutations and the neurotoxicity and inclusions induced by markedly decreasing tetramers suggest that decreased α -helical tetramers and increased unfolded monomers initiate pathogenesis. Tetramer-stabilizing compounds should prevent this.

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α -Synuclein (α S) accumulates as insoluble protein aggregates in Lewy bodies and Lewy neurites, the hallmarks of familial and sporadic Parkinson's disease (PD) and several other fatal diseases¹. α S missense mutations, copy number variants and upregulated expression have each been associated with familial PD^{2–6}. Unexpected findings from our⁷ and one other laboratory⁸ suggested that α S can occur physiologically not only as unfolded monomers but in large part as multimers, principally tetramers, that have α -helical conformation and resist aggregation. Crosslinking analyses of endogenous α S in living cells⁹ supported this new model: in primary neurons and other cells, cell-penetrant crosslinkers disuccinimidyl glutarate (DSG) or dithiobis(succinimidyl propionate) (DSP) trapped α S in abundant species larger than monomer, especially at ~ 60 kDa, the size of four *N*-acetylated α S monomers ($4 \times 14,502$ daltons = 58,010 daltons). Known monomeric proteins (for example, cytosolic tubulin; Parkin; Ran) were not altered by the crosslinking, while known multimeric proteins (for example, DJ-1; voltage-dependent anion channel (VDAC); dynamin-related protein 1 (Drp1)) were trapped as the expected multimers. We systematically ruled out artifactual induction of these multimers by the crosslinking, abnormally migrating monomers, and hetero-oligomers with other proteins⁹. In addition to the major ~ 60 kDa apparent tetramer (α S60), smaller amounts of endogenous α S were trapped as multimers of ~ 80 and ~ 100 kDa (α S80 and α S100) that have the same isoelectric point as the monomer and may be conformers of the tetramer or higher-order multimers (for example, hexamer and octamer)⁹. All multimers were depolymerized to monomers when crosslinkers were applied after cell lysis, unless the lysis was done at high protein concentrations ('molecular crowding')⁹. Other endogenous multimeric proteins did not show this striking cell lysis sensitivity, except for the homolog β -synuclein (β S)⁹. These findings led us to postulate that dynamic intracellular populations of α S monomers and metastable multimers co-exist normally^{7,9}. Such a dynamic equilibrium has been proposed for well-known tetrameric proteins such as p53 (refs 10,11) and haemoglobin¹².

Some labs subsequently published data they interpreted as supporting the earlier model of α S existing as natively unfolded monomers in cells and brain. However, these studies either did not employ crosslinking of intact cells¹³ or considered any crosslinked oligomeric α S to be non-specific¹⁴. The possibility that the endogenous multimers we trap by crosslinking are pathological is made highly unlikely by biophysical evidence of α -helical tetramers (~ 58 kDa) purified natively from normal human cells^{7,15}, the abundance of trapped tetramers in healthy, non-transfected cells^{9,16} and the trapping in analogous tetrameric forms of the non-pathogenic β S and of α S lacking its aggregation-prone NAC domain⁹.

This unresolved but central debate about the existence and biological relevance of α S tetramers led us to use additional approaches. Here we first examined α S in freshly biopsied normal human brain, the most physiologically relevant source of the protein. To do so, we developed a method for intact-cell crosslinking of fresh brain tissue that reveals the endogenous state of α S. Second, we asked whether α S missense mutations that unequivocally cause PD might alter the tetramer:monomer ratio in human neurons. Despite almost two decades of study, the fundamental mechanism by which these mutants initiate α S aggregation, Lewy body formation and PD remains unclear. To this end, we rigorously standardized our intact-cell crosslinking method until it was precise enough to quantify accurately the effects of each of the five PD-causing α S missense mutations. And third, we applied fluorescent protein complementation to

independently confirm our key findings in living cells. Using both live-cell methods (chemical crosslinking and yellow fluorescent protein (YFP) complementation), we found that PD-causing α S missense mutations partially shifted cellular α S from tetramers/multimers to monomers. This shift became pronounced when we amplified the effect of the E46K PD mutation by making similar E-to-K substitutions in two adjacent KTKEGV repeat motifs, leading to marked reduction of cellular tetramers, decreased α S solubility, formation of round inclusions and cytotoxicity.

Results

Abundant α S tetramers occur in normal human and mouse brain.

We previously reported that the crosslinking pattern of endogenous α S in intact cells differed from the stochastic crosslinking of recombinant monomeric α S *in vitro*, in that the former strongly favoured the trapping of tetramers, with few or no intermediate dimers or trimers⁹. We now sought to crosslink α S in its endogenous state in normal brain tissue. We achieved this by mincing fresh brain with a mechanical tissue chopper, performing gentle centrifugal washes on the intact tissue pieces and then subjecting only these washed brain bits to crosslinking, thereby largely avoiding the tetramer-destabilizing effects of breaking cells open⁹ (see Methods and Supplementary Fig. 1). We applied this protocol to an optimal source of physiological α S: a fresh brain biopsy (elective surgery for focal epilepsy) from the cerebral cortex of a young patient free of any neurodegenerative process. After the mincing and centrifugal washes, we applied DSG (1 mM solution) at an increasing ratio of volume of crosslinking solution to brain protein, or else applied the reducible crosslinker DSP (2 mM solution) at a fixed ratio. The crosslinked tissue bits were then sonicated and separated (100,000 g spin) into PBS ('cytosol') and Triton X-100 (TX-100; 'membrane') fractions. Immunoblotting confirmed efficient crosslinking had occurred, as the cytosolic protein DJ-1 and the transmembrane protein VDAC were each trapped in their native dimeric states in a DSG dose-dependent manner (Fig. 1a, upper panels). As before, we fixed blots in paraformaldehyde¹⁷ to improve α S retention during blot washing¹⁶. Total α S levels were compared using the DSP-crosslinked-and-reduced sample, which we had shown previously was optimal for quantifying total cellular α S levels in crosslinking studies¹⁶. Enrichment of DJ-1 and absence of VDAC and synaptobrevin-2 established cytosol purity (Fig. 1a, upper panels). Immunoblotting confirmed our earlier findings in cultured neurons⁹: α S was detected in the biopsied human brain primarily as tetramers (α S60) and related multimers (α S80, α S100) by mAbs Syn1 and 15G7 (Fig. 1a). Antiserum C20 preferentially detected monomers, although its α S60:14 ratio was still 1:1 versus 2:1 using mAb Syn1 (Fig. 1a, lower panels). Including the α S80 and α S100 multimers with the α S60 tetramers showed the clear preponderance of α S multimers over monomers (3:1) in normal human brain. Similar to our findings in primary neurons⁹, the multimers were found overwhelmingly in the human brain cytosol (Fig. 1a, lower panels). In the membrane fraction, virtually only monomer was detected.

Next, we obtained closely similar results in minced fresh wild-type (WT) mouse brain using antibodies to α S and, importantly, to β S (Fig. 1b). Applying increasing DSG concentrations to mouse brain bits, human erythroleukemia (HEL) cells (Fig. 1c) or cultured primary neurons (Fig. 1d) did not completely trap α S at discrete multimeric positions. Instead, α S60 levels plateaued at a certain DSG concentration; further depletion of free 14 kDa monomer only added to high molecular weight (HMW) smears of α S immunoreactivity, probably the result of non-specific overcrosslinking (Fig. 1c). Similar to α S, known tetrameric proteins such as p53 (refs 18,19) and Drp1 (ref. 20) exhibited HMW smears as well as oligomeric bands higher than their

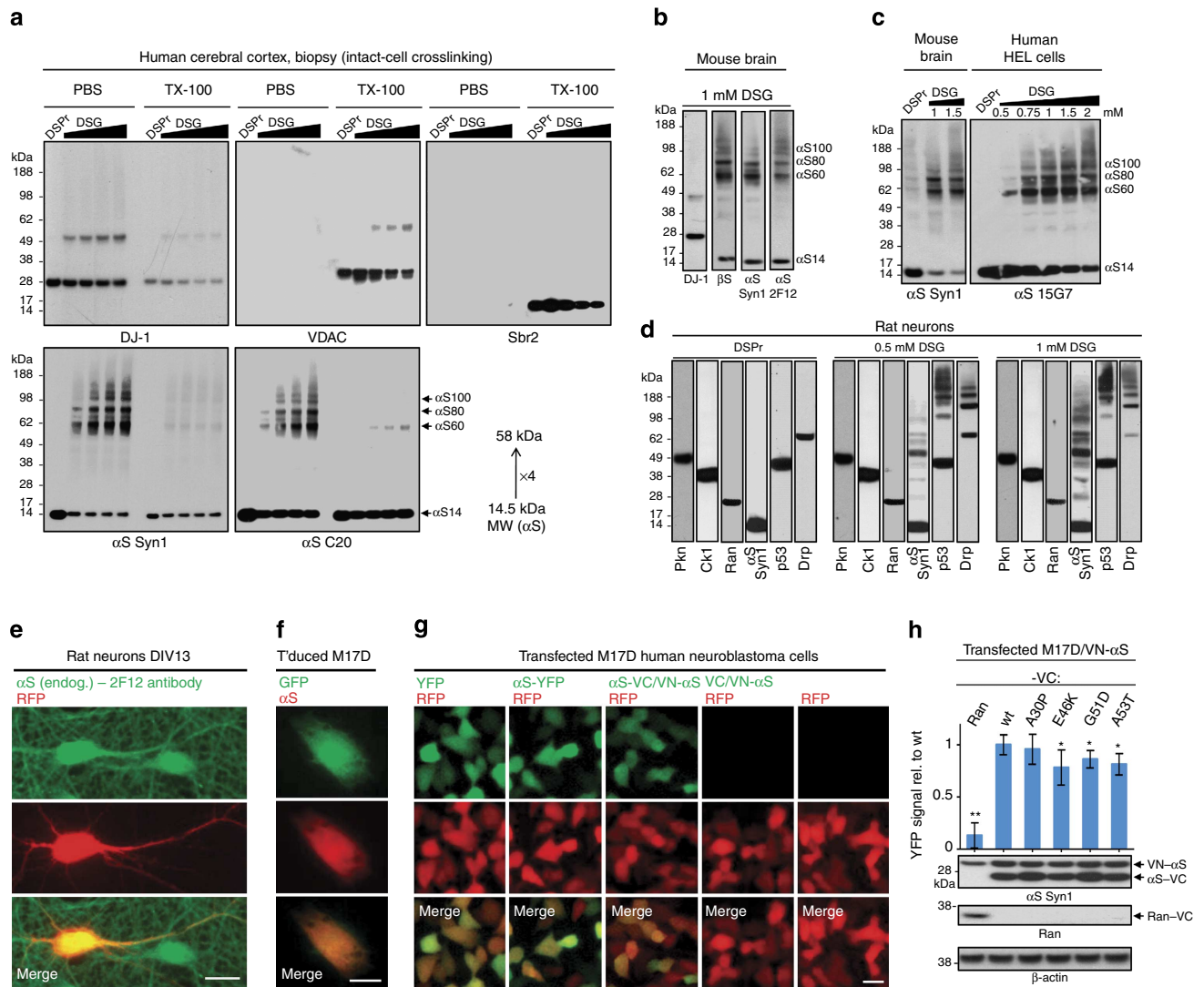


Figure 1 | α S multimers in normal brain tissues and neural cells. (a) A fresh human cortical biopsy was crosslinked with either 1.75 mM DSP and reduced by β ME (DSPr) or else with 1 mM DSG at increasing volume-to-protein ratio, followed by sequential extraction of PBS- and TX-100-soluble fractions. Each lane is one technical replicate from the biopsy. (b) Mouse brain, 1 mM DSG, PBS fraction; blots represent five independent experiments from different WT mice. (c) Mouse brain and human erythroleukemia (HEL) cells. DSG concentration gradients applied as indicated, and PBS fractions prepared; blots represent at least three independent experiments. (d) DIV13 rat neurons. 1.75 mM DSP/ β ME (DSPr, left panel), 0.5 mM DSG (middle) and 1 mM DSG (right) were applied, and PBS fractions prepared. Western blots for α S (Syn1 mAb), the monomeric proteins Parkin (Pkn), casein kinase 1 α (CK1) and Ran, and the tetrameric proteins p53 and Drp1 (Drp); blots represent at least 3 independent experiments from different primary cultures. (e) Fluorescence microscopy of DIV13 rat neurons: endogenous α S in green (mAb 2F12) and transfected RFP (red), plus merged image; scale bar, 20 μ m. (f) Fluorescence microscopy of virally transduced M17D cells: α S in red (mAb 2F12), GFP in green; scale bar, 5 μ m. (g) Fluorescence microscopy of M17D cells transiently transfected with RFP plus YFP or α S-YFP or YFP α S complementation pairs, as indicated (empty vector control on far right). YFP in green, RFP in red, merge below; scale bar, 10 μ m. (h) Quantification of multiple Venus-YFP complementation assays. A stable cell line M17D/VN- α S was transfected with DNA constructs expressing VC fused to α S (WT, A30P, E46K, G51D or A53T) or Ran (negative control). YFP complementation intensity relative to WT α S-VC transfection from N = 8 independent experiments on 4 days using different DNA preparations; * P < 0.05, ** P < 0.01, Student's t -test; relative to WT in this and subsequent figures, unless stated otherwise; error bars, s.d. Below are representative western blots for α S (Syn1 mAb), Ran, and the loading control β -actin.

expected tetramer positions, especially at high DSG (Fig. 1d). Interestingly, crosslinking of the known tetrameric protein p53 was similar to α S in that intermediate dimers were of low abundance, whereas Drp1 showed a more ladder-like oligomer pattern (Fig. 1d). Monomeric cytosolic proteins such as Parkin, Ran and casein kinase 1 α were always unaffected by crosslinking, as expected (Fig. 1d; Supplementary Fig. 2 for uncropped blots). Compared with the known tetrameric proteins, crosslinking of the known dimeric proteins DJ-1 or VDAC led to less HMW smears (Fig. 1a). These multiple control proteins support the

ability of our crosslinking protocol to detect endogenous tetramers of proteins like p53, α S and β S in intact cells and brain tissue and not to induce artifactual oligomers of known monomeric proteins.

These data support a model in which α S exists predominantly as cytosolic multimers at steady state, while a smaller cytosolic portion and most α S on membranes (together representing a minority of total cellular α S) appears to be monomeric. To address the localization of intracellular α S without disrupting the cell membrane, we performed immunofluorescence microscopy

of endogenous α S in primary rat neurons (Fig. 1e) and also virally expressed WT α S in human M17D neuroblastoma cells, which have very low endogenous α S (Fig. 1f). In both cell types, α S strongly co-localized with transiently expressed red fluorescent protein (RFP; Fig. 1e) or green fluorescent protein (GFP; Fig. 1f), which are known to be soluble and cytoplasmic. Next, we transfected M17D cells with α S fused to either intact YFP (Fig. 1g, second column) or else split YFP, that is, we co-transfected VN- α S (N-terminal half of Venus-YFP fused to N-terminus of an α S monomer) and α S-VC (C-terminal half of Venus-YFP fused to the C-terminus of an α S monomer) (Fig. 1g, third column). YFP complementation assays monitor the interaction of intracellular proteins in real-time and are based on the principle that interactions of the tagged protein (here α S) bring VN and VC into close proximity, leading to Venus-YFP complementation and thus fluorescence detectable by microscopy or plate reader. The living cells showed strong YFP fluorescence that co-localized with free cytosolic RFP and was distributed similarly to YFP alone (Fig. 1g, first column). These controls confirmed previously reported fluorescent complementation of WT α S using such an assay²¹ and underscored the cytosolic nature of these multimeric species. VC alone (C-terminal half of Venus-YFP) did not complement with VN- α S, ruling out an intrinsic affinity of VC and VN as responsible for the observed complementation (Fig. 1g: fourth versus fifth columns).

Two independent methods to quantify cellular α S multimers.

To standardize the fluorescent complementation assay for repeated measurements, we generated a VN- α S stable M17D line, enabling highly consistent complementation signals by transiently transfecting this line with various WT or mutant α S-VC constructs. Using an automated plate reader to quantify YFP fluorescence, we found that VN- α S complementation with VC-tagged Ran (a control cytosolic protein) was negligible compared with α S-VC (quantified in Fig. 1h: first two lanes). Additional controls for signal specificity, including the need for specific fusion positions of α S relative to the fluorescent protein fragments and the negligible self-complementation of the split YFP fragments alone (see Fig. 1g, fourth lane), were reported previously for α S GFP-complementation²² and YFP-complementation²¹. Despite the rapid and strong self-interaction we detected for WT α S (Fig. 1h, second lane) and the widespread use of fluorescent protein complementation to study native protein interactions, the α S field has heretofore interpreted such complementation as indicative of pathological oligomerization, given the long-standing assumption that the normal state of cellular α S is monomeric¹⁴. We analysed the effects of the known familial PD-linked missense mutations and observed significant reductions of fluorescent complementation for the E46K, G51D and A53T mutants versus WT α S (Fig. 1h). A30P complementation was similar to WT, but we consistently observed increased expression of A30P and also G51D by immunoblotting (Fig. 1h; top western blot), indicating that these two variants lead to enhanced α S-VC levels, and thus normalized values might detect reduced complementation for A30P as well. The decreased α S complementation seen here with three of the four familial PD (fPD) mutants studied supports the physiological nature of the multimers (otherwise, fPD mutations should increase complementation). There are certain disadvantages of this system: α S is modified by large tags; determining the sizes of the multimers is not possible; YFP complementation may be irreversible²³ and thus disturb multimer:monomer equilibria; and crosslinking trapped abundant dimers but less tetramers of VN- α S + α S-VC (Supplementary Fig. 3), a pattern different

from endogenous (untagged) α S. The latter result may be due to the relative steric hindrance we previously reported for tagged α S⁹ and/or decreased access by the crosslinker into any tagged tetramers.

In light of these disadvantages of relying solely on YFP complementation for α S multimer quantification, we sought to rigorously standardize our intact-cell crosslinking protocol as a second independent method (Methods). We transfected M17D cells with increasing amounts of WT α S cDNA and applied a constant DSG amount at 40 hr post transfection. By confirming equal DJ-1 dimer:monomer ratios in the same cytosols as an internal standard for crosslinking efficiency in all experiments (Fig. 2a, left panel), we observed a constant α S60:14 ratio across the range of α S expression levels we tested (Fig. 2a, right panel). Plotting the relative intensities of α S60 and α S14 in multiple independent experiments revealed a highly linear relationship of the two ($R^2=0.97$) (Fig. 2b), and the apparent α S60:14 ratio (using Syn1 for detection) was a constant 1.2 in this series of experiments (Fig. 2c). (For the three multimer species combined (α S60 + 80 + 100), their ratio to monomer was 2.8.) We next tested the reducible crosslinker DSP (Fig. 2d) and again found a highly linear correlation ($R^2=0.97$) between α S60 and α S14 levels (Fig. 2e), here with a constant ratio of 0.9 (Fig. 2f). Analysis of α S80 and α S100 revealed a similarly linear ratio to monomer across this range of α S expression (Supplementary Fig. 4), but we focused our further analyses on the α S60 tetramer as our standard readout for four reasons: an independent biophysical method (analytical ultracentrifugation) originally revealed that the principal α -helical multimer purified from fresh human erythrocytes under non-denaturing conditions has a molecular weight of 58 kDa, exactly that of four monomers⁷; all of our many intact-cell crosslinking experiments to date identified α S60 as the most abundant and consistent cellular multimer^{7,9,16}; mass spectrometry confirmed here that α S60 contains only α S (Supplementary Fig. 5); and for quantification, α S60 is representative of all physiological α S multimers we detect (for example, Supplementary Fig. 4). The constant α S60:14 ratio across a wide range of cellular α S levels (Fig. 2c,f) was important for our subsequent analysis of fPD mutations: even if α S expression levels differ somewhat among mutants for biological or technical reasons, comparisons of the ratios should be meaningful.

The linear correlation of α S60 and α S14 levels across the expression range we tested suggests that these levels are theoretically in the same range as the apparent equilibrium constant. However, protein assembly systems in living cells are not in a true equilibrium but rather in a dynamic flux influenced by factors like biosynthetic and degradation rates and changes in subcellular localization. In this context, it is noteworthy that the α S concentrations we obtained by transient expression in human neuroblastoma cells (0.15–1.65 ng α S per μ g of total cytosolic protein when using a range of 1–8 μ g DNA transfected into 6-cm dishes) did not exceed the α S concentration in lysates of freshly biopsied human brain (1.8 ng μ g⁻¹), as quantified by α S enzyme-linked immunosorbent assay (ELISA) and confirmed by immunoblotting (Fig. 2g). Indeed, our measured concentrations are lower than the endogenous neuronal concentration in brain because brain lysate includes proteins from many non-neuronal cells having low α S. Overall, we achieved generally similar PBS-soluble (cytosolic) α S levels when we repeatedly expressed WT or five α S mutants in M17D cells (Fig. 2h). Despite a putative function in membrane vesicle homeostasis, α S has been reported to be predominantly cytosolic^{13,24,25}, consistent with our data in primary neurons⁹ and human brain (Fig. 1a). In accord, our ELISA detected a very high portion (>85%) of cellular α S in the cytosol; the TX-100-soluble membrane fractions contained minor

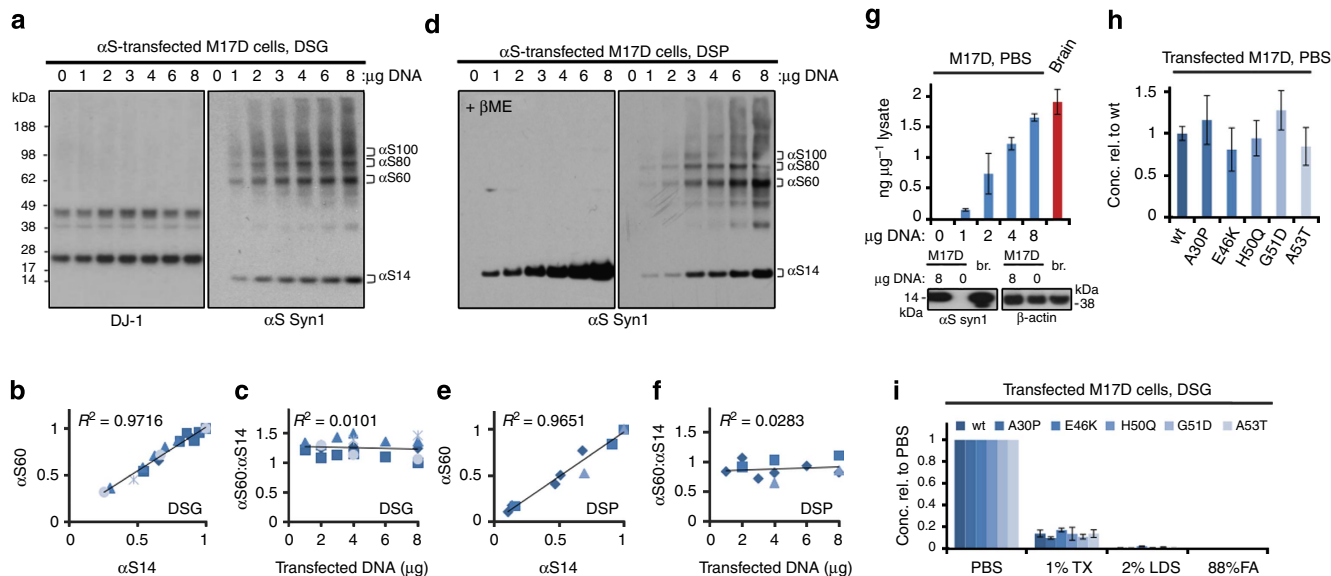


Figure 2 | Intact-cell crosslinking of α S expressed at varying levels. (a) DSG-crosslinking analysis of an α S DNA gradient (0–8 μ g per 6-cm culture dish). Western blots for α S (Syn1) and endogenous DJ-1. (b) DSG samples: α S60 intensities plotted against α S14 (densitometry). Highest value in each series was set to 1; graph shows mean data for $N = 5$ independent experiments of 1, 2, 3, 4, 6, 8 μ g DNA (2 exps.), 2, 4, 8 μ g DNA (1 exp.) and 4, 8 μ g DNA (2 exps.); data points generated in the same experiment are indicated by identical symbols. (c) α S60: α S14 ratios for the same samples as in 2b. (d) DSP-crosslinking analysis of an α S DNA gradient (0; 1–8 μ g); western blots (Syn1) for α S in non-reduced and β ME-reduced samples (e) Quantification of DSP samples: α S60 versus α S14. Highest value in each series was set to 1; $N = 3$ independent experiments of 1, 2, 3, 4, 6, 8 μ g DNA (1 exp.), 2, 4, 8 μ g (1 exp.) and 4, 8 μ g (1 exp.). (f) α S60:14 ratios for the same samples as in (e). (g) ELISA analysis of α S DNA gradients (1, 2, 4, 8 μ g) transfected into M17D cells compared to human cortical homogenate (PBS fraction). Below: western blots for 0 and 8 μ g DNA transfection versus human brain homogenate (red). $N = 2$ for gradients (different days, different DNA preps) and brain homogenates. (h) ELISA of α S WT and fPD mutant transfected cells (8 μ g per 6-cm dish), PBS fraction. Graph: concentrations versus α S WT set to 1 ($N = 10$ independent transfections on 4 different days using at least 4 different DNA preps per α S variant). t'ed, transfected. (i) ELISA of α S WT and fPD mutant transfected cells after 1 mM DSG crosslinking and sequential extraction (PBS \rightarrow PBS/1% Triton \rightarrow 2% LDS \rightarrow 88% formic acid = FA). Graph: concentrations relative to the PBS fractions of the respective α S variant set to 1.

amounts (<15%) (Fig. 2i). Highly insoluble cellular fractions (that is, sequential 2% SDS and 88% formic acid extracts) contained very minor α S amounts, with no obvious effects of the 5 fPD mutations. Our ELISA was validated as detecting both normal and aggregated forms of α S (Supplementary Fig. 6). Western blots likewise detect only minor α S amounts in the 2% SDS-soluble fraction⁹. These relative distributions across fractions were true for both WT and fPD-mutant α S even after intact-cell crosslinking (Fig. 2i). Therefore, in our many iterative experiments below, we focused on the PBS-soluble (cytosol) major fraction but included some analyses of the TX-100-soluble minor fraction.

fPD missense mutations lower the α S multimer:monomer ratio.

To determine whether the fPD-causing α S mutations A30P, E46K, G51D and A53T (tested by fluorescence complementation in Fig. 1h) and the recently discovered H50Q²⁶ alter the tetramer:monomer ratio in intact cells, we initially transfected human M17D neural cells with WT α S or one of these five fPD mutants and performed DSG crosslinking and quantitative western blots of the cytosols (Fig. 3a, top panel). Trapping of the endogenous DJ-1 dimer confirmed equal crosslinking across samples (Fig. 3a, middle panel; Supplementary Fig. 7 for uncropped blots). We identified Syn1 as the optimal antibody to detect α S because it is widely used and commercially available (unlike 15G7 or our 2F12), it does not show a preference for monomeric or multimeric α S (C20 reacts more with monomers, and 211 and LB509 do not detect multimers⁹), and it gave the clearest blots of the α S transfectants. Thus, statistical analyses of the experiments that follow used Syn1, but importantly, other α S antibodies confirmed

all key findings in this section. In many iterative experiments, we detected similar or slightly reduced α S expression levels for the mutants versus WT, except for A30P and G51D, which showed variable but modestly higher α S levels (Fig. 3a,b; compare with Fig. 1h). These variations were well within the α S expression range found not to alter the α S60:14 ratio (see Fig. 2). Densitometry of multiple independent experiments after intact-cell crosslinking with DSG (exemplified by Fig. 3a) or DSP (exemplified by Fig. 3b) and a meta-analysis of the two agents revealed highly significant decreases ($P < 0.01$, $n = 25$, one-sided analysis of variance (ANOVA), percentages \pm s.d.; statistical details in Fig. 3 legend and Methods) in the α S60:14 ratio for A30P (77.5 \pm 15.5% of WT, that is, a 22.5% decrease), E46K (61.7 \pm 10.1% of WT, a 38.3% decrease), G51D (47.8 \pm 10.0% of WT, a 52.2% decrease) and A53T (66.5 \pm 10.4% of WT, a 33.5% decrease). H50Q caused a smaller (83.7 \pm 14.4% of WT, 16.3% decrease) but still significant ($P < 0.05$) reduction (Fig. 3c, upper panel). The same significant effects were observed when the ratio of all multimeric species (α S60 + 80 + 100 combined) relative to α S14 was analysed (Fig. 3c, lower panel). We observed decreases versus WT in α S60 levels (Fig. 3d, upper panel) or α S60 + 80 + 100 levels (Fig. 3d, lower panel) for E46K, G51D and A53T, while the α S60 levels for A30P and H50Q were not significantly changed from WT. α S14 monomer levels increased significantly versus WT for A30P and G51D (Fig. 3e). Strikingly, engineered compound fPD mutants '3x' (H50Q + G51D + A53T) and '4x' (H50Q + G51D + A53T + E46K) further decreased the α S60:14 ratio compared with G51D alone, causing clear-cut elevations of free monomers (Fig. 3f,g). We then tested the overall finding—reduction of relative cellular multimer levels by fPD mutants—in a system that is closer to the steady-state situation in neurons,

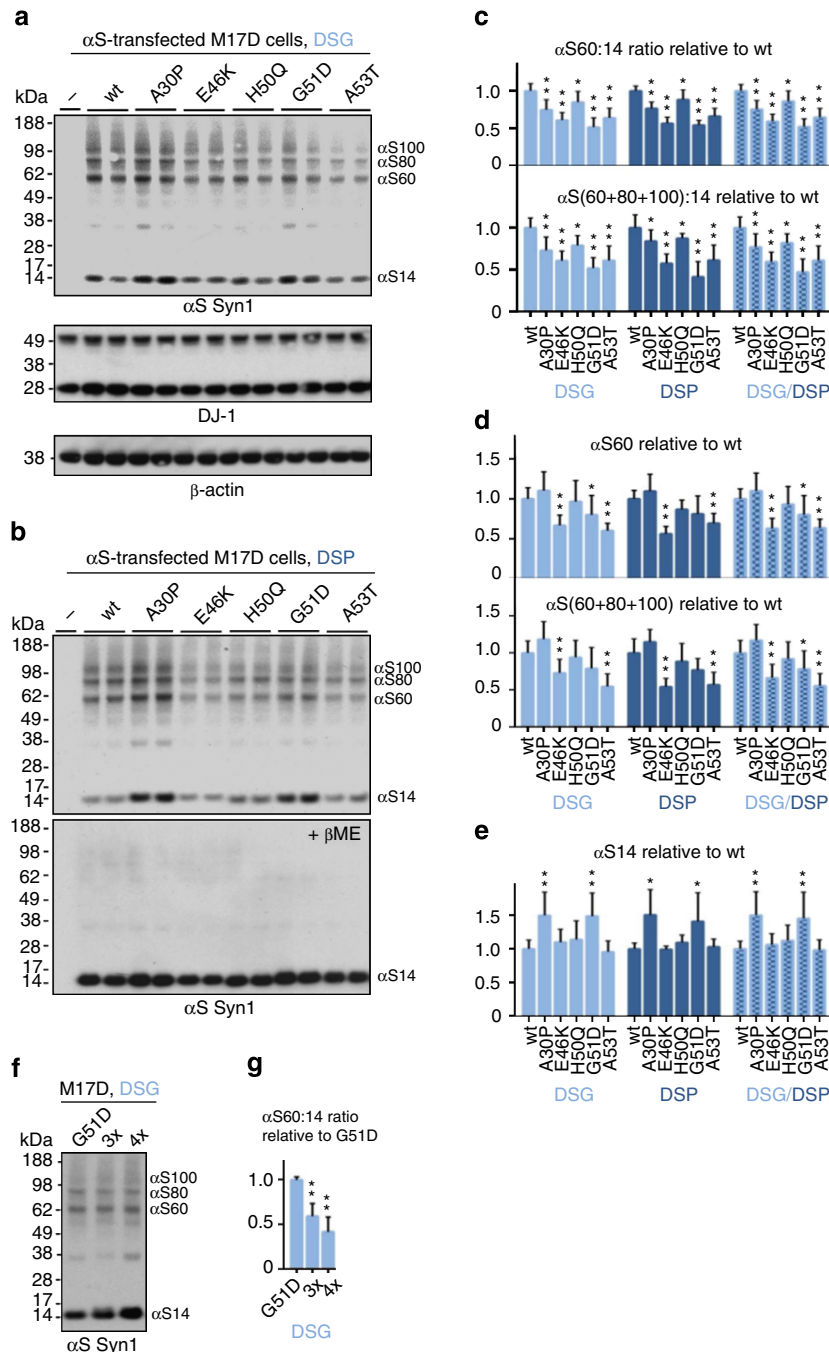


Figure 3 | Intact-cell crosslinking of fPD-linked α S missense mutations. (a) DSG crosslinking analysis of M17D cells transiently transfected with α S WT or the indicated mutations. Western blots for endogenous DJ-1 and transfected α S in duplicate (Syn1); each lane is one transfection. (b) Analogous to Fig. 2a, but using the reducible crosslinker DSP: upper panel, non-reduced; bottom panel, β ME-reduced (Syn1). (c) DSG and DSP crosslinking, plus meta-analysis of both: intensity of α S60 alone (upper panel) or α S60 + 80 + 100 (lower panel) is graphed relative to WT α S (set to 1) (DSG: $N = 8$ experiments each done in biological duplicates ($n = 2$) on different days, total $n = 16$; DSP: $N = 4$, $n = 9$). For better visibility in this and the following graphs, only one-direction error bars are shown. (d) DSG and DSP crosslinking plus meta-analysis: levels of α S60 alone (upper panel) or α S60 + 80 + 100 together (lower) relative to WT α S; (e) level of α S14 alone relative to WT α S. (f) Representative western blots of DSG crosslinking of 3x (H50Q-G51D-A53T) and 4x (3x + E46K) compound fPD mutants relative to G51D alone. (g) Quantification of α S60:14 ratio for 3x and 4x compound fPD mutants relative to G51D alone ($N = 4$, $n = 8$). * $P < 0.05$, ** $P < 0.01$; one-sided ANOVA (see Methods) for all quantifications shown; error bars, s.d.

namely stable lentivirus transduction of the neural M17D cells with WT, E46K or G51D α S. The fPD mutants again reduced the cytosolic multimer:monomer ratio (due to both a decrease in multimers and increase in monomers versus WT), and sequential extractions again showed that non-cytosolic fractions contained only minor α S amounts (Supplementary Fig. 7).

Decreased multimers in hA53T mice and A53T iPSC neurons. Our method of crosslinking fresh, minced brain tissue (Fig. 1; Supplementary Fig. 1; Methods) presented the opportunity to analyse the effects on α S tetramers of a PD-causing α S mutation expressed in mouse brain. We examined the brains (Fig. 4a) of young α S^{-/-} mice expressing relatively low levels of human WT

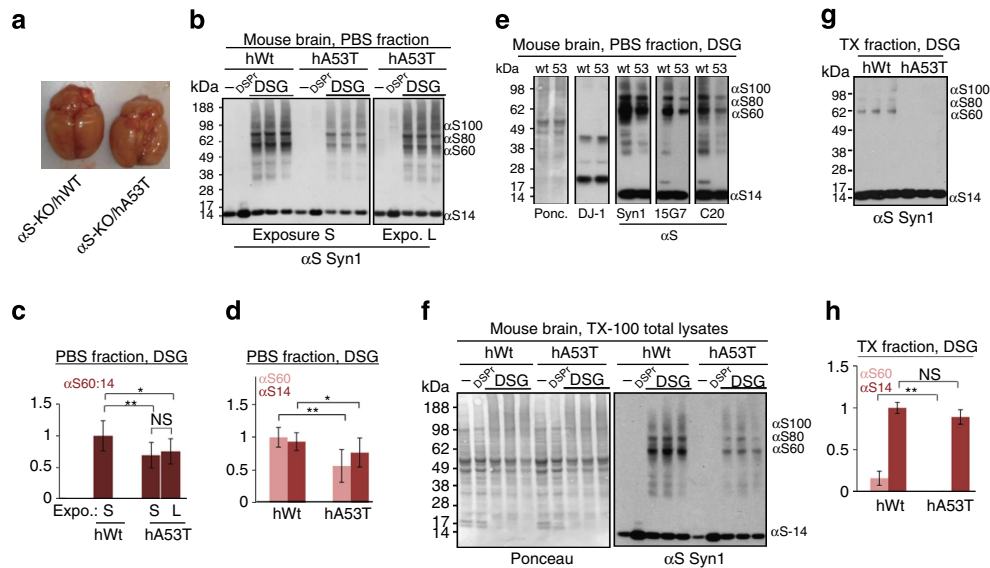


Figure 4 | Transgenic hA53T versus hWT α S expressed in α S^{-/-} mouse brain. (a) Whole brains from both genotypes immediately before mincing. (b) Minced brain bits from both mouse genotypes were subjected to crosslinking, and PBS-soluble ('cytosolic') fractions blotted (Syn1). Untreated (-), DSP/ β ME-treated (DSPr) and DSG-treated samples (in technical triplicate) are shown at identical short (S) exposures of hWT and hA53T samples (left panel) or a longer (L) exposure of the hA53T samples (right panel: exposure matched to α S14 intensity of hWT α S in the left panel). (c) Densitometry of the cytosolic α S60:14 ratios (relative to hWT, set to 1) based on both S and L exposures ($N = 3$ mice of each genotype analysed on different days in triplicates of separate brain-bit samples, total $n = 9$); NS, not significant. (d) Densitometry of cytosolic α S60 and α S14 bands in both genotypes based on identical exposures ($N = 3$, $n = 9$); values relative to hWT α S60. (e) DSG crosslinked mouse brain samples: cytosols blotted for α S (Syn1, 15G7, C20) and DJ-1; Ponceau-staining of the blot membrane is on left. DJ-1 served as control for equal crosslinking efficiency and equal loading. (f) Minced brain bits from both genotypes: TX-100 total homogenates (cytosolic and membrane proteins). Untreated (-), DSP/ β ME-treated (DSPr) and DSG-treated samples in triplicates (Syn1 mAb, right panel). Left panel, Ponceau-staining of the membrane. (g) DSG crosslinking of PBS-insoluble TX-100-soluble (TX) fractions of brain from both genotypes in triplicates. (h) Densitometry of α S60 and α S14 in the TX fractions ($N = 2$ mice of each genotype analysed on different days in triplicates of separate brain bits, total $n = 6$); values relative to those of hWT α S14. * $P < 0.05$, ** $P < 0.01$; Student's t -test (see Methods) for all quantifications shown; error bars, s.d.

(hWT) or A53T (hA53T) α S²⁷. Comparing total α S levels among samples using both untreated and DSP + β -mercaptoethanol (β ME)-treated samples confirmed that DSP + β ME facilitates the quantitative detection of total α S immunoreactivity on blots (Fig. 4b)¹⁶. Consistent with the brain expression data reported for these mouse lines²⁷, PBS extracts ('cytosol') of the hA53T brain showed somewhat lower total α S than the hWT brain (Fig. 4b). The α S60:14 ratio was significantly reduced ($P < 0.05$, Student's t -test, $n = 9$) in hA53T brain cytosols (Fig. 4c), due to a greater decrease in α S60 tetramer than α S14 monomer in hA53T versus hWT (Fig. 4d). To show that this ratio difference was not an artifact of the somewhat lower total α S levels in the hA53T brain, we included longer (L) exposures for hA53T that were adjusted to match the α S14 intensity of the hWT sample (Fig. 4b: 'expo. L'). Densitometry on both exposures led to closely similar results of >20% reduction, namely, an α S60:14 ratio for hA53T versus hWT of $69.4 \pm 20.2\%$ ('S') or $75.3 \pm 20.1\%$ ('L'), ($P < 0.05$, $n = 9$, Student's t -test), with no significant difference in this result between the two exposures (Fig. 4c). This lower α S60:14 ratio in the hA53T mouse brain cytosol was consistently observed with Syn1 and confirmed with antibodies 15G7 and C20 (Fig. 4e). As expected, the hA53T α S60:14 ratio was also reduced when we analysed total lysates of whole mouse brain (that is, homogenizing the crosslinked tissue directly in TX-100) (Fig. 5f). In the membrane (TX-100) fractions, we found no significant reduction of α S14 monomer levels in hA53T versus hWT; as in the fresh human brain (Fig. 1a), tetramers/multimers were very low in abundance in this fraction and were almost undetectable for hA53T (Fig. 4g,h).

Given that the human A53T mutation corresponds to rodent WT α S at that codon, possibly masking pathological effects in the mouse brain, we sought to validate the A53T effect directly in a disease-relevant human cell model. To preclude experimental variation resulting from random genomic integration of a transgene, we initially used human embryonic stem cells (hESCs) that express human α S from a defined genetic 'safe harbour' locus under the control of a doxycycline (Dox) inducible promoter, using a zinc-finger nuclease (ZFN)-based gene editing strategy^{28,29} (Methods). These hESCs carrying either WT or A53T α S were differentiated into neural precursor cells (NPCs), which have low endogenous α S expression (Methods). Adding Dox for 4 days produced robust expression of the WT and A53T transgenes (Fig. 5a). These human NPCs closely reproduced our mouse brain observation: a ~30% lower α S60:14 ratio for A53T than WT ($67.9 \pm 9.1\%$ of WT, $n = 6$, $P < 0.05$, Student's t -test; Fig. 5b), and a reduction in total A53T α S immunoreactivity (Fig. 5a) due to lower α S60 levels (Fig. 5c). Moreover, this NPC system indicated that neither long expression (>4 days) nor full neuronal differentiation is necessary for α S tetramer formation.

We then conducted quantitative analyses in neurons differentiated from an induced pluripotent stem cell (iPSC) line of a living PD patient harbouring the A53T mutation versus its corrected isogenic line (WT_{corr}; ref. 30; Methods). Abundant microtubule-associated protein 2 (MAP2) expression in both lines indicated equal neuronal differentiation (Fig. 5d, left panel). Following intact-cell crosslinking and sequential extraction, the cytosols showed similar crosslinking efficiency based on DJ-1 dimer levels, and we again detected lower α S60:14 ratios for A53T than WT using three α S antibodies (Fig. 5d-f). Quantification of

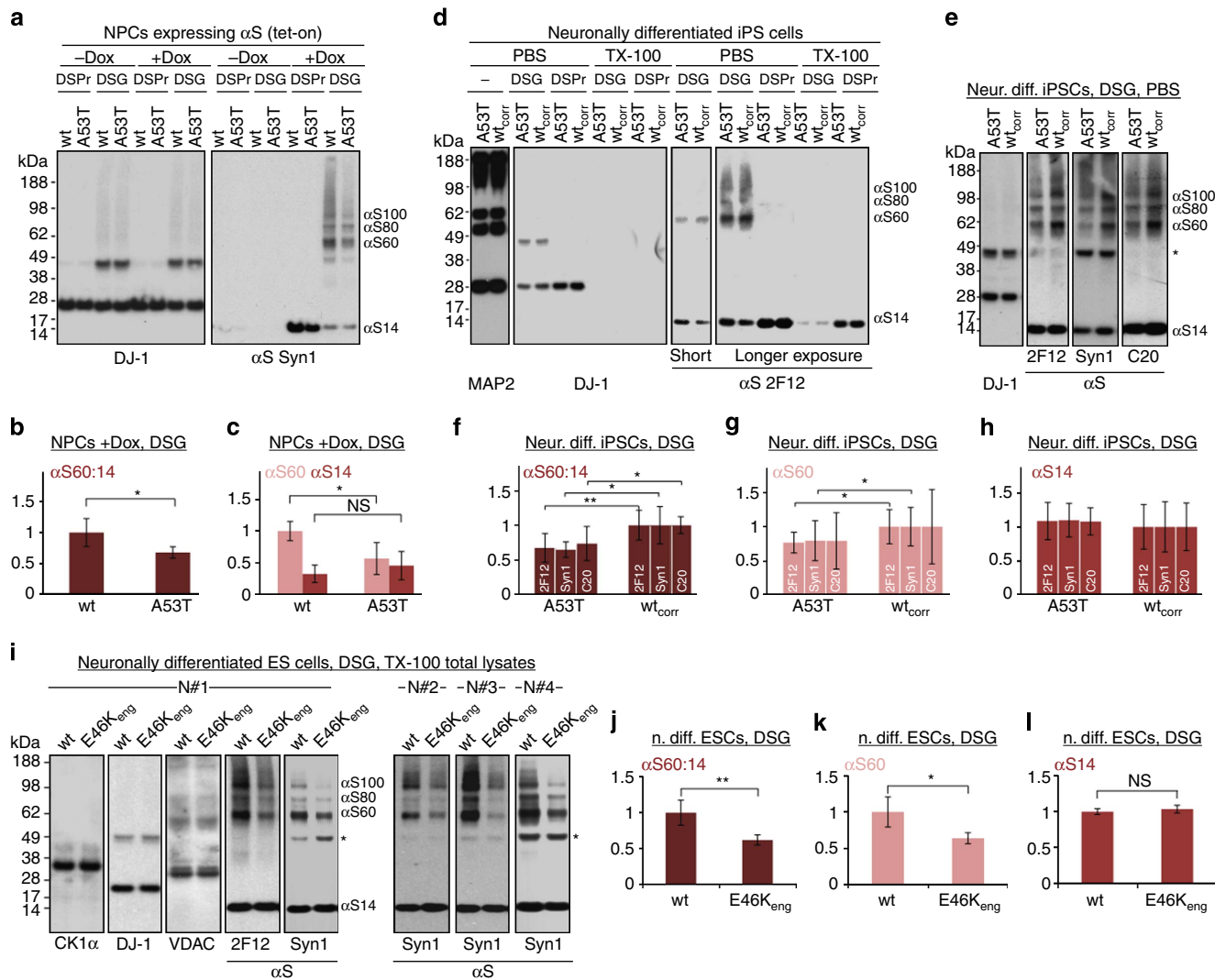


Figure 5 | A53T versus WT α S in hESC- and hiPSC-derived neurons. (a) Crosslinking analysis of non-induced (-Dox) and induced (+Dox) hESC-derived NPCs expressing WT or A53T α S: PBS-soluble fraction. DSP/ β ME-treated (DSPr) and DSG-treated samples were blotted for DJ-1 and α S (Syn1). (b) Quantification of the cytosolic α S60:14 ratios ($N=3$ different cultures, each analysed in parallel in duplicates, total $n=6$); ratios relative to WT α S. (c) Quantification of cytosolic α S60 and α S14 for both genotypes (normalized to WT α S60); NS, not significant. (d) Crosslinking analysis of neurons differentiated from human A53T iPSCs versus their genetically corrected isogenic WT line (WT_{corr}). PBS and TX-100 fractions of untreated (-), DSP/ β ME-treated and DSG-treated cells were probed for DJ-1 and α S (2F12). (e) DSG-crosslinked samples: cytosols blotted for α S (2F12, C20, Syn1) and DJ-1; * non-specific band detected only by Syn1 (ref. 9). (f) Densitometry of the cytosolic α S60:14 ratios (relative to WT) as detected by 2F12, C20 and Syn1 ($N=4$ cultures grown independently and analysed on different days in biological duplicates or triplicates; total $n=10$). (g) Densitometry of cytosolic α S60 (relative to WT) for both genotypes. (h) Densitometry of cytosolic α S14 (versus WT) for both genotypes. (i) Crosslinking analysis of neurons differentiated from WT hESCs or from a genetically engineered isogenic E46K line (E46K_{eng}). TX-100 total protein lysates of DSG-treated cells were probed with casein kinase 1 α (cytosolic monomer), DJ-1 (cytosolic dimer), VDAC (membrane-associated dimer) and α S (mAbs 2F12 and Syn1). The five panels on the left are from one experiment (N#1), the three panels on the right show the Syn1 western blots from three independent experiments (N#2-4, each quality-controlled by DJ-1 and VDAC western blots). *non-specific band detected only by Syn1 (ref. 9). (j) Densitometry of the cytosolic α S60:14 ratios (relative to WT) as detected by Syn1 mAb ($N=4$ cultures grown independently). (k) Densitometry of cytosolic α S60 (versus WT) for both genotypes. (l) Densitometry of cytosolic α S14 (versus WT) for both genotypes. * $P<0.05$, ** $P<0.01$; Student's t -test (see Methods) for all quantifications shown; error bars, s.d.

3 independent experiments done on different days in biological duplicates or triplicates ($n=10$) confirmed a >25% significantly reduced α S60:14 ratio in the hA53T neurons using three different antibodies ($66.9 \pm 21.0\%$ of WT_{corr} for 2F12; $64.4 \pm 11.3\%$ of WT_{corr} for Syn1; and $73.3 \pm 24.8\%$ of WT_{corr} for C20; $P<0.05$ or <0.01 depending on antibody, Student's t -test; Fig. 5f). This was again due to a lower α S60 level (Fig. 5g,h), a finding entirely consistent across antibodies and experiments. In a similar way, we confirmed the effect of E46K (Fig. 5i), as initially determined in

M17D cells (Fig. 3), in neurons derived from isogenic hESCs in which the E46K mutation had been inserted by genome editing³⁰: the α S60:14 ratio was reduced by >35% ($61.1 \pm 8.7\%$ of WT, $P<0.01$, $n=4$, Student's t -test; Fig. 5j), mostly due to strongly reduced tetramer levels (Fig. 5k,l). Interestingly, the decrease in tetramers in these lines was closely similar to that seen in the earlier systems, even though these human neurons are heterozygous for A53T or E46K α S, suggesting a possible dominant-negative effect on tetramer formation.

Amplifying E46K leads to less multimers and induces toxicity. Whereas A53T occurs in a unique stretch within α S, E46K is located within a canonical KTKEGV motif, which occurs at least six times in α S with slight variation (Fig. 6a), resembles motifs in apolipoprotein A1, and may facilitate interactions with lipids³¹. These facts suggested an intriguing opportunity to validate our

central hypothesis: we reasoned that the multimer-destabilizing effect of E46K (Figs 1h and 3) might be amplified by analogous mutations in other KTKEGV motifs. We analysed α S constructs with one, two or three E46K-like substitutions, targeting the highly conserved KTKEGV motifs #3 and #5 flanking the fPD E46K-harboring motif #4 (Fig. 6a). Intact-cell crosslinking of

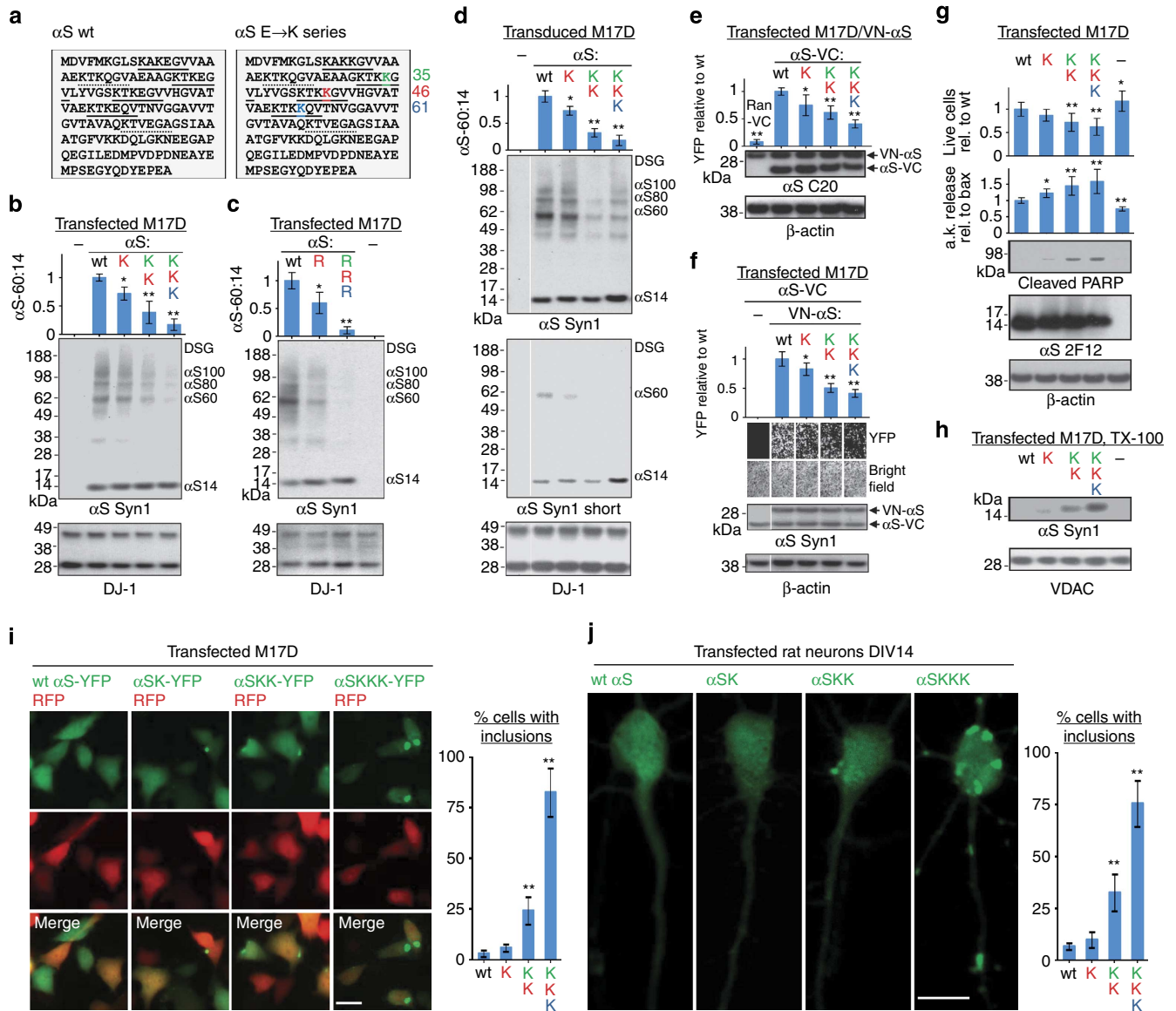


Figure 6 | Multimers/toxicity/inclusions with 1-3 E46K-like mutations. (a) Schematic of human α S sequence showing engineered mutations (color-coded). Highly conserved KTKEGV motifs underlined, less conserved motifs dotted. (b) DSG-treated M17D cells expressing the indicated α S variants lysed in PBS/1% TX-100; graph (α S60:14 ratios by western blot densitometry) and western blots (Syn1, anti-DJ-1) represent $N = 3$ experiments on different days. For this and the following graphs, WT set to 1 unless stated otherwise. (c) Analogous to (b) but 1 or 3 E \rightarrow R substitutions. (d) Analogous to (b) using lentiviral pools; long and short western blot exposures, blots cut once as indicated. (e) Venus-YFP complementation assay by automated fluorescence reading. M17D/VN- α S cells transfected with α S-VC WT or mutant or Ran-VC (negative control). YFP fluorescence relative to WT; $N = 6$ independent experiments on 3 different days using different DNA preparations. Representative western blots for α S (pAb C20) plus loading control β -actin. (f) Venus-YFP complementation assay by fluorescence microscopy; α S-VC (always WT) and 3 indicated VN- α S mutants were co-expressed (or not: -) in M17D cells; representative bright-field or fluorescent images and corresponding western blots (Syn1; β -actin as a loading control; blots cut as indicated). $N = 8$ independent transfections on 3 different days. (g) Cytotoxicity assays: trypan blue exclusion for live cell count ($N = 18$) relative to WT α S, Toxilight assay for adenylate kinase (a.k.) release relative to Bax ($N = 12$), and western blot for cleaved PARP (representative of 6 independent experiments), each transfected as indicated or mock (-); plus western blots for α S (2F12) and β -actin (total lysates PBS/1% TX-100). (h) Western blots for α S (Syn1) and VDAC in TX-100-soluble fractions of M17D cells transfected as indicated. (i) Fluorescence microscopy of live M17D cells co-expressing RFP plus indicated α S-YFP variants; scale bar, 10 μ m. Percentages of cells with inclusions were counted in a blinded fashion (right: $N = 3$; 100 cells each). (j) Fluorescence microscopy of rat neurons (DIV14) transfected with indicated untagged α S variants; immunofluorescence with human-specific mAb 15G7; scale bar, 20 μ m. Percentages of cells showing inclusions, blinded counting (right: $N = 3$; 100 cells each). * $P < 0.05$, ** $P < 0.01$; one-sided ANOVA (see Methods) for all quantifications shown; error bars, s.d.

transfected M17D cells revealed a striking ‘dose-dependent’ destabilization of α S60 by each additional E→K substitution, with the triple-K mutant almost abolishing cellular tetramers (Fig. 6b; Supplementary Fig. 9 for all uncropped blots in this section). To exclude an artificial effect of these lysine substitutions on the lysine-directed DSG crosslinking reaction itself, we expressed analogous arginine mutations (that is, 1R = E46R, and 3R), which also change the negative charge to positive but do not offer additional sites for our lysine-directed DSG crosslinker. Like E→K, the E→R substitutions in these three adjacent α S repeat motifs caused similar sharp reductions of the α S60:14 ratio (Fig. 6c). Importantly, the α S14 monomer remained well expressed for all constructs. We confirmed these findings in independent pools of lentivirus-transduced E→K M17D cells (Fig. 6d), obtaining closely similar decreases in the α S60:14 ratio despite some variability in total α S expression. The stepwise decrease in tetramer level was associated with a stepwise increase in monomer level, strongly supporting our overall hypothesis.

These consistent decreases in multimer formation documented by crosslinking offered an opportunity to independently quantify the E→K effect using our Venus-YFP complementation assay (Fig. 1g,h) and thus further cross-validate both methods. As a first step, we used the same protocol as in Fig. 1h by transfecting WT or E→K mutated α S-VC constructs into our VN- α S WT stable M17D line, followed by automated quantification of YFP-fluorescence. We observed a stepwise decrease in α S complementation with each additional E→K substitution, and with no consistent differences in total α S expression as detected by immunoblotting (Fig. 6e). Next, we used the opposite setup, introducing the E→K mutants into VN- α S constructs and co-expressing them in M17D cells with stable WT α S-VC. To quantify the resultant complementation, we chose areas from de-identified (‘blinded’) cultures having closely similar cell densities by bright-field microscopy and examined many fields by fluorescence microscopy (Fig. 6f, upper panels). As always, stronger fluorescent signals (white) indicate greater complementation. There was a striking stepwise decrease of fluorescence with each additional E46K-like substitution in the repeat motifs (Fig. 6f). Immunoblotting with different α S and control antibodies excluded differences in protein levels (Fig. 6f, lower panels), and expressing α S-VC alone produced no fluorescence (Fig. 6f). In primary rat neurons, co-expression of α SKKK-VC and VN- α SKKK led to very low complementation that was virtually below detection limit, while both variants were clearly expressed, as shown by immunofluorescence using human α S-specific antibody 15G7 (Supplementary Fig. 10).

The analysis of single fPD mutants in acute cell culture has not produced consistent effects as regards their cytotoxicity³¹, in keeping with fPD being a very gradual disease of relatively late onset, but we reasoned that our ‘amplified’ E46K tetramer-abrogating effect might produce measurable cytotoxicity in cultured neural cells. We thus expressed WT α S and all three E→K variants (1, 2 and 3K) in human M17D cells and observed a highly significant stepwise reduction in cell viability with each E→K substitution added to E46K, as documented by both trypan blue exclusion and an adenylate kinase release assay (Methods); the effect of E46K alone versus WT was noticeable but relatively weak (Fig. 6g, upper two panels). Importantly, these decreases in cell viability were accompanied by opposite increases in cleaved PARP, a widely used indicator for the activation of apoptosis (Fig. 6g, third panel), and again the single E46K alone had a weak but noticeable effect. These results were not due to differences in expression levels (Fig. 6g, fourth and fifth panels). When we asked whether this increase in neurotoxicity was paralleled by changes in α S solubility, we found increased total α S immunoreactivity in PBS-insoluble fractions such as the

TX-100-soluble fraction (Fig. 6h) with each additional E→K substitution, starting with just 1K. Moreover, in live M17D cells, YFP-tagged α S-KKK showed a strong tendency to form round cytoplasmic inclusions (in $79 \pm 8.1\%$ of cells; Fig. 6i), and this was weaker ($23.5 \pm 5.9\%$ of cells) but still highly significant ($P < 0.01$) in α S-KK and less so but still noticeable ($4.5 \pm 1.0\%$ of cells; $P > 0.05$) in α S-K versus α S-WT ($2.3 \pm 0.6\%$), whereas the co-transfected soluble RFP control protein remained uniformly cytosolic (Fig. 6i). Importantly, we obtained highly similar results when we expressed untagged WT α S and the E→K mutants in primary rat neurons (DIV 12) and quantified the prevalence of cells harbouring discrete round inclusions in cell bodies and/or neurites (Fig. 6j).

Discussion

α S accumulates in β -sheet-rich fibrils in numerous neurodegenerative diseases. For nearly two decades, α S was believed to exist normally as a natively unfolded, 14.5 kDa monomer^{14,32}, and most papers on α S have emphasized this assumption in their introductions. In contrast, we recently reported that cellular α S can occur physiologically in partially α -helical, aggregation-resistant ~ 60 kDa tetramers⁷ and that these may be in a complex equilibrium with monomers also normally present in neurons⁹. This unexpected finding has been controversial^{13,14}. Here we document that α S exists as a ~ 60 kDa tetramer in fresh, normal human brain tissue. Then, we show in multiple systems that all fPD-causing α S missense mutations significantly decrease tetramer:monomer ratios in neural cells, including in human neurons derived directly from a patient with the A53T mutation or WT human neurons genetically engineered to express E46K. This invariant effect by mutations known to unequivocally cause clinical PD strongly suggests that a tetramer-to-monomer destabilization facilitates subsequent pathological aggregation of monomers into disease-mediating assemblies. We validate this concept in Fig. 6: by inserting the PD-causing mutation at E46K into the analogous sites of first one and then two adjacent repeat motifs, we simultaneously link a stepwise decrease in tetramers/multimers and increase in monomers inside neurons (quantified by two methods) to a stepwise increase in neurotoxicity (quantified by three independent assays) and accumulation of α S in round cytoplasmic inclusions. This elucidation of a genotype-to-phenotype relationship for five fPD mutations, the initial pathogenic mechanisms of which have remained unclear, provides a compelling new model for how α -synucleinopathy can be initiated in PD and related disorders.

Each fPD-causing mutation reduced the intracellular α S60:14 ratio by 10–40% depending on the mutant, and engineered triple (H50Q-G51D-A53T) and quadruple (triple + E46K) fPD mutants produced even larger decreases, with sharp rises in free monomers. YFP complementation (analysed in a blinded fashion) showed highly significant effects of α S mutations in the same direction. We confirmed the fPD-mutant effect in the most relevant available systems: α S-/- mouse brain having low transgenic expression of human A53T α S, iPSC-derived neurons obtained from an A53T patient and engineered E46K hESC-derived neurons. This consistency across multiple cell/tissue sources and with two distinct techniques validates the mutational effects. The multimer-destabilizing effect and concurrent slight neurotoxicity of the E46K fPD mutation (Fig. 6g) was significantly ‘amplified’ by adding just one or two E46K-like mutations in the adjacent KTKEGV motifs. Our observation that intact six-residue repeats contribute to the normal α S tetrameric structure is of interest because analogous repeats occur in apolipoprotein A1 (ref. 31), which forms native low-*n* multimers in its lipid-free state^{33–35} and was crystallized as a tetramer³⁶. Our multiple cellular and

brain tissue experiments suggest that all of the clinical missense mutations destabilize the physiological tetramer, eventually leading to higher relative levels of unfolded monomer, the form believed to be the starting point for pathological α S aggregation³⁷. Our results across the five fPD mutations showed a destabilization of tetramers most clearly, while the accumulation of monomers in our short-term experiments varied and achieved significance for A30P and G51D (Fig. 3e). Neurons are likely to compensate for unfolded α S monomers by degrading or clearing them in the short-term, but over longer periods or a human lifetime, this may not suffice to control the potential aggregation of excess monomers arising chronically from destabilized tetramers. In this context, we previously found that the native, helical tetramer purified from WT human erythrocytes showed no aggregation upon extended *in vitro* incubation, whereas unfolded monomers readily aggregated into β -sheet-rich fibrils (see Fig. 3d in ref. 7).

Two technical advances over our previous protocol for intact-cell crosslinking⁹ enabled these new findings. First, we developed a procedure to efficiently crosslink intracellular proteins in fresh brain samples that largely excludes broken cells. The latter step is key to studying α S because upon cell lysis, tetramers of α S or β S are depolymerized and can no longer be trapped quantitatively in their *in vivo* state⁹. Applying this new protocol to human brain immediately after biopsy confirmed the major observations of our intact-neuron crosslinking⁹: an abundant α S60 species (plus some α S80 and α S100 multimers) in the PBS-soluble fraction, with only minor and primarily monomeric α S in the membrane fraction. The fresh, normal human brain showed tetramers and monomers but essentially no intermediate dimer and trimer species, entirely distinct from what would be seen with artifactual (non-specific) crosslinking of the highly abundant α S protein, which in unfolded recombinant form produces a stochastic ladder of monomers > dimers > trimers > tetramers and so on. (see Fig. 4c in ref. 9). Of note, the known tetrameric protein p53 behaved similarly to α S in both our (Fig. 1d) and previous¹⁸ crosslinking assays: pronounced tetramers, few dimer or trimer intermediates, some apparent higher assemblies, and smears on over-crosslinking. And as before⁹, known monomeric proteins were not made into artifactual oligomers by our crosslinking (Fig. 1). Our new tissue protocol also revealed abundant α S tetramers in normal mouse brain, and the non-pathogenic homolog β S showed a closely similar pattern. Collectively, these results substantiate that the cellular tetramer detected by crosslinking is a physiological, not pathological or artifactual, assembly.

Second, by stringently standardizing our protocol to make it quantitative, we addressed novel questions relevant to PD pathogenesis: are cellular tetramer:monomer ratios affected by α S expression levels and by PD-causing α S mutations? We generated data indicating that (a) the cellular tetramer:monomer ratio is constant over a relatively wide range of α S expression levels that were similar to but always somewhat lower than those in human brain tissue; and (b) mutations which cause fPD shift the ratio away from the tetramer. The steady rise in tetramer levels with increasing α S expression (Fig. 2) suggests that physiological tetramerization is either an intrinsic feature of the α S polypeptide chain or any transacting co-factors needed for tetramer assembly and stabilization (for example, membrane vesicles, free lipids and so on) are not limiting in our cells at these α S expression levels.

Two recent studies confirm the existence of physiological multimers (including tetramers) in neurons^{21,38}, and one of these²¹ provides functional evidence that a portion of α S multimers can localize to synaptic vesicles, cluster them and prevent their exocytosis, which could downregulate neurotransmitter release. Nonetheless, data from our and several other^{7,8,15,39} labs suggest that native α S multimers are mainly

present in the cytosol. Westphal and Chandra¹⁵ confirmed the purification of α -helical α S assemblies from erythrocytes using our methods and underscored their soluble nature. A study on γ -synuclein suggested that this α S homolog is tetrameric unless it binds to heterologous binding partners or membranes⁴⁰, in agreement with our finding that cytosolic but not membrane-associated α S molecules are recovered as multimers. Wang *et al.*⁸ reported NMR analyses of an N-terminally modified α S construct expressed in *E. coli* and purified under non-denaturing conditions that formed α -helical tetramers. Their CD spectroscopy of WT and fPD recombinant proteins revealed decreasing α -helical content in the order WT > A30P > A53T > E46K, closely resembling the relative order of the α S60:14 ratios in our assays (see Supplementary Fig. 11). The reported ages of symptom onset in humans carrying fPD missense mutations may agree in general with the relative decrease in our α S60:14 ratios. G51D causes very early onset PD similar to α S triplication^{41,42}, while symptoms from H50Q and A30P begin somewhat later^{3,26,43}; we find the greatest lowering of the tetramer:monomer ratio for G51D and the least for A30P and H50Q (Fig. 3).

Tetramers and monomers are likely to be in a complex dynamic equilibrium in living cells, as proposed for p53 (refs 10,11). The α S60 species (which we confirmed here by mass spectrometry to contain only α S on purification (Supplementary Fig. S3)) is representative of the α S80 and α S100 species (Supplementary Fig. S2), which may be conformers of the tetramer (discussed in ref. 9). When all α S-immunoreactive mid-MW bands (60, 80 and 100 kDa) are considered together, the multimer content of cultured cells and brain substantially exceeds the monomer content (2–3:1), as judged by antibodies that react similarly with both. And if some of the α S14 we observe represents ‘false-positive’ monomers present because the crosslinking is not fully efficient, then the multimer:monomer ratio *in vivo* is even higher than 3:1. Whatever the absolute multimer:monomer ratios are *in vivo*, the consistent detection of multimers of endogenous α S and β S in normal cells by both *in situ* crosslinking and fluorescence complementation contrasts sharply with the long-held view that cellular α S assemblies above monomer are exclusively pathological.

How can our findings be synthesized into an integrated model for the conversion of physiological α S into pathogenic forms? The field has long accepted that contact between the ‘natively unfolded monomer’ and negatively charged lipid vesicles *in vitro* induces α -helical structure⁴⁴. If this finding has any *in vivo* relevance, we hypothesize that after their synthesis on the ribosome, unfolded α S monomers undergo a helical conversion and multimeric assembly, perhaps by transient contact with lipid vesicles followed by rapid detachment, thus ending up principally in the cytosol, where we recover the tetramers quantitatively after *in vivo* crosslinking. Alternatively, an unknown small ligand, for example, a free lipid, could induce and/or maintain the folding and assembly state. Our inability to detect by mass spectrometry any other proteins as a component of the gel-purified α S60 tetramer does not exclude the presence of one or more lipids in the assembly. Our findings (sequential extractions that leave α S60 principally in the cytosol (Fig. 1a); diffuse cytosolic signal of the multimers by YFP complementation (Fig. 1e–g)) argue against a persistent close contact between membranes and most tetramers/multimers at steady state.

Pathological polymerization may occur when the storage capacity of the cell’s native tetrameric population is somehow exceeded, for example, destabilization by missense mutations leading to relatively more monomers, or else chronic elevation of all forms of the protein (for example, increased gene dosage⁵), leading to absolutely more monomers. In sporadic PD, different neuronal stressors could, over time, destabilize a small fraction

of the WT tetrameric population to monomers, for example, through post-translational modifications such as phosphorylation, dopamine induced conformational changes, oxidative stress/free radical accumulation with age, chronic mitochondrial dysfunction, altered chaperone systems, metal ion dyshomeostasis, or extraneuronal amyloid- β accumulation (in Alzheimer's disease). In such pathogenic scenarios, which could underlie cases of 'idiopathic' synucleinopathies, pathological α S aggregates would represent a secondary effect, whereas in α S mutation carriers, they would be considered primary.

The drastic alteration in α S multimerization state on cell lysis⁹ makes addressing its function challenging. α S cannot be easily studied in its native state outside of cells by conventional biochemical approaches because of the apparent dynamic flux between multimers and monomers that exists in intact cells⁹. Only techniques that reveal the intracellular state of α S and are also sufficiently sensitive to quantify the range of endogenous assembly forms should be used. At present, this means relying on quantifiable *in vivo* crosslinking with cell-penetrant agents that can trap α S and β S in their native multimeric states, and imaging of intact cells expressing tagged forms, although the latter approach has the twin disadvantages of modifying the α S structure and not distinguishing different-sized multimers. We have reported clear evidence that multi-step purification of endogenous α S from human cells and brain allows a portion of the purified protein to remain tetrameric during the native purification^{7,45}.

The central therapeutic implication of our work is similar to that achieved for transthyretin⁴⁶, a physiological tetramer stabilized by a natural ligand (thyroxine), destabilized by amyloidogenic missense mutations, and therapeutically stabilized by certain small molecules (for example, tafamidis). Similar strategies are being discussed for the cancer-relevant physiological tetramer of p53 (refs 47–49). The stabilization by small, brain-penetrant molecules of the α S tetramer is now a rational and attractive goal.

Methods

All materials mentioned were purchased from Invitrogen unless stated otherwise.

Brain samples. The experimental use of discarded human brain samples was approved under protocol number 1999P001180 ('Aging in the Brain: Role of the Fibrous Proteins') by the Partners Human Research Committee, the Institutional Review Board of Partners Research Management. Informed consent for the use of brain tissue was obtained from the subject undergoing brain biopsy. Rodent samples were acquired under protocol number 05022 ('Mouse Models for Parkinson's Disease'), approved by the appropriate IACUC, the Harvard Medical Area Standing Committee on Animals. Wt mice (C57BL/6; 12 wk old) were from Charles River, Wilmington, MA, and transgenic mice (ref. 27; age-matched pairs, male, 6–9-week old) from Jackson labs, Bar Harbor, ME. The human cerebral cortex biopsy was obtained fresh from a patient undergoing focal ablative surgery for epilepsy who was otherwise healthy and had no synucleinopathy or other neurodegenerative disease; the sample was obtained from unaffected, discarded tissue and analysed fresh (storage on ice about 2 h).

Cell culture and transfection. Cells were cultured at 37 °C in 5% CO₂. Human erythroid leukemia cells (HEL; ATCC number TIB-180) were cultured in RPMI 1640 (ATCC modification) supplemented with 10% fetal bovine serum (Sigma), 50 units per ml penicillin, and 50 μ g ml⁻¹ streptomycin. Human neuroblastoma cells (BE(2)-M17, called M17D; ATCC number CRL-2267) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units per ml penicillin, 50 μ g ml⁻¹ streptomycin, and 2 mM L-glutamine. M17D cells were transfected using Lipofectamine 2000 according to manufacturer's directions. Cells were normally collected 48 h after transfection. Primary neurons were cultured from E18 Sprague-Dawley rats (Charles River, Wilmington, MA). Rats were euthanized with CO₂ followed by cervical dislocation. Embryonic cortices were isolated and dissociated with trypsin and trituration. Cells were plated in DMEM supplemented with 10% FBS, 50 units per ml penicillin, 50 μ g ml⁻¹ streptomycin, and 2 mM glutamine at 680 cells per mm² on BioCoat poly-D-lysine-coated culture dishes (BD Biosciences). After 4 h, medium was changed to Neurobasal medium supplemented with B-27, 2 mM GlutaMAX, and 50 μ g ml⁻¹ gentamicin. Half of the

medium was replaced every 4 days; on the first medium change, 5-fluoro-2-deoxyuridine (Sigma) and uridine (Sigma) were added to concentrations of 100 and 500 μ g l⁻¹, respectively.

cDNA cloning. Single-mutation α S expression plasmids were generated from the pcDNA4/ α S plasmid⁹ using the QuikChange II site-directed mutagenesis kit and appropriate primers. Multiple-mutation constructs were synthesized as GeneArt Strings DNA fragments (GeneArt/Life Technologies), and inserted into pcDNA4 with the In-Fusion HD Cloning Kit (Clontech). pcDNA3/VN- α S and pcDNA3/ α S-VC WT and β P mutant constructs were gifts of T.F. Outeiro, Goettingen). VN and VC constructs for insertion into pcDNA4/TO/myc-his A (pcDNA4) were synthesized by GeneArt, excised directly from the vector in which the constructs were delivered, and ligated into pcDNA4. α S tagged constructs were created by linearizing the resulting construct in pcDNA4 and inserting α S cDNA by In-Fusion. Full-length YFP was reconstituted from the VN and VC tags and inserting into pcDNA4; full-length tagged constructs were subsequently made by insertion of cDNA upstream of the tag. Lentiviral constructs pLenti6/ α S WT, G51D, E46K, 2K and 3K were generated by In-Fusion with the pLenti6 vector and respective cDNA templates. Lentiviral construct pWPXL/ α S-WT was generated by In-Fusion from pWPXL/eGFP (pW), a gift of Didier Trono (Addgene plasmid # 12257), without removing eGFP. pCAX/dsRed and pcDNA3.1/Bax were gifts from T. Young-Pearse and M. LaVoie, respectively. The following primers were used. To reconstitute full-length YFP and infused into pcDNA4: VN-to-PP_FW 5'-AGTGTGGTGGGA ATTCTGCAGATGGTGAGCAAGGGCGAGG-3', VNVC-combine_FW 5'-CTAT ATCACCCGCGACAAGCAGAAGAAGCGGCATCAAGGCC-3', VNVC-to-YFP_Rev 5'-GCCCTCTAGACTCGAGTTACTGTACAGCTCGTCCATG-3'. To insert α S into pW (digested with BamHI and MluI): pW- α S-infu-BamHI-MluI_FW 5'-T TAAACTACGGGATCCATGGATGTATTTCATGAAAGGA-3' pW- α S-infu-Bam HI-MluI_Rev 5'-TAGCGCTAGGACGCGTTAGGCTTCAGGTTTCGTAGTC-3'. To insert α S variants into pcDNA4/VN- α S: α S-VN-FW_Infu 5'-TGGAGGTG GTGGATCCCTTAAGGATGTATTTCATGAAAG-3', α S-VN-Infu 5'-GCGGC CGCCGATATCTTAGGTTTCAGGTTTCGTAG-3'. To insert α S into pcDNA4/VC: α S-VC-fw_Infu 5'-AGTGTGGTGAATTCTGCAGATGGATGATTATTCAGAA AGG-3', α S-VC-Infu 5'-TGCCGTTCTTCTCGAGGGCTTCAGGTTTCGTAGTA GTC. To insert α S into pcDNA4/YFP p4-BamHI- α S-Infu_FW 5'-TACCGAGCT CCGATCCATGGATGTATTTCATGAAAGGA-3', p4- α S-PstI-FP-fus_Rev 5'-CCC TTGCTCACCATCTGCAGGGCTTCAGGTTTCGTAGTC-3'. To insert Ran into pcDNA4/VC: p4-Ran-VC-Infu_FW 5'-AGTGTGGTGAATTCTGCAGATGGCT GCGCAGGGAGAG-3', p4-Ran-VC-Infu_Rev 5'-TGCCGTTCTTCTCGAGCAG GTCATCATCTCATCC-3'. To insert α S WT, E46K, KK and KKK into pLenti6: pLenti6- α S_FW 5'-GGATCCACTAGTATGGATGTATTTCATGAAAGG-3', pLenti6- α S_Rev 5'-GCCCTCTAGACTCGAGTTAGGCTTCAGGTTTCGTAG-3'.

Stable cell pools and cell lines. Lentiviral particles derived from pLenti6 were generated as recommended by the manufacturer. Lentiviral particles derived from pWPXL were generated with plasmids psPAX2 and pMD2.G (also gifts from D. Trono. Addgene plasmids #12260 and 12259, respectively). M17D cells were transduced with viral particles according to the respective recommended protocols. The stable monoclonal cell line M17D/VN- α S was generated by transfection of M17D cells with pcDNA3/VN- α S, followed by G418 selection and isolation of single clones. Stable pools of pLenti6 transduced cells were generated by blasticidin selection, and stable pools of pWPXL transduced cells were selected for residual GFP expression with FACS.

Human ES cell and iPSC cultures. hiPSCs and the hESC lines WIBR3 (Whitehead Institute Center for Human Stem Cell Research, Cambridge, MA) and BG01 (NIH code: BG01; BresaGen, Inc., Athens, GA) were maintained on mitomycin C-inactivated mouse embryonic fibroblast feeder layers in hESC medium (DMEM/F12 supplemented with 15% FBS (Hyclone), 5% KnockOut Serum Replacement, 1 mM glutamine, 1% nonessential amino acids, 0.1 mM β -mercaptoethanol (Sigma) and 4 ng ml⁻¹ FGF2 (R&D systems))³⁰. Cultures were passaged every 5–7 days either by trituration or enzymatically with collagenase type IV (Invitrogen; 1.5 mg ml⁻¹).

Gene-edited pluripotent stem cell lines. hESCs lines expressing human α S (WT or A53T mutant) under the control of Dox from a defined genetic 'safe harbor' locus were generated by a previously described ZFN-based genome-editing strategy. ZFNs were linked to WT FokI which was linked to an obligate heterodimer form of the FokI endonuclease (ELD-KKR)^{28,29}. hESCs were simultaneously targeted in both alleles of the AAVS1 locus with two distinct donor plasmids, each containing a distinct selection marker (neomycin or puromycin resistance gene) followed by either a CAGGS promoter driving constitutively expressed reverse tetracycline transactivator (M2rtTA) or the α S transgene (WT or A53T) driven by a Dox-responsive element. Correctly targeted hESC clones derived from individual cells with unique integration of each donor plasmid in one of the AAVS1 alleles and lack of additional random donor integrations were identified by Southern blotting as described²⁸. α S expression in NPC was induced by supplementing the medium with Dox at a final concentration of 2 μ g ml⁻¹. The A53T iPSCs plus genetically corrected isogenic line (WT_{corr}) as well as the genetically engineered

E46K hESC line plus parental WT α S hESC line had been generated before by the ZFN-based genome-editing strategy, as published in detail³⁰.

NPC culture and terminal differentiation. Differentiation into NPCs and then terminally differentiated neurons was according to reported protocols with slight modifications^{28,50}. Briefly, hESC/hiPSC colonies were collected using 1.5 mg ml^{-1} collagenase type IV, separated from mouse embryonic fibroblast feeder cells by gravity, gently triturated and cultured for 8 days in non-adherent suspension dishes (Corning) in EB medium (DMEM with 20% KnockOut Serum Replacement, 0.5 mM glutamine, 1% nonessential amino acids, 0.1 mM β -ME (Sigma)) supplemented with 50 ng ml^{-1} human recombinant Noggin (PeproTech) and 500 nM dorsomorphin (Stemgent). Subsequently, human EBs were plated onto dishes coated with poly-L-ornithine ($15 \mu\text{g ml}^{-1}$, Sigma), laminin (Sigma) and fibronectin (Sigma) in N2 medium supplemented with 50 ng ml^{-1} human recombinant Noggin, 500 nM dorsomorphin and FGF2 (20 ng ml^{-1} , R&D Systems). After 8 days, neural rosette-bearing embryonic bodies (EBs) were microdissected, dissociated with 0.05% trypsin/EDTA solution and expanded on poly-L-ornithine, laminin and fibronectin coated dishes at a density of 5×10^5 cells per cm^2 in N2 medium supplemented with FGF2 (20 ng ml^{-1}). Proliferating NPCs were passaged 2–3 times before induction of terminal differentiation into neurons by growth factor withdrawal in N2 medium supplemented with ascorbic acid (Sigma). Differentiated neurons were used for chemical crosslinking 45–55 days after induction of terminal differentiation.

Immunocytochemistry. Cells were grown on poly-D-lysine-coated surfaces or coverslips, rinsed with HBSS with divalent cations, fixed 25 min at RT with 4% paraformaldehyde/PBS, then washed three times for 5 min with PBS⁵¹. Cells were then blocked and permeabilized with 5% BSA/0.25% Triton X-100/PBS. Cells were incubated with primary antibody in block-permeabilizing buffer for 2 h at RT or overnight at 4 °C. After incubation with primary antibody, cells were washed $3 \times$ for 5 min with PBS, then incubated 1–2 h at RT with Alexa Fluor 488- and Alexa Fluor 568-coupled secondary antibodies diluted 1:2,000 in 5% BSA/PBS (no Triton). Cells were washed $3 \times 10 \text{ min}$ at RT with PBS, then analysed directly in the dish (Fig. 6j) or after mounting with Mowiol (Sigma) and coverslips (Fig. 1e,f).

Intact-cell crosslinking of cultured cells. Crosslinkers were stored at 4 °C with desiccant. Cells were collected by trituration (HEL and M17D) or scraping (primary neurons), washed with PBS, and resuspended in PBS with Complete Protease Inhibitor, EDTA-free (Roche Applied Science). Crosslinkers were prepared at $50 \times$ final concentration in DMSO immediately before use. Samples were incubated with crosslinker for 30 min at 37 °C with rotation. The reaction was quenched by adding Tris, pH 7.6, at 50 mM final concentration and incubated for 15 min at RT. After quenching, proteins were extracted (see below). Before crosslinking, one representative aliquot of the cells (for example, one extra 6-cm dish cultured for this purpose) was lysed in a volume of PBS/PI that was estimated to generate a protein concentration in the cytosol fraction of $1.5 \mu\text{g ml}^{-1}$ (lysis by sonication; 20,000 g spin, 30 min, 4 °C to collect cytosol). Then, the actual protein concentration was determined by BCA assay. On the basis of this result, the final volume of crosslinking buffer (PBS/PI + 1 mM DSG or 1.75 mM DSP) for all the cell samples was adjusted so as to guarantee a $1.5 \mu\text{g ml}^{-1}$ cytosolic protein concentration in every sample at time of crosslinking. In general, samples with a measured concentration of < 1.3 or $> 1.7 \mu\text{g ml}^{-1}$ were excluded from analyses. As an alternative, samples (for example, cells from one 10-cm dish) were split into different tubes of 1 mM DSG at different volumes (for example, 300, 400, 500 μL), followed by protein extraction, BCA assay and matching of samples that have similar protein concentration as an indication of similar protein-to-crosslinker ratios. The most important criterion for data inclusion was equal crosslinking observed for the DJ-1 control blots (no apparent differences in DJ-1 dimer:monomer ratios was a pre-established criterion⁹). Before crosslinking and further processing, sample order was randomized.

Intact-cell crosslinking of fresh brain tissue. For intact-cell crosslinking of fresh brain, samples (mouse whole brain, human cortical biopsy) were finely minced by two rounds on a McIlwain Tissue Chopper (model MTC/2E, Mickle Laboratory Engineering Co., Gomshall, UK; blade interval: 100 μm); the sample was turned 90° after the first round. Minced samples were transferred to 15 ml tubes containing 5 ml PBS/PI, and the solution was resuspended by gentle shaking. Aliquots were transferred to 1.5 ml tubes and spun at 1,500 g for 5 min at RT. Supernatants were discarded, and the pellets (intact tissue bits) underwent crosslinking routinely at a ratio of 1 ml 1 mM DSG per 100 mg tissue or 1 ml 1.75 mM DSP per 100 mg tissue, while for DSG gradients we used volumes of 0.4–2 ml of 1 mM DSG per 100 mg tissue. The tissue suspensions were incubated at 37 °C for 30 min with shaking, followed by spinning at 1,500 g for 5 min at RT. After this intact-cell crosslinking, the supernatant was discarded, and the brain bits in the pellet were resuspended in PBS/PI (in 0.25 ml per 100 mg tissue), followed by protein extraction as described below.

Protein extraction. Cells were lysed by sonication (Sonic Dismembrator model 300; microtip setting = 40; $2 \times 15 \text{ s}$). Spinning at 800 g for 5 min at 4 °C yielded the postnuclear supernatant (step was occasionally omitted), and this was centrifuged at 20,000 g for 30 min or ultracentrifuged at 100,000 g for 60 min at 4 °C to collect the supernatant ('cytosol') and pellet (membranes). The latter pellet was resolubilized by sonication ($2 \times 15 \text{ s}$, amplitude 40) in PBS/1% TX-100 (same volume as cytosols), followed by centrifugation at 20,000 g for 30 min or ultracentrifugation at 100,000 g for 60 min at 4 °C. The resultant supernatant contained extracted membrane proteins; in some experiments, we added a 1 h 2% sarkosyl incubation of the TX-100-insoluble or a 4 h incubation of the LDS-insoluble pellet in 88% formic acid with shaking.

Immunoblotting. Protein concentrations were determined by BCA assay (Thermo Scientific) according to the manufacturer's directions. Samples were prepared for electrophoresis by the addition of NuPAGE LDS sample buffer and boiling for 10 min. If not stated otherwise, 30 μg of total protein were loaded per lane. Samples were electrophoresed on NuPAGE 4–12% Bis-Tris gels with NuPAGE MES-SDS running buffer and the SeeBlue Plus2 MW marker. After electrophoresis, gels were electroblotted onto Immobilon-Psq 0.2 μm PVDF membrane (Millipore) for 1 h at 400 mA constant current at 4 °C in 25 mM Tris, 192 mM glycine, 20% methanol transfer buffer. After transfer, membranes were incubated in 0.4% paraformaldehyde, PBS for 30 min at RT, rinsed twice with PBS, stained with 0.1% Ponceau S in 5% acetic acid, rinsed with water, and blocked in 0.2% IBlock solution (PBS containing 0.1% (v/v) Tween 20 (PBS-T) and 0.2% (w/v) IBlock) for either 30 min at RT or overnight at 4 °C. After blocking, membranes were incubated in primary antibody in 0.2% IBlock with 0.02% sodium azide for either 1 h at RT or overnight at 4 °C. Membranes were washed $3 \times 10 \text{ min}$ in PBS-T at RT and incubated (45 min at RT) in horseradish peroxidase-conjugated secondary antibody (GE Healthcare) diluted 1:10,000 in 0.2% IBlock solution. Membranes were then washed $3 \times 10 \text{ min}$ in PBS-T and developed with ECL Prime (GE Healthcare-Amersham Biosciences) or SuperSignal West Dura (Thermo Scientific) according to manufacturers' instructions.

Antibodies. Antibodies used were monoclonals Syn1 to α S (Clone 42, Becton-Dickinson; 1:3,000 in western blotting for transfectants, 1:400 in western blotting for endogenous α S), 2F12 to α S⁹ ($0.09 \mu\text{g ml}^{-1}$ for transfectants and 0.9 μg per ml for endogenous in western blotting; $9 \mu\text{g ml}^{-1}$ in immunocytochemistry), SOY1 to α S (used in ELISA, see below for details), 15G7 to α S⁵² (hybridoma supernatants were 1:500 in western blotting for transfectants, 1:50 for endogenous α S, 1:5 in ICC), 71.1 to GAPDH (Sigma; 1:5,000 in western blotting), DLP1 to DRP-1 (Becton-Dickinson; 1:1,000 in western blotting), sc-126 to p53 (Santa Cruz, 1:1,000 in western blotting), EP1537Y to β S (Novus Biologicals, 1:3,000 in western blotting) and PRK8 to Parkin (Santa Cruz, 1:1,000 in western blotting), as well as polyclonal antibodies C20 to α S (Santa Cruz; 1:1,000 for endogenous α S and 1:5,000–1:10,000 for transfectants in western blotting), ab8227 to β -actin (Abcam; 1:5,000 in western blotting), ab5392 to MAP2 (Abcam; 1:1,000 in western blotting), PA1-954A to VDAC (Affinity Bioreagents; 1:1,000 in western blotting), D64E10 to cleaved PARP (Asp214) (Cell Signaling; 1:1,000 in western blotting), sc-126 anti-synaptobrevin-2 (Synaptic Systems, Göttingen, Germany; 1:1,000 in western blotting), anti-Ran (4462, Cell Signaling; 1:1,000 in western blotting), anti-kinase 1 α (Sc-6477, Santa Cruz; 1:2,000 in western blotting), anti-DJ-1 (ref. 53, 1:3,000 in western blotting) and anti-GFP (T-19, Santa Cruz, 1:500 in ICC). 2F12 and SOY1 were generated by immunizing α S^{-/-} (KO) mice with native α S purified from human erythrocytes. Hybridoma cell lines were generated by fusion of mouse splenocytic B lymphocytes with X63-Ag8.653 myeloma cells. Antibodies were purified from hybridoma supernatant by Cell Essentials (Boston, MA).

α S-specific ELISA. Multi-Array High Bind plates (96-well plates; MSD, Meso Scale Discovery, Rockville, MD) were coated with the capture antibody 2F12 diluted ($6.7 \text{ ng } \mu\text{l}^{-1}$) in Tris-buffered saline with 0.05% Tween-20 (TBS-T) in 30 μL per well and incubated at 4 °C overnight. Liquid was removed and plates were blocked for 1 h at RT in blocking buffer (5% MSD Blocker A; TBS-T). After 3 washes with TBS-T, samples diluted in TBS-T with 1% MSD Blocker A and 0.5% NP40 were incubated at 4 °C overnight. Sulfo-tagged SOY1 mAb (for detection) was generated using Sulfo-Tag-NHS-Ester (MSD), diluted in blocking buffer ($6.7 \text{ ng } \mu\text{l}^{-1}$), added to the plate (30 μL per well) and shaken for 1 h at RT. After three washes, MSD Reader buffer was added, and plates were immediately measured using a MSD Sector 2400 imager.

Densitometry and statistical analyses. Scanned western blots were analysed with the ImageJ software, version 1.47 (ref. 54). Pictures were inverted and usually the background signal from an empty lane was subtracted to obtain specific signals for each lane. R^2 values (Fig. 2 and S4) were calculated using Microsoft Excel. WT α S was normalized to 1 in each independent experiment (N) to allow comparison between experiments done on different days. For statistical analyses of WT versus A53T (Figs 4,5), we performed Student's *t*-test (tails = 2, type = 2; Microsoft Excel). For all other statistical analyses (Figs 1,3,6), one-way ANOVA was routinely performed to determine significance versus WT α S using GraphPad Prism

Version 6.05 under the program's guidelines (Tukey's multiple comparison's test, calculation of adjusted *P*-values, 'repeated measures' correction where applicable). Where necessary, standard deviations for controls were obtained by calculating the deviation of duplicates or triplicates from their mean in each independent experiment. Normal distribution and similar variances were observed for both WT and mutant α S values. Graphs are means \pm s.d. Criteria for significance: $P < 0.05$, * $P < 0.01$, ** $P < 0.001$. Sufficient experiments and replicates were run to achieve statistical significance.

Cytotoxicity assays. The ToxiLight Non-destructive Cytotoxicity BioAssay Kit (Lonza) was used according to directions to measure adenylate kinase release; signals from untransfected cells were subtracted for normalization. For trypan blue assays, cell suspensions were mixed 1:1 with 0.4% Trypan Blue (Sigma) and counted on a TC10 automated cell counter (Bio-Rad).

YFP complementation and microscopy. The principle of the YFP complementation assay, including controls for the specificity of the VN- α S/ α S-VC interaction, has been described^{22,23,55,56}. We routinely used a stable cell line M17D/VN- α S into which we transfected VC-tagged plasmids (1 μ g DNA per well of a 24-well dish), followed by automated fluorescence detection (excitation 505 nm, emission 535 nm; Synergy H1 HybridReader, BioTek) 40 h post transfection. Alternatively, we co-expressed a constant WT α S-VC plasmid (4 μ g per 6-cm dish) and a variable VN- α S plasmid (WT or point mutants; 2 μ g per 6-cm dish) for 40 h in M17D cells followed by bright-field and fluorescence microscopy of live cells in culture dishes (AxioVert 200 microscope; AxioCam MRm camera; AxioVision Release 4.8.2; all by Zeiss, Jena, Germany). Images of YFP were collected using a GFP/FITC filter cube and are pseudo-colored green. GFP detection was enhanced by using an anti-GFP antibody. Microscopy analyses were performed in a blinded fashion by assigning random numbers to culture dishes by one investigator before representative images for each culture were taken or cells (+/- inclusions) were counted by another investigator. Confocal images were obtained on a Zeiss LSM710 system.

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Author contributions

U.D., A.J.N., F.S., R.J., T.B. and D.S. designed the research; U.D., A.J.N., F.S., E.S.L., N.C.K., V.E.v.S., J.B.S., and T.B. performed the research; U.D., A.J.N., E.S.L., N.C.K., V.E.v.S., J.B.S., F.S., R.J., T.B. and D.S. analysed the data; U.D., A.J.N., F.S., T.B. and D.S. wrote the manuscript.

Additional information

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