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Pre-fibrillar α-Synuclein Variants with Impaired ß-Structure Increase Neurotoxicity in Parkinson`s Disease Models

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1st Editorial Decision 16 April 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three reviewers, whose comments are attached below. As you will see, these referees acknowledge your comprehensive approach of structure-based rational design and subsequent pathogenicity analysis of alpha-synuclein mutants in principle. At the same time, however, they also each raise a number of substantive concerns with regard to the various different aspects of the study. While such an extensive list of criticisms and comments would under normal circumstances appear to preclude publication, I realize that it is in this case partially owed to the comprehensive nature of the study, with different issues arising for each of the different sections. In this light, I would therefore be inclined to nevertheless offer you the possibility to respond to the reviewers' criticisms in a revised version of the manuscript. With regard to the more major issues, the referees criticize that the pathophysiological relevance of the designed mutants is unclear, and that no new insights into toxicity mechanisms of the pre-fibrillar aggregates are offered - I realize that given the already very data-dense manuscript, an extension into this direction would probably be beyond the scope of the present study; however, what would certainly be needed to be addressed in some form is the related concern that aggregation characteristics of the designed mutants have not been determined in any of the relevant in vivo models (primary neurons, fly, worm), in order to fully support the conclusions on the proposed correlation. Other important issues to consider are referee 2's comments on the biophysical experiments, as well as the request for control of alpha-synuclein protein levels. Finally, it would clearly also strengthen the manuscript if you could provide some further insight into the specificity of mutant alpha-synuclein neurotoxicity, as suggested in referee 1's major point 5.

Should you feel confident that you might be able to adequately address these key points as well as, of course, the more specific technical issues, we should be happy to consider a revised manuscript further for publication. In this respect, please bear in mind that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you answer to all the points raised at this stage if you wish for the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Although pre-fibrillar a-synuclein species are widely thought to be the major toxic principle underlying a-synuclein-mediated neurotoxicity, there is a paucity of evidence supporting their direct association. Based on rational design, Karpinar and colleagues have generated several a-synuclein mutants that exhibit a propensity to form protofibrils, but a significantly reduced tendency to fibrillize into amyloidogenic aggregates. Using a variety of cell and animal models of Parkinson's disease (PD), the authors investigated the cytotoxicity of the various artificial a-synuclein mutants alongside the wild type protein and two disease-associated a-synuclein mutants. Their results support the notion that oligomeric a-synuclein species represent the major toxic principle in PD.

Overall, the study is logical and comprehensive. Importantly, the findings provide support for the role of a-synuclein protofibrils in PD pathogenesis. However, I do have some comments/suggestions/queries regarding the results in their present form, as discussed below.

1. Notwithstanding the utility of the various a-synuclein proline mutants used in the current study in addressing the toxicity of a-synuclein-generated protofibrils, the pathophysiological significance of these artificial mutants is debatable, as none of them are found in PD patients. Further, whