Excess membrane binding of monomeric alpha-, beta-, and gamma-synuclein is invariably associated with inclusion formation and toxicity

Tae-Eun Kim1, Andrew J. Newman1, Thibaut Imberdis1, Lisa Brontesi1, Arati Tripathi1, Nagendran Ramalingam1, Saranna Fanning1, Dennis Selkoe1, and Ulf Dettmer1

1Ann Romney Center for Neurologic Diseases, Department of Neurology, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115 USA

© The Author(s) 2021. Published by Oxford University Press.
*to whom correspondence should be addressed.

Ulf Dettmer, PhD
Ann Romney Center for Neurologic Diseases
Brigham and Women’s Hospital
Building for Transformative Medicine, Room 10002-M
60 Fenwood Road
Boston, MA 02115
USA

Phone: +1-617-525-5761
Abstract

α-synuclein (αS) has been well-documented to play a role in human synucleinopathies such as Parkinson’s disease (PD) and dementia with Lewy bodies (DLB). First, the lesions found in PD/DLB brains - Lewy bodies and Lewy neurites - are rich in aggregated αS. Second, genetic evidence links missense mutations and increased αS expression to familial forms of PD/DLB. Third, toxicity and cellular stress can be caused by αS under certain experimental conditions. In contrast, the homologs β-synuclein (βS) and γ-synuclein (γS) are not typically found in Lewy bodies/neurites, have not been clearly linked to brain diseases, and have been largely non-toxic in experimental settings. In αS, the so-called non-amyloid-β component of plaques (NAC) domain, constituting amino acids 61 to 95, has been identified to be critical for aggregation in vitro. This domain is partially absent in βS and only incompletely conserved in γS, which could explain why both homologs do not cause disease. However, αS in vitro aggregation and cellular toxicity have not been firmly linked experimentally, and it has been proposed that excess αS-membrane binding is sufficient to induce neurotoxicity. Indeed, recent characterizations of Lewy bodies have highlighted the accumulation of lipids and membranous organelles, raising the possibility that βS and γS could also become neurotoxic if they were more prone to membrane/lipid binding. Here, we increased βS and γS membrane affinity by strategic point mutations and demonstrate that these proteins behave like membrane-associated monomers, are cytotoxic, and form round cytoplasmic inclusions that can be prevented by inhibiting stearoyl-CoA desaturase.

Introduction

Ever since α-synuclein (αS) was identified to be a primary component of Lewy bodies and Lewy neurites (1) in synucleinopathies such as Parkinson’s disease (PD), dementia with Lewy Bodies (DLB), and multiple-system atrophy (MSA), the endeavors of many labs have coalesced around elucidating αS native structure, physiological function, aggregation propensity and cell toxicity. Our understanding of the remaining two homologous proteins in
the synuclein family, β-synuclein (βS) and γ-synuclein (γS), however, remains more nebulous than our knowledge of αS. No reports have directly implicated βS or γS mutations and associated aggregation to a synucleinopathy, and neither protein is typically considered to be a major presence in Lewy bodies or Lewy neurites (2–4). However, occasional reports have identified βS and/or γS in synucleinopathy lesions, albeit not classical Lewy bodies (5–7). Moreover, a study that screened and sequenced DLB patient and family samples identified two mutations to βS, V70M and P123H, to potentially be associated with αS aggregation (8). The authors speculated that either mutant might interfere with an unknown molecular pathway in which normal βS prevents αS aggregation (8). γS finds itself most distant from synucleinopathy research because, unlike αS and βS (9), it is prevalent in the peripheral nervous system (10,11). In addition, γS has been identified in liver, breast carcinoma, and possibly ovarian tumors (11,12).

Despite being localized in various cell types and involved in various diseases, all three synucleins have a considerable degree of sequence and structural similarities (Fig. 1A,B). They generally share a similar N-terminal region, of which αS has been purported to form a single or two distinct α-helices when interacting with the lipid bilayer of membranes (13–17), and a C-terminal region that is populated with negatively-charged residues and prolines (Fig. 2). Amino acid (aa) positions 61-95 were identified in αS as the ‘non-amyloid-β component’ (NAC) of Alzheimer’s disease amyloid plaques (18,19). In particular, aa 71-82 within the αS NAC region have been proposed to be critical for β-sheet-rich aggregation in vitro (20,21). This region consists of VTGVTAVAQKTV in αS, FSGAGNIAATG in βS, and VSSVNTVAEKTG in γS (Fig. 1A; conserved aa relative to αS are underlined).

Giasson et al. postulated that the 71-82 stretch is both necessary and sufficient for αS fibrillization based on the following observations (21): 1) aa 71-82 are not conserved in βS, and βS does not assemble into filaments in vitro; 2) introducing a single charged aa within these 12 residues reduces αS in vitro aggregation, and a deletion of the 12 aa abrogates it; 3) the stretch resists proteolytic digestion in αS filaments; and 4) synthetic VTGVTAVAQKTV peptides form filaments in vitro and promote fibrillization of full-length αS.

While key elements of the NAC domain - including the 71-82 aa region - seem to be homolog-specific, a defining feature of all synucleins is an 11-aa repeat with the core consensus motif KTKEGV. The 11-aa repeat spans several times in αS, βS and γS throughout the N-terminus, albeit with slight differences (Fig. 1A,B). In αS, repeats #1-5, and 7 are highly conserved, in βS repeats #1-5. γS seems to contain conserved repeats #1-5 and 7 plus an additional
relatively conserved KTKEGV motif near its C-terminus that is not mirrored in αS or βS (Fig. 1A, B). There have been several studies relating αS aggregation with cell toxicity and pathology from the perspective of the repeat motif (22–26) as the motif seems to be critical for αS’s normal dynamic behavior in cells (reviewed in (27)). Our group has contributed to an increasingly nuanced understanding of αS dynamics in which the KTKEGV motif plays a central role (28–30). We proposed that: 1) αS physiologically exists in dynamic and sensitive equilibria between cytoplasmic vs. membrane-associated species as well as monomeric vs. multimeric species; 2) disruption of these dynamic αS equilibria results in cell toxicity; 3) specific missense mutations to the αS KTKEGV repeat motif can alter the equilibria to the cell’s detriment by stabilizing αS amphipathic helices at membranes and rendering αS unable to multimerize; and 4) cytotoxic αS KTKEGV variants cause vesicle- and lipid-droplet-rich inclusions largely devoid of apparent fibrillar material, as assessed by electron microscopy (EM), while crosslinking and YFP complementation analyses were consistent with monomeric - not aggregated - αS being present in the inclusions (30–32). The hypothesis that we have maintained confers the KTKEGV repeat motif as an important component in αS membrane binding, multimerization, and toxicity.

Here we report that rationally designed KTKEGV repeat motif mutants in αS, βS and γS can invariably be associated with inclusion formation and toxicity in neural cells. The biochemical characterization of the engineered mutants suggests the accumulation of monomeric proteins at membranes. Our observations are consistent with a membrane-mediated mechanism of synuclein toxicity that is independent of the αS NAC domain and may also (at least initially) be independent of proteinaceous, β-sheet-rich aggregation.

Results

Design of αS, βS and γS variants with strong effects on membrane binding and multimerization. We have recently shown that strategic missense mutations to the repeat motif of αS (exaggerations of fPD-linked mutants or strategic designs) can alter the protein’s membrane interactions and assembly state. Four such αS mutant variants that we extended to βS and γS are relevant for this study (Fig. 3A): 1) three KTKEGV→KTKKGV mutations which we named “3K”. In the case of αS, this variant is an amplification of the αS familial PD (fPD) E46K mutant (E35K + E46K + E61K). 3K presumably stabilizes membrane-induced synuclein helices by providing additional attraction
between positively charged lysine residues and negatively charged lipid headgroups (33,34); see Fig. 2. We had previously found decreased αS cytosol:membrane ratios for αS 3K, which were accompanied by decreased multimer:monomer ratios, increased inclusion formation, and increased toxicity (31). We now generated βS 3K and γS 3K (both are E35K + E46K + E61K) for comparison (Fig. 3A, second column); 2) Six KTKEGV→KLKEGV mutations that we named “KLK” (30). KLK presumably strengthens hydrophobic interactions with membrane lipids by replacing threonine with leucine residues, thereby increasing the hydrophobicity of the membrane-embedded half of the synuclein amphipathic helix and giving rise to additional steric interactions. We had previously found strongly decreased αS cytosol:membrane ratios for αS KLK that were accompanied by decreased multimer:monomer ratios, increased inclusion formation, and increased toxicity (30). We now generated βS KLK and γS KLK for comparison (Fig. 3A, third column); 3) Seven KTKEGV→KTKEIV mutations which we named “EIV” (30). Like KLK, this variant increases the hydrophobicity of the membrane helix (Fig. 2A, fourth column); and 4) six KTKEGR mutations which we named “EGR” (30). EGR has additional positive arginine charges in the hydrophobic half of the helix (Fig. 3B, last column, top). Based on previous work on similar helix-disrupting αS mutations (35,36) and a reported mechanism of αS multimer formation that involves transient membrane interactions (37), it is plausible that αS EGR is repelled from membranes and accumulates in the cytosol as an (unfolded) monomer (Fig. 3B, last column, bottom). Here, we generated βS EGR and γS EGR for comparison with αS EGR.

Given similar sequences across all synuclein species, and given our previous report demonstrating that wt αS, βS, and γS (Fig. 3A, first column) all exhibit a pattern of apparent multimers and monomers upon crosslinking (30), it seemed reasonable to hypothesize that the phenotypic differences among the various missense mutations introduced in αS might also hold true if we introduced the analogous mutations in βS and γS (Fig. 3A).

**The conserved KTKEGV repeat motifs mediate αS, βS, and γS membrane interaction and assembly.** To test whether the above-described effects of αS 3K, KLK, EIV, and EGR mutagenesis might apply to all synuclein homologs, we expressed the respective αS, βS, and γS variants in M17D neuroblastoma cells by lipofection. We previously demonstrated the multimerization of endogenous neuronal αS and βS by intact-cell crosslinking (30). Moreover, we showed by sequential extraction that endogenous neuronal βS is less membrane-associated than αS,
while both homologs are present in cytosol and membrane fractions in relevant amounts (38). To enable direct comparisons across all three synuclein proteins and their variants in the present study, we fused our constructs with C-terminal FLAG₃ tags. 48 hours post transfection, we subjected the cells to sequential protein extraction and analyzed PBS-soluble (‘cytosol’) and detergent-soluble (1% TX-100; ‘membrane’) fractions by Western blot (WB).

As expected based on our previous work, we found αS 3K, KLK, and EIV to be enriched in the membrane fraction, whereas EGR accumulated in the cytosol (Fig. 4). Overall, we observed similar effects in the respective βS and γS mutations, with only γS 3K not being significantly different from wt under the chosen conditions (Fig. 4). 3K had weaker effects than KLK or EIV with regard to increasing αS membrane binding, which might be explained by only three residues being changed in comparison to six and seven in the cases of KLK and EIV, respectively. Next, we asked whether introducing the 3K, KLK, EIV, or EGR mutations into βS and γS would also affect multimer:monomer ratios, as we had observed for αS (30,31,39). As in our previous studies, we tested for synuclein assembly by intact-cell crosslinking using 1 mM disuccinimidyl glutarate (DSG) and WB (Fig. 5). We observed that: 1) all wt and EGR variants exhibited both multimers (30 kDa, 60 kDa, 80 kDa, 100 kDa) and monomers (14 kDa), but the multimer:monomer ratios of the EGR variants were reduced relative to wt; 2) all 3K variants had lower levels of multimers when compared to their wt counterparts; 3) KLK and EIV variants were predominantly monomeric as expected from our previous αS studies (30,39). To confirm one of our key observations in the absence of protein tags, we subjected untagged αS and βS wt and 3K variants to DSG crosslinking followed by sequential extraction. In both homologs, the 3K variants were more membrane-associated and exhibited reduced 60/80/100 kDa multimers, which were mostly found in the cytosolic fractions for both wt and 3K (Fig. S1).

**Decreased solubility and multimerization in αS, βS, and γS are associated with toxicity.** We previously reported that inclusion formation due to certain engineered αS KTKEGV repeat motif mutations is associated with cell stress/toxicity (30,31,39,40), and we planned to verify if this also held true when analogous mutations were introduced into the repeat motifs in βS and γS. We therefore transfected M17D cells with all of our FLAG₃-tagged variants and observed by live-cell imaging how overexpression of the various synuclein variants might affect cell confluence as a proxy for cell health. 48 hours post-transfection across all synuclein homologs, the confluence of M17D cells that were transfected with wt synuclein was not significantly different from those that were transfected
with vector alone. However, the relative confluence of all 3K, KLK, and EIV transfectants was significantly lower (Fig. 6A). The reduced confluence was accompanied by changes in cell morphology: many cells appeared rounded and detached, similar to cells that were treated with the strong toxin staurosporine (Fig. 6A, top left panel). This indicated to us that 3K, KLK, and EIV variants were toxic for all synuclein homologs. The EGR variant was generally less toxic than the other variants (as indicated by higher confluence levels and fewer numbers of small and rounded cells); its confluence level was moderately less than wt (Fig. 6A). We next transfected DIV14 rat neurons with the respective YFP-tagged proteins (wt, 3K, KLK, EGR) and recorded YFP signals in the IncuCyte live-cell microscope after 96 h. Due to the relatively low transfection efficiency, we were able to identify single YFP+ neurons and categorized them into intact and disintegrated neurons (see Fig. 6B, top panel for examples). Blinded analysis revealed that for all synuclein homologs, about 80% of wt YFP+ neurons were intact, a value that was strongly reduced in the case of 3K and KLK (p < 0.0001), and slightly reduced in the case of EGR variants (p < 0.05). In the case of 3K and KLK, even seemingly intact neurons displayed changes in cellular αS distribution (inclusion formation), which we addressed in detail for shorter transfection times (next paragraph).

**Decreased multimerization and solubility in αS, βS, and γS are associated with cytoplasmic inclusion formation.** Previously, we showed that transfecting M17D cells with αS 3K, KLK, and EIV variants resulted in inclusion formation, whereas αS EGR, like wt αS, remained diffuse throughout the cytosol. We had also observed that primary rat neurons transiently transfected with αS 3K, KLK, and EIV variants displayed multiple inclusions of various sizes in both somata and neurites (30,31); electron microscopy identified the inclusions as rich in vesicles and lipid droplets (32,39). Here, we first transiently transfected M17D cells with YFP-tagged wt and variant αS, βS, and γS. We observed by IncuCyte live-cell imaging that all wt synuclein homologs remained diffuse throughout the cytosol, and all 3K, KLK, and EIV variants formed round inclusions of various sizes 48 hours post transfection (Fig. 7A). We verified our observations by co-transfecting DIV 13 mouse neurons with YFP-tagged synuclein variants and RFP (cytosolic marker and control for cell integrity): using fluorescence microscopy of fixed cells, we observed that after 48 hours all wt and EGR constructs remained diffuse and cytosolic like RFP, whereas 3K, KLK, and EIV expression resulted in the appearance of multiple round inclusions in somata and neurites (Fig. 7B). Of note, the 3K, KLK, and EIV inclusions (green signal) did not seem to co-localize with RFP (red signal). This is unlike the
wt and EGR synuclein homologs, which overlapped with RFP (merged signals). Transient transfections of DIV 14 primary rat neurons with FLAG-tagged wt and variants of all three homologs followed by immunofluorescence led to similar results (Fig. 7C), thereby confirming our previous observations: wt and EGR of all synuclein homologs appeared diffuse throughout the neuron, whereas 3K, KLK, and EIV variants formed round inclusions.

**Pharmacological alteration of (membrane) lipid composition rescues synuclein inclusions.** We recently identified by lipidomic profiling that αS cytotoxicity is accompanied by elevated levels of monounsaturated fatty acids and oleic acid (OA) in particular (41). Moreover, we also identified lipid saturation as a modifier of αS inclusion formation in a small-molecule screen (42). This prompted us to speculate about a bi-directional scenario in which αS leads to elevated OA levels, which – after incorporation into membrane lipids – increase αS membrane association and toxicity (43). Indeed, we and others have demonstrated that loss/inhibition of the OA-generating enzyme stearoyl-CoA desaturase (SCD) rescues αS-related toxicity in yeast (41,44), *C. elegans* (41,45), primary rodent neurons (41), and neuroblastoma cells (42). The increased cellular viability was found to be accompanied by the following changes in αS homeostasis: 1) αS serine-129 phosphorylation of αS E46K and excess wt αS was reduced upon SCD inhibition (41,42); 2) αS E46K solubility, as assessed by cytosol:membrane ratio in sequential extraction experiments, was increased (41,42); and 3) inclusion formation of αS 3K (amplified E46K) was reduced (41,42). We therefore tested if putatively beneficial effects of SCD inhibition would also hold true for the other homologs. Specifically, we focused on inclusion formation of the respective 3K variants because their intermediate effects in our experiments (Figs. 3-6) indicated to us the potential for reversibility. YFP alone as well as YFP-tagged αS 3K, βS 3K, and γS 3K were expressed in control vs. 24-hour SCD inhibitor-pretreated M17D cells (10 μM MF-438; Fig. 8A). We observed a phenotypic rescue of both inclusion-bearing (αS, βS, γS) and rounded (βS, γS) cells, as confirmed by blinded statistical analysis (Fig. 8B): while SCD inhibition did not affect the appearance of YFP-only expressing cells, the percentage of flat, inclusion-free cells was markedly increased by the SCD inhibitor for all 3K transfectants. Conversely, the count of abnormal (i.e., inclusion-positive and rounded) cells was reduced (Fig. 8B) while all 3K transfectants displayed similar levels of transgene expression (Fig. 8C).

**Discussion**
αS is considered a toxic protein. Its homologs βS and γS are not. In the present study we asked if αS is truly unique among the synucleins with regard to the ability to cause cellular stress/toxicity. We found that three defined aa substitutions (E35K+E46K+E61K = ‘3K’) are sufficient to render all synucleins significantly more toxic than their wt counterparts. In addition to 3K, which changes three αS repeat motifs from KTKEGV to KTKKGV, we saw similar effects for motif variants KLKEGV (‘KLK’; 6 substitutions) as well as KLKEIV (‘EIV’; 7 substitutions). All three variants have in common the expected effect of facilitating αS amphipathic helix formation at membranes (Fig. 2), thereby increasing αS-membrane interactions (Fig. 3). Indeed, upon sequential extraction we found significantly higher amounts of 3K, KLK, and EIV relative to wt in detergent-soluble fractions for all synucleins, consistent with enhanced membrane binding (Fig. 4). The increase in membrane-interaction was paralleled by reduced multimer formation, as evidenced by intact-cell crosslinking (Fig. 5). Moreover, 3K, KLK, and EIV expression in neural cells triggered synuclein toxicity (Fig. 6) and inclusion formation (Fig. 7) for all three homologs. A key motif that has been associated with αS aggregation are aa 61-95 (NAC domain), particularly aa 71-82 (20,21). The aa 71-82 stretch (VTGVTAVAQKTV in αS) is largely deleted in βS, and more hydrophilic in γS (VSSYNTVAEKT). Thus, our new data are consistent with a membrane-associated mechanism of synuclein toxicity that is independent of the NAC domain and at least initially not mediated by proteinaceous β-sheet-rich protein aggregation. Related to this notion, a study by Volles et al. compared αS in vitro fibrillization and yeast toxicity by screening a library of random point mutants (46). A lack of correlation between the two aspects suggested that fibrillization is not necessary for synuclein-induced toxicity in yeast. A second yeast screen in the same study identified 25 non-toxic αS sequence variants, which reduced membrane binding, and a toxic point mutation, which increased membrane binding (46). The authors hypothesized that yeast toxicity is caused by αS binding directly to membranes at levels sufficient to non-specifically disrupt homeostasis. A recent study performed deep-mutational αS scanning in the yeast model and likewise came to the conclusion that membrane binding mediates αS toxicity (47). Previous studies had characterized the membrane-associated toxicity of αS in detail: wt αS expression in yeast causes vesicle clustering/aggregation (48) and vesicle trafficking defects (49) in the absence of apparent amyloid formation. The relevance of these findings for PD pathogenesis was highlighted when similar trafficking defects were observed in patient-derived αS A53T and triplication iPS cell cultures (50). In contrast to
the yeast system, no acute toxicity was observed in the iPSC neurons, possibly due to a better ability of mammalian cells to compensate αS-induced cellular dyshomeostasis. Interestingly, a recent characterization of Lewy body pathology came to the conclusion that excess αS interaction with lipids/membranes also plays a major role in these hallmark lesions of synucleinopathy (51).

The hypothesis that our observed synuclein toxicity is mediated by an excess of synuclein-membrane interaction is further supported by our SCD inhibitor experiments (Fig. 8). SCD inhibition increases the proportion of saturated fatty acids in the membrane, while the proportion of mono-unsaturated fatty acids decreases. Higher levels of saturated fatty acids decrease membrane fluidity and thus αS-membrane interaction via the formation of amphipathic helices (52). Consequently, we detected reduced inclusion formation of the membrane-affine 3K variants upon treatment with SCD inhibitor MF-438 (Fig. 8). SCD inhibition has emerged as a potential new treatment for synucleinopathies in several studies (41,42,44). The present work suggests that SCD inhibition could also overcome βS- and γS-related cytotoxicity. Yet, a disease mechanism based on βS or γS excess or misfolding that would be independent of αS has not been proposed. Our study, however, highlights that increasing βS or γS membrane binding could theoretically render these proteins toxic as well. Changing one KTKEGV motif to KLKEGV or KTKEIV might be sufficient to strengthen membrane interaction in such a way that toxicity occurs within the lifetime of a human, and it will be interesting to see if such a mutation will ever be found. In fact, two potentially DLB-linked mutations to βS (V70M and P123H) were recently suggested to have higher membrane-binding affinity compared to wt βS (53). However, instead of causing neurodegeneration via βS aggregation, an indirect effect on αS aggregation had been proposed for these two mutants (8). This raises important questions about the interactions among the synuclein homologs in the cell. αS-βS interactions have been observed, most notably the aggregation-reducing effect that wt βS has over wt αS (54–57) and familial-PD αS mutants (58). However, there are no explicit reports on mixed helical multimers consisting of both αS and βS (or γS), and more work will be needed to establish or rule out their existence.

In our present study, we also included the engineered αS variant “EGR”. Based on the helical-wheel model of αS-membrane interaction, this variant is expected to enhance αS cytosol localization (Figs. 2, 3). Indeed, we observed an accumulation in the cytosol upon sequential extraction (Fig. 4). EGR also reduces multimer:monomer ratios
relative to wt αS, consistent with a model in which a specific level of αS-membrane interaction is needed to keep cellular αS equilibria at bay (31). Of note, it has been suggested that native αS multimerization is a cellular mechanism of preventing αS aggregation (28). Transient αS-membrane interaction seems to be critical for native αS assembly (37). Human genetics tells us that both a lack (A30P, G51D) and an increase (E46K) in αS-membrane interaction is associated with PD/DLB (59). Interestingly, we observe significant toxicity for αS and γS EGR, but not for βS EGR upon expression in neural cells (Fig. 6). It is tempting to speculate that the EGR-like, cytosolic αS toxicity may be mediated by ‘classical’ proteinaceous aggregation, which is facilitated by the presence of the hydrophobic αS NAC-domain that is less hydrophobic in γS and partially deleted in βS (Fig. 2). However, we do not observe signs of obvious YFP-tagged or FLAG3-tagged αS EGR aggregation in neuroblastoma cells or rodent neurons: Fig. 6 does not show focal αS accumulation, consistent with no or only small aggregates. Intact-cell crosslinking of EGR suggests an accumulation of 14-kDa αS monomers, but it may be worth following up on an apparent increase in αS dimer formation (Fig. 5). Lastly, the analysis of EGR is further complicated by the fact that this variant apparently enhances αS expression levels (Fig. 5), precluding a firm conclusion about its toxicity relative to wt synuclein. In contrast, the αS, βS, and γS 3K variants increase αS toxicity (Fig. 6) despite lower expression (Fig. 5), underlining the rapid toxicity caused by synuclein accumulation at (vesicle) membranes and/or lipid droplets.

Materials and Methods

cDNA cloning. FLAG3-tagged (3K, KLK, EIV, EGR) and untagged (wt, 3K) αS, βS, and γS mutant variant constructs were designed using GeneArt Strings DNA Fragments (Life Technologies) and ligated into pcDNA4/TO/myc-His plasmids using the In-Fusion HD cloning system (Takara) according to manufacturer instructions. YFP-tagged βS and γS variants were cloned using specific primers as described for αS (30).

Primary mouse and rat neuron harvest. Primary neurons were acquired under protocol number 05022, approved by the appropriate Institutional Animal Care and Use Committee, the Harvard Medical Area Standing Committee on Animals. Embryos from anesthetized pregnant CD-1 mouse and Sprague-Dawley rats (Charles-River Laboratories) at embryonic day 18 were harvested by cesarean microdissection. Dissected cortices were collected...
in HBSS on ice and dissociated with Accumax (Innovative Cell Technologies) and DNase (40 U/μl) at 37 °C for 25 min followed by gentle trituration with a Pasteur pipette in Neurobasal Medium (Life Technologies) supplemented with B27 (Life Technologies), glutamine (0.5 mM), β-mercaptoethanol (25 μM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were then filtered through a 70 μM cell strainer to remove tissue debris, and clumps and were seeded in flat-bottom poly-D-lysine coated polystyrene plates (Lab-Tek 70378–81) at a density of 40,000 cells (per well of 96-well plate) and 200,000 cells (per well of 24-well plate). The cells were cultured by keeping half the volume of existing supplemented Neurobasal Medium in the wells and adding a fresh half of supplemented Neurobasal Medium.

**Cell culture and transfection.** Human BE(2)-M17 neuroblastoma cells (called M17D, ATCC number CRL-2267) and primary CD-1 mouse and Sprague-Dawley rat neurons (Charles River, Wilmington, MA) were cultured at 37 °C in 5% CO₂. M17D cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, penicillin (50 units per mL), streptomycin (50 μg per mL), and 2 mM L-glutamine. Cells were transfected with Lipofectamine 2000 or 3000 (Invitrogen) following the manufacturer’s instructions in Opti-MEM (Gibco) for M17D cells and in unsupplemented Neurobasal Medium (Gibco) for primary cells.

**Intact-cell crosslinking.** Transfected cells were collected by trituration, washed with PBS, and then resuspended in PBS with EDTA-free Complete Protease Inhibitor (Roche). Next, cells were crosslinked in 1 mM final concentration of disuccinimidyl glutarate (DSG) (Thermo Scientific) in DMSO for 30 min at 37 °C while rotating (60). The reaction was then quenched by adding 50 mM Tris, pH 7.6, and incubating for 15 min at RT. Afterwards, cytosolic and membrane proteins were extracted by lysing the cells in 1% Triton-X 100 detergent (Sigma) by briefly vortexing followed by incubation on ice for 20 min. Then, the lysed samples were centrifuged at 100,000 x g for 1 hour at 4 °C to collect the supernatant.

**Immunoblotting.** Protein concentrations were determined by BCA assay (Thermo Scientific), and then 20 μg of each protein sample were boiled in 1X NuPAGE LDS Sample (Thermo Fisher) for 10 min. The samples were then run on a NuPAGE 4–12% Bis-Tris gel (Thermo Fisher). SeeBlue Plus2 marker (Invitrogen) was used as a protein ladder. Gels were electroblotted onto an Immobilon-Psq 0.2 μm PVDF membrane (Millipore) for 90 min at 400 mA
at 4 °C in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). Then, the proteins were fixed onto the membranes with 0.4% paraformaldehyde in PBS for 30 min at RT, rinsed with PBS, and blocked in 0.2% I-Block solution (Invitrogen) for either 30 min at RT or overnight at 4 °C. Afterwards, the membranes were incubated in primary antibody (in 0.2% I-Block with 0.02% sodium azide) for either 1 hour at RT or overnight at 4 °C. Membranes were then washed three times for 30 min in PBS (containing 0.1% Tween) at RT. Then, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody (GE Healthcare; diluted 1:10,000 in PBS/0.2% Tropix I-Block) for 45 min at RT. Membranes were then washed three times for 30 min in PBS + 0.1% Tween and developed with SuperSignal West Dura (Thermo Scientific).

Sequential protein extraction. Cells were first lysed by hypotonic shock (addition of H2O containing protease inhibitors to cell pellets, 10 min of incubation at RT while shaking). Then 10x PBS was added for a final concentration of 1x. Lysates were spun at 4 °C (> 12,000 g). The supernatant was collected (PBS fraction, cytosol). The pellet was lysed in PBS/1% Triton-X 100 by sonication, followed by centrifugation at 4 °C (> 12,000 g). The supernatant was collected (TX-100 fraction, membranes).

Immunofluorescence. Primary rodent neurons transfected (described above) for 48 hours were washed twice with HBSS (Thermo Fisher), and fixed with a PBS solution containing 4% paraformaldehyde and 0.02% glutaraldehyde for 25 min at RT. The cells were then blocked and permeabilized with 5% BSA/0.25% Triton X-100/PBS for 1 hour. Cells were then incubated with primary antibody (with 0.2% I-block) for 2 hrs at RT or overnight at 4 °C. Afterwards, the cells were washed three times with PBS followed by incubating the cell with either Alexa Fluor 488- or Alexa Fluor 568-coupled secondary antibodies (diluted 1:2000 in 5% BSA/PBS) for 1-2 hours at RT. Finally, cells were washed three times in PBS for 10 min each at RT before visualizing fluorescence.

Fluorescence microscopy. Visualizing cells by fluorescence microscopy was performed on an AxioVert 200 microscope (AxioCam MRm camera; AxioVision Release 4.8.2; all by ZEISS). Images of YFP fluorescence were collected using a GFP/FITC filter cube and are pseudo-colored green. Confocal images were obtained on a Zeiss LSM710 system.
**Antibodies.** Antibodies used were monoclonal antibodies M2 to the FLAG tag (Sigma, 1:10,000 in WB, 1:1000 in ICC) 71.1 to GAPDH (Sigma; 1:5,000 in WB) as well as polyclonal antibodies ab22595 to Calnexin (abcam; 1:200 in ICC, 1:1000 in WB), ab84036 to TfR (abcam; 1:1000 in WB) and anti-DJ-1 (61), 1:3000 in WB).

**Cell toxicity assay based on density.** M17D cells were plated on 96 well plates and 24 hours later treated with 0.1% DMSO or 10 µM SCD inhibitor (MF-438; see Fig. S2 for structure). After this pretreatment, the cells were transfected (as described above) and cell confluency was measured using the IncuCyte Zoom 2000 platform (Essen Biosciences) and an IncuCyte processing definition as described before (SI Appendix, Table S2 in (42)). Quantification was performed using GraphPad Prism Version 7.

**Neuron integrity assessment.** YFP-tagged wt, 3K, KLK, or EGR variants for all human synuclein homologs were transfected into DIV14 rat cortical neurons via lipofection (Lipofectamine 2000 following the manufacturer’s protocol, except that unsupplemented Neurobasal media was used instead of OPTIMEM) and cells were monitored in the IncuCyte Zoom 2000 platform (Essen Biosciences). Transfection efficiency <5% allowed for assessing integrity of single transfected neurons 96 hours post transfection. After image acquisition and blinding, cells were categorized into “intact” and “disintegrated” and the relative percentage of intact neurons was calculated.

**Statistical analyses.** Blinded analyses were performed by assigning random numbers to dishes or images by one investigator before representative images were taken or features were counted by another investigator. We performed one-way ANOVA including Tukey’s or Dunnett’s or unpaired two-tailed t-tests using GraphPad Prism Version 7 following the program’s guidelines. Normal distribution and similar variance were observed for all values. Graphs are means +/- S.D. Criteria for significance, routinely determined relative to wt αS: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Sufficient experiments and replicates were analyzed to achieve statistical significance and these judgements were based on earlier, similar work.

**Acknowledgments**

We thank Elizabeth Terry-Kantor in our group as well as Silke Nuber (BWH/HMS) and Tim Bartels (University College London) for helpful discussions and critical revision of the manuscript. This work was supported by the
Conflict of Interest Statement

DS is a director and consultant to Prothena Biosciences. The other authors declare no conflict of interest.

References

1. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R. and Goedert, M. (1997) Alpha-synuclein in Lewy bodies. Nature, 388, 839–840.
2. Lavedan, C., Buchholtz, S., Auburger, G., Albin, R. L., Athanassiadou, A., Blancato, J., Burguera, J. A., Ferrell, R. E., Kostic, V., et al. (1998) Absence of mutation in the beta- and gamma-synuclein genes in familial autosomal dominant Parkinson’s disease. DNA Res. Int. J. Rapid Publ. Rep. Genes Genomes, 5, 401–402.
3. Lincoln, S., Crook, R., Chartier-Harlin, M. C., Gwinn-Hardy, K., Baker, M., Mouroux, V., Richard, F., Becquet, E., Amouyel, P., et al. (1999) No pathogenic mutations in the beta-synuclein gene in Parkinson’s disease. Neurosci. Lett., 269, 107–109.
4. Baba, M., Nakajo, S., Tu, P. H., Tomita, T., Nakaya, K., Lee, V. M., Trojanowski, J. Q. and Iwatsubo, T. (1998) Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson’s disease and dementia with Lewy bodies. Am. J. Pathol., 152, 879–884.
5. Galvin, J. E., Giasson, B., Hurtig, H. I., Lee, V. M. and Trojanowski, J. Q. (2000) Neurodegeneration with brain iron accumulation, type 1 is characterized by alpha-, beta-, and gamma-synuclein neuropathology. Am. J. Pathol., 157, 361–368.
6. Galvin, J. E., Uryu, K., Lee, V. M. and Trojanowski, J. Q. (1999) Axon pathology in Parkinson’s disease and Lewy body dementia hippocampus contains alpha-, beta-, and gamma-synuclein. Proc. Natl. Acad. Sci. U. S. A., 96, 13450–13455.
7. Nishioka, K., Wider, C., Vilariño-Güell, C., Soto-Ortolaza, A. I., Lincoln, S. J., Kachergus, J. M., Jasinska-Myga, B., Ross, O. A., Raiput, A., et al. (2010) Association of alpha-, beta-, and gamma-Synuclein with diffuse lewy body disease. Arch. Neurol., 67, 970–975.
8. Ohtake, H., Limprasert, P., Fan, Y., Onodera, O., Kakita, A., Takahashi, H., Bonner, L. T., Tsuang, D. W., Murray, I. V., J., et al. (2004) Beta-synuclein gene alterations in dementia with Lewy bodies. Neurology, 63, 805–811.
9. Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H. A., Kittel, A. and Saitoh, T. (1995) The precursor protein of non-A beta component of Alzheimer’s disease amyloid is a presynaptic protein of the central nervous system. Neuron, 14, 467–475.
10. Buchman, V. L., Hunter, H. J., Pinon, L. G., Thompson, J., Privalova, E. M., Ninkina, N. N. and Davies, A. M. (1998) Persyn, a member of the synuclein family, has a distinct pattern of expression in the developing nervous system. J. Neurosci. Off. J. Soc. Neurosci., 18, 9335–9341.
11. Lavedan, C., Leroy, E., Dehejia, A., Buchholtz, S., Dutra, A., Nussbaum, R. L. and Polymeropoulos, M. H. (1998) Identification, localization and characterization of the human gamma-synuclein gene. Hum. Genet., 103, 106–112.
12. Ji, H., Liu, Y. E., Jia, T., Wang, M., Liu, J., Xiao, G., Joseph, B. K., Rosen, C. and Shi, Y. E. (1997) Identification of a breast cancer-specific gene, BCSG1, by direct differential cDNA sequencing. Cancer Res., 57, 759–764.

13. George, J. M., Jin, H., Woods, W. S. and Clayton, D. F. (1995) Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. Neuron, 15, 361–372.

14. Davidson, W. S., Jonas, A., Clayton, D. F. and George, J. M. (1998) Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. J. Biol. Chem., 273, 9443–9449.

15. Chandra, S., Chen, X., Rizo, J., Jahn, R. and Südhof, T. C. (2003) A broken alpha-helix in folded alpha-synuclein. J. Biol. Chem., 278, 15313–15318.

16. Jao, C. C., Hegde, B. G., Chen, J., Haworth, I. S. and Langen, R. (2008) Structure of membrane-bound alpha-synuclein from site-directed spin labeling and computational refinement. Proc. Natl. Acad. Sci. U. S. A., 105, 19666–19671.

17. Georgieva, E. R., Ramllall, T. F., Borbat, P. P., Freed, J. H. and Eliezer, D. (2008) Membrane-bound alpha-synuclein forms an extended helix: long-distance pulsed ESR measurements using vesicles, bicus, and rodlike micelles. J. Am. Chem. Soc., 130, 12856–12857.

18. Han, H., Weinreb, P. H. and Lansbury, P. T. (1995) The core Alzheimer’s peptide NAC forms amyloid fibrils which seed and are seeded by beta-amyloid: is NAC a common trigger or target in neurodegenerative disease? Chem. Biol., 2, 163–169.

19. Irizarry, M. C., Kim, T. W., McNamara, M., Tanzi, R. E., George, J. M., Clayton, D. F. and Hyman, B. T. (1996) Characterization of the precursor protein of the non-A beta component of senile plaques (NACP) in the human central nervous system. J. Neuropathol. Exp. Neurol., 55, 889–895.

20. Waxman, E. A., Mazzulli, J. R. and Giasson, B. I. (2009) Characterization of hydrophobic residue requirements for alpha-synuclein fibrillization. Biochemistry, 48, 9427–9436.

21. Giasson, B. I., Murray, I. V., Trojanowski, J. Q. and Lee, V. M. (2001) A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. J. Biol. Chem., 276, 2380–2386.

22. Rivers, R. C., Kumita, J. R., Tartaglia, G. G., Dedmon, M. M., Pawar, A., Vendruscolo, M., Dobson, C. M. and Christodoulou, J. (2008) Molecular determinants of the aggregation behavior of alpha- and beta-synuclein. Protein Sci. Publ. Protein Soc., 17, 887–898.

23. Tashiro, M., Kojima, M., Kihara, H., Kasai, K., Kamiyoshihara, T., Uéda, K. and Shimotakahara, S. (2008) Characterization of fibrillation process of alpha-synuclein at the initial stage. Biochem. Biophys. Res. Commun., 369, 910–914.

24. Harada, R., Kobayashi, N., Kim, J., Nakamura, C., Han, S.-W., Ikebukuro, K. and Sode, K. (2009) The effect of non-amyloid acid substitution in the imperfect repeat sequences of alpha-synuclein on fibrillation. Biochim. Biophys. Acta, 1792, 998–1003.

25. Zariviv, Y., Simhi-Haham, D., Israeli, E., Elhadi, S. A., Grigoletto, J. and Sharon, R. (2014) Lysine residues at the first and second KTKEGV repeats mediate α-Synuclein binding to membrane phospholipids. Neurobiol. Dis., 70, 90–98.

26. Xu, L., Nussinov, R. and Ma, B. (2016) Coupling of the non-amyloid-component (NAC) domain and the KTK(E/Q)GV repeats stabilize the α-synuclein fibrils. Eur. J. Med. Chem., 121, 841–850.

27. Yeboah, F., Kim, T. E., Bill, A. and Dettmer, U. (2019) Dynamic behaviors of α-synuclein and tau in the cellular context: New mechanistic insights and therapeutic opportunities in neurodegeneration. Neurobiol. Dis., 132, 104543.

28. Bartels, T., Choi, J. G. and Selkoe, D. J. (2011) α-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. Nature, 477, 107–110.

29. Dettmer, U., Newman, A. J., Luth, E. S., Bartels, T. and Selkoe, D. (2013) In vivo cross-linking reveals principally oligomeric forms of α-synuclein and β-synuclein in neurons and non-neural cells. J. Biol. Chem., 288, 6371–6385.

30. Dettmer, U., Newman, A. J., von Saucken, V. E., Bartels, T. and Selkoe, D. (2015) KTKEGV repeat motifs are key mediators of normal α-synuclein tetramerization: Their mutation causes excess monomers and neurotoxicity. Proc. Natl. Acad. Sci. U. S. A., 112, 9596–9601.
31. Dettmer, U., Newman, A. J., Soldner, F., Luth, E. S., Kim, N. C., von Saucken, V. E., Sanderson, J. B., Jaenisch, R., Bartels, T. and Selkoe, D. (2015) Parkinson-causing α-synuclein missense mutations shift native tetramers to monomers as a mechanism for disease initiation. Nat. Commun., 6, 7314.

32. Ericsson, M., von Saucken, V., Newman, A. J., Doehr, L., Hoesch, C., Kim, T.-E. and Dettmer, U. (2021) Crowded organelles, lipid accumulation, and abnormal membrane tubulation in cellular models of enhanced α-synuclein membrane interaction. Brain Res., 1758, 147349.

33. Perlmutter, J. D., Braun, A. R. and Sachs, J. N. (2009) Curvature dynamics of alpha-synuclein familial Parkinson disease mutants: molecular simulations of the micelle- and bilayer-bound forms. J. Biol. Chem., 284, 7177–7189.

34. Kovacs, M., Powers, A. E., Jiang, H., Pitino, J. C., Fonseca-Ornelas, L., Patel, D. S., Achille, A., Langen, R., Varkey, J. and Bartels, T. (2019) E46K-like α-synuclein mutants increase lipid interactions and disrupt membrane selectivity. J. Biol. Chem.

35. Wang, L., Das, U., Scott, D. A., Tang, Y., McLean, P. J. and Roy, S. (2014) α-synuclein multimers cluster synaptic vesicles and attenuate recycling. Curr. Biol. CB, 24, 2319–2326.

36. Burre, J., Sharma, M. and Sridh, T. C. (2015) Definition of a Molecular Pathway Mediating α-Synuclein Neurotoxicity. J. Neurosci. Off. J. Soc. Neurosci., 35, 5221–5232.

37. Rovere, M., Sanderson, J. B., Fonseca-Ornelas, L., Patel, D. S. and Bartels, T. (2018) Refolding of helical soluble α-synuclein through transient interaction with lipid interfaces. FEBS Lett.

38. Ramalingam, N. and Dettmer, U. (2021) Temperature is a key determinant of alpha- and beta-synuclein membrane interactions in neurons. J. Biol. Chem., 100271.

39. Dettmer, U., Ramalingam, N., von Saucken, V. E., Kim, T.-E., Newman, A. J., Terry-Kantor, E., Nuber, S., Ericsson, M., Fanning, S., et al. (2017) Loss of native α-synuclein multimerization by strategically mutating its amphipathic helix causes abnormal vesicle interactions in neuronal cells. Hum. Mol. Genet., 26, 3466–3481.

40. Terry-Kantor, E., Tripathi, A., Imberdis, T., LaVoie, Z. M., Ho, G. P. H., Selkoe, D., Fanning, S., Ramalingam, N. and Dettmer, U. (2020) Rapid Alpha-Synuclein Toxicity in a Neural Cell Model and Its Rescue by a Stearoyl-CoA Desaturase Inhibitor. Int. J. Mol. Sci., 21.

41. Fanning, S., Haque, A., Imberdis, T., Baru, V., Barrasa, M. I., Nuber, S., Termine, D., Ramalingam, N., Ho, G. P. H., et al. (2018) Lipidomic Analysis of α-Synuclein Neurotoxicity Identifies Stearoyl CoA Desaturase as a Target for Parkinson Treatment. Mol. Cell.

42. Imberdis, T., Negri, J., Ramalingam, N., Terry-Kantor, E., Ho, G. P. H., Fanning, S., Stirtz, G., Kim, T.-E., Levy, O. A., et al. (2019) Cell models of lipid-rich α-synuclein aggregation validate known modifiers of α-synuclein biology and identify stearyl-CoA desaturase. Proc. Natl. Acad. Sci. U. S. A.

43. Fanning, S., Selkoe, D. and Dettmer, U. (2020) Parkinson’s disease: proteinopathy or lipidopathy? NPJ Park. Dis., 6, 3.

44. Vincent, B. M., Tardiff, D. P., Piotrowski, J. S., Aron, R., Lucas, M. C., Chung, C. Y., Bacherman, H., Chen, Y., Pires, M., et al. (2018) Inhibiting Stearoyl-CoA Desaturase Ameliorates α-Synuclein Cytotoxicity. Cell Rep., 25, 2742–2754.e31.

45. Maulik, M., Mitra, S., Basmayor, A. M., Lu, B., Taylor, B. E. and Bulit-Ito, A. (2019) Genetic Silencing of Fatty Acid Desaturases Modulates α-Synuclein Toxicity and Neuronal Loss in Parkinson-Like Models of C. elegans. Front. Aging Neurosci., 11, 207.

46. Volles, M. J. and Lansbury, P. T. (2007) Relationships between the sequence of alpha-synuclein and its membrane affinity, fibrillation propensity, and yeast toxicity. J. Mol. Biol., 366, 1510–1522.

47. Newberry, R. W., Leong, J. T., Chow, E. D., Kampmann, M. and DeGrado, W. F. (2020) Deep mutational scanning reveals the structural basis for α-synuclein activity. Nat. Chem. Biol., 16, 653–659.

48. Soper, J. H., Roy, S., Stieber, A., Lee, E., Wilson, R. B., Trojanowski, J. Q., Burd, C. G. and Lee, V. M.-Y. (2008) Alpha-synuclein-induced aggregation of cytoplasmic vesicles in Saccharomyces cerevisiae. Mol. Bi. Cell, 19, 1093–1103.

49. Cooper, A. A., Gitter, A. D., Cashikar, A., Haynes, C. M., Hill, K. J., Bhullar, B., Liu, K., Xu, K., Strathcarn, K. E., et al. (2006) Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson’s models. Science, 313, 324–328.
Chung, C. Y., Khurana, V., Auluck, P. K., Tardiff, D. F., Mazzulli, J. R., Soldner, F., Baru, V., Lou, Y., Freyzon, Y., et al. (2013) Identification and rescue of α-synuclein toxicity in Parkinson patient-derived neurons. *Science*, **342**, 983–987.

Shahmoradian, S. H., Lewis, A. J., Genoud, C., Hench, J., Moors, T. E., Navarro, P. P., Castaño-Díez, D., Schweighauser, G., Graff-Meyer, A., et al. (2019) Lewy pathology in Parkinson’s disease consists of crowded organelles and lipid membranes. *Nat. Neurosci.*, **22**, 1099–1109.

Nuscher, B., Kamp, F., Mehnert, T., Odoy, S., Haass, C., Kahle, P. J. and Beyer, K. (2004) Alpha-synuclein has a high affinity for packing defects in a bilayer membrane: a thermodynamics study. *J Biol Chem*, **279**, 21966–75.

Sharma, K., Mehra, S., Sawner, A. S., Markam, P. S., Panigrahi, R., Navalkar, A., Chatterjee, D., Kumar, R., Kadu, P., et al. (2020) Effect of Disease-Associated P123H and V70M Mutations on β-Synuclein Fibrillation. *ACS Chem. Neurosci.*, **11**, 2836–2848.

Janowska, M. K., Wu, K.-P. and Baum, J. (2015) Unveiling transient protein-protein interactions that modulate inhibition of alpha-synuclein aggregation by beta-synuclein, a pre-synaptic protein that co-localizes with alpha-synuclein. *Sci. Rep.*, **5**, 15164.

Brown, J. W. P., Buell, A. K., Michaels, T. C. T., Meisl, G., Carozza, J., Flaggmeier, P., Vendruscolo, M., Knowles, T. P. J., Dobson, C. M. and Galvagnion, C. (2016) β-Synuclein suppresses both the initiation and amplification steps of α-synuclein aggregation via competitive binding to surfaces. *Sci. Rep.*, **6**, 36010.

Williams, J. K., Yang, X., Atieh, T. B., Olson, M. P., Khare, S. D. and Baum, J. (2018) Multi-Pronged Interactions Underlie Inhibition of α-Synuclein Aggregation by β-Synuclein. *J. Mol. Biol.*, **430**, 2360–2371.

Yang, X., Williams, J. K., Yan, R., Mouradian, M. M. and Baum, J. (2019) Increased Dynamics of α-Synuclein Fibrils by β-Synuclein Leads to Reduced Seeding and Cytotoxicity. *Sci. Rep.*, **9**, 17579.

Leitao, A., Bhumkar, A., Hunter, D., Gamin, Y. and Sierecki, E. (2018) Unveiling a Selective Mechanism for the Inhibition of α-Synuclein Aggregation by β-Synuclein. *Int. J. Mol. Sci.*, **19**, 334.

Dettmer, U. (2018) Rationally Designed Variants of α-Synuclein Illuminate Its in vivo Structural Properties in Health and Disease. *Front. Neurosci.*, **12**, 623.

Imberdis, T., Fanning, S., Newman, A., Ramalingam, N. and Dettmer, U. (2019) Studying α-Synuclein Conformation by Intact-Cell Cross-Linking. *Methods Mol. Biol. Clifton NJ*, **1948**, 77–91.

Baulac, S., LaVoie, M. J., Strahle, J., Schlossmacher, M. G. and Xia, W. (2004) Dimerization of Parkinson’s disease-causing DJ-1 and formation of high molecular weight complexes in human brain. *Mol. Cell. Neurosci.*, **27**, 236–246.

**Legends to Figures**
Figure 1: A, Schematic of aligned αS, βS, and γS aa sequences. Bottom, aa were aligned from N-terminus to C-terminus and demarcated with the up to ten repeat motifs. Aa highlighted in black are conserved with regard to the KTKEGV core repeat motif. Top, close-up of the region that is called ‘NAC domain’ for αS, with a special emphasis of aa 71-82 that had been reported to be essential for αS aggregation. βS is characterized by a deletion relative to αS and γS in this region, and we are displaying both an alignment with and without a gap. 

B, Schematic of aligned αS, βS, and γS aligned by repeat motif, color-coded. Analogous to A, but color-coded: blue indicates basic (light blue: histidine), red: acidic, purple: polar uncharged, black: non-polar, green: proline residues.
Figure 2: Left, Schematic of $\alpha$S, $\beta$S, and $\gamma$S aligned by the first seven repeat motifs. $\alpha$S, $\beta$S and $\gamma$S are depicted as a series of seven repeats R1-R7, and aa are color-coded. Blue indicates basic (light blue: histidine), red: acidic, purple: polar uncharged, and black: non-polar residues. Right, $\alpha$S helix formation at membranes ($\alpha$S, $\beta$S, $\gamma$S helical wheels embedded in membrane lipids). The formation of 3-11 helices (3 turns per 11 aa) at membranes is
driven by hydrophobic non-polar amino acids in αS that interact with fatty-acid tails of membrane lipids (large black curved lines), and by positively-charged lysine (K) residues that interact with negatively-charged lipid head groups of membrane lipids (large red circles). Arrows indicate aa substitutions relative to αS that are expected to lower membrane binding by either reducing electrostatic (yellow) or hydrophobic interactions (green).
### A

|     | wt      | 3K      | KLK     | EIV     | EGR     |
|-----|---------|---------|---------|---------|---------|
| αS  |         |         |         |         |         |
| 1   | MVEKMKL | MVEKMKL | MVEKMKL | MVEKMKL | MVEKMKL |
| 2   | SEKREGVA | SEKREGVA | SEKREGVA | SEKREGVA | SEKREGVA |
| 3   | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA |
| 4   | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG |
| 5   | SEKRCVHOFVA | SEKRCVHOFVA | SEKRCVHOFVA | SEKRCVHOFVA | SEKRCVHOFVA |
| 6   | SEKREKING | SEKREKING | SEKREKING | SEKREKING | SEKREKING |
| 7   | SEKRCAGSTA | SEKRCAGSTA | SEKRCAGSTA | SEKRCAGSTA | SEKRCAGSTA |
| 8   | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL |
| 9   | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL |
|     | MPSEEGYQDIVEEPA | MPSEEGYQDIVEEPA | MPSEEGYQDIVEEPA | MPSEEGYQDIVEEPA | MPSEEGYQDIVEEPA |

|     | wt      | 3K      | KLK     | EIV     | EGR     |
|-----|---------|---------|---------|---------|---------|
| βS  |         |         |         |         |         |
| 1   | MVEKMKF | MVEKMKF | MVEKMKF | MVEKMKF | MVEKMKF |
| 2   | SEKREGVA | SEKREGVA | SEKREGVA | SEKREGVA | SEKREGVA |
| 3   | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA |
| 4   | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG |
| 5   | SEKRCVQGAVA | SEKRCVQGAVA | SEKRCVQGAVA | SEKRCVQGAVA | SEKRCVQGAVA |
| 6   | SEKREKSQGASA | SEKREKSQGASA | SEKREKSQGASA | SEKREKSQGASA | SEKREKSQGASA |
| 7   | SEKREKLVLG | SEKREKLVLG | SEKREKLVLG | SEKREKLVLG | SEKREKLVLG |
| 8   | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL |
| 9   | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL |
|     | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE |

|     | wt      | 3K      | KLK     | EIV     | EGR     |
|-----|---------|---------|---------|---------|---------|
| γS  |         |         |         |         |         |
| 1   | MVEKMKF | MVEKMKF | MVEKMKF | MVEKMKF | MVEKMKF |
| 2   | SEKREGVA | SEKREGVA | SEKREGVA | SEKREGVA | SEKREGVA |
| 3   | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA |
| 4   | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA | SEKRCGAPA |
| 5   | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG | SEKRCGLYVG |
| 6   | SEKRCVQGAVA | SEKRCVQGAVA | SEKRCVQGAVA | SEKRCVQGAVA | SEKRCVQGAVA |
| 7   | SEKREKLVLG | SEKREKLVLG | SEKREKLVLG | SEKREKLVLG | SEKREKLVLG |
| 8   | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL |
| 9   | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL | SEKREKVEKQDL |
|     | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE | MPSEEGYDVPDQEE |

### B

#### B1

**Synuclein variant**

- **wt**: Wild type
- **3K**: 3-repeat
- **KLK**: Klonoic
- **EIV**: Eilev
- **EGR**: Egric

#### B2

**Predicted effect**

- **wt**: Wild type
- **3K**: 3-repeat
- **KLK**: Klonoic
- **EIV**: Eilev
- **EGR**: Egric

- **Synuclein variant**
  - wt
  - 3K
  - KLK
  - EIV
  - EGR

- **Predicted effect**
  - wt
  - 3K
  - KLK
  - EIV
  - EGR
Figure 3: A, Schematic of wt and strategic missense mutations of αS, βS, and γS. Sequences are aligned by their KTKEGV repeat motifs. Aa that fully conform to the KTKEGV repeat are highlighted in black. Aa in red are the strategic missense mutations that disrupt equilibria towards membrane-bound monomers. Aa in green are missense mutations that are expected to disrupt equilibria towards cytosolic monomers. B, Schematic of predicted changes in equilibria. Among cytosolic monomer, cytosolic multimer, and membrane-bound monomer for all synuclein species, 3K, KLK, and EIV are expected to strongly reduce cytosolic species levels (K interacts with phospholipid head, L and I interact with lipid tails), whereas EGR is predicted to remain largely cytosolic (R repels αS from membrane lipid tails).
Figure 4: WB of sequential protein extraction and quantification. All human synuclein homologs, wt and the indicated variants, were transiently transfected in M17D neuroblastoma cells. Cytosolic (PBS-soluble) and membrane proteins (TX-100-soluble) were separated by sequential extraction. Synucleins were detected using anti-FLAG (first and second WB rows, respectively). Controls for cytosolic and membrane fractions were GAPDH and Calnexin, respectively. WB is representative of N = 3 independent transfections on different days. Cytosol:membrane ratios were quantitated relative to αS wt, which was set to 1. We determined if cytosol:membrane ratios were significantly different vs. the respective wt. Graph shows mean data for N = 3 independent experiments and SEM. One-way ANOVA analysis, Tukey's multiple comparisons test, *, p<0.05; **, p<0.01; ***, p<0.001.
transfected M17D cells, 1 mM crosslinker DSG, PBS fraction

\[ \alpha_\text{S-FLAG}_3 \quad \beta_\text{S-FLAG}_3 \quad \gamma_\text{S-FLAG}_3 \]

|            | wt | 3K | KLK | EIV | EGR |
|------------|----|----|-----|-----|-----|
| \( \alpha_\text{S-FLAG}_3 \) |    |    |     |     |     |
| *          |    |    |     |     |     |
| ****       |    |    |     |     |     |
| ***        |    |    |     |     |     |

|            | wt | 3K | KLK | EIV | EGR |
|------------|----|----|-----|-----|-----|
| \( \beta_\text{S-FLAG}_3 \) |    |    |     |     |     |
| n.s.       |    |    |     |     |     |
| ****       |    |    |     |     |     |
| *          |    |    |     |     |     |

|            | wt | 3K | KLK | EIV | EGR |
|------------|----|----|-----|-----|-----|
| \( \gamma_\text{S-FLAG}_3 \) |    |    |     |     |     |
| n.s.       |    |    |     |     |     |
| ****       |    |    |     |     |     |
| *          |    |    |     |     |     |

---

\[ \text{FLAG (M2)} \]

- 188
- 97
- 62
- 49
- 38
- 28
- 17
- 14

**kDa**

---

**1-mer**

**2-mer**

**DJ-1**
Figure 5: WB and quantitation of αS60/80/100:αS14 ratios. Synuclein homologs and variants were transfected in M17D cells. Anti-FLAG WB after intact cell crosslinking (top). Blotting for DJ-1 served as a control (bottom). The ratios of Syn60/80/100 and Syn14 were normalized to αS wt and quantitated relative to each respective wt. Graph shows mean data for N=3 independent experiments performed on different days in duplicates (n=6) and SEM. One-way ANOVA analysis, Tukey's multiple comparisons test. *, p<0.05; ***, p<0.001.

Figure 6: A, Live-cell imaging of M17D cell confluence. M17D cells transfected with FLAG3-tagged wt, 3K, KLK, EIV, or EGR variants for all human synuclein homologs. Staurosporine-treated (a strong toxin, positive control, set to 0 viability), non-transfected, and vector-transfected cultures (both negative controls) are shown in the left column. Images were taken 48 hours post-transfection by IncuCyte live-cell imaging. Cells were identified and their confluence quantified by using a custom IncuCyte algorithm that identifies areas occupied by cells (displayed in orange). Images are representative of N=4 independent experiments performed in quadruplicates (n=16). Graph shows mean data and stdev. All statistics relative to vector only (red). One-way ANOVA analysis, Tukey's multiple comparisons test. *, p<0.05; **, p<0.01; ***, p<0.001. B, Assessing neuron integrity upon transfection of YFP-tagged synuclein variants. YFP-tagged wt, 3K, KLK, or EGR variants for all human synuclein homologs were
transfected into DIV14 rat cortical neurons. Transfection efficiency <5% allowed for assessing integrity of single transfected neurons 96 hours post transfection. After image acquisition and blinding, cells were categorized into “intact” and “disintegrated” and the relative percentage of intact neurons was calculated (N = 3 independent experiments, n = 2 transfected wells per experiment and variant, 36 fields per well). Graph shows mean data and stdev. All statistics relative to the respective wt variant. One-way ANOVA analyses, Tukey's multiple comparisons test. *, p<0.05; **, p<0.01; ****, p<0.0001.
A) transfected M17D neuroblastoma cells

mock  YFP

αS

βS

γS

B) transfected mouse neurons DIV13

αS::YFP

βS::YFP

γS::YFP

C) transfected rat neurons DIV14

αS

βS

γS

anti-FLAG antibody (M2)
Figure 7: Fluorescence microscopy images of cells transfected with wt and variants of synuclein homologs. 

A, M17D cells were transfected with YFP-tagged wt, 3K, and KLK variants for all homologs. YFP was also transfected as a control. Images were taken 48 hours post-transfection. B, DIV 13 primary mouse neurons were co-transfected with YFP-tagged variants for all homologs and RFP as a control. Images were taken 48 hours post-transfection. C, DIV 14 rat neurons were transfected with YFP-tagged variants for all homologs. Images were taken 48 hours post-transfection. All images are representative of N = 3 independent experiments done on different days.
A  
transfected M17D cells

|          | YFP       | αS-3K::YFP | βS-3K::YFP | γS-3K::YFP |
|----------|-----------|------------|------------|------------|
| +SCDi    | ![Image](#) | ![Image](#) | ![Image](#) | ![Image](#) |
| -SCDi    | ![Image](#) | ![Image](#) | ![Image](#) | ![Image](#) |

B  
flat, inclusion(-)

|          | YFP       | αS-3K::YFP | βS-3K::YFP | γS-3K::YFP |
|----------|-----------|------------|------------|------------|
| +SCDi    | ![Bar](#) | ![Bar](#) | ![Bar](#) | ![Bar](#) |
| -SCDi    | ![Bar](#) | ![Bar](#) | ![Bar](#) | ![Bar](#) |

flat, inclusion(+)

|          | YFP       | αS-3K::YFP | βS-3K::YFP | γS-3K::YFP |
|----------|-----------|------------|------------|------------|
| +SCDi    | ![Bar](#) | ![Bar](#) | ![Bar](#) | ![Bar](#) |
| -SCDi    | ![Bar](#) | ![Bar](#) | ![Bar](#) | ![Bar](#) |

rounded

|          | YFP       | αS-3K::YFP | βS-3K::YFP | γS-3K::YFP |
|----------|-----------|------------|------------|------------|
| +SCDi    | ![Bar](#) | ![Bar](#) | ![Bar](#) | ![Bar](#) |
| -SCDi    | ![Bar](#) | ![Bar](#) | ![Bar](#) | ![Bar](#) |

n.s. 
1.0 | 0.8 | 0.6 | 0.4 | 0.2 | 0.0

*** | *** | *** | *** | *** |

0.2 | 0.4 | 0.6 | 0.8 | 1.0

*** | *** | *** | *** | *** |

0.2 | 0.4 | 0.6 | 0.8 | 1.0

0.2 | 0.4 | 0.6 | 0.8 | 1.0

0.2 | 0.4 | 0.6 | 0.8 | 1.0

0.14 | * | * | * | * |

C  
transfected M17D cells

|          | YFP       | αS-3K::YFP | βS-3K::YFP | γS-3K::YFP |
|----------|-----------|------------|------------|------------|
| +SCDi    | ![Image](#) | ![Image](#) | ![Image](#) | ![Image](#) |
| -SCDi    | ![Image](#) | ![Image](#) | ![Image](#) | ![Image](#) |

62 38 28

kDa

YFP

GAPDH
**Figure 8:** Pretreating M17D cells with SCD inhibitor rescues cellular inclusion formation and toxicity. A, IncuCyte live-cell images of M17D cells transfected with either YFP alone, YFP-tagged αS-3K, βS-3K, or γS-3K after 48 hours. Another set of M17D transfectants was pretreated with SCD inhibitor MF-438 (10 µM) and also imaged. Arrows point at inclusions, arrowheads point at rounded cells. B, Quantification of the number of cells without inclusions (left graph), with inclusions (middle graph), and rounded (right graph) 48 hours post-transfection and with/without 24 h pretreatment of SCD inhibitor (y-axis: fraction). Graph shows mean data for N = 3 independent experiments (n = 8 each) and SEM. One-way ANOVA analysis (comparisons as indicated), Tukey's multiple comparisons test. *, p<0.05; **, p<0.01; ***, p<0.001. C, WB of M17D cells transfected with either YFP alone, YFP-tagged αS-3K, βS-3K, or γS-3K with/without pretreatment of SCD inhibitor. WB represents 3 independent experiments.

**Abbreviations**

aa, amino acid; αS, α-synuclein; βS, β-synuclein; DIV, days in vitro; γS, γ-synuclein; kDa, kilodalton; LDS, lithium dodecyl sulphate; mAb, monoclonal antibody; min, minutes; pAb, Polyclonal antibody; MW, molecular weight; PFA, paraformaldehyde; RFP, red fluorescent protein; RT, room temperature; YFP, yellow-fluorescent protein.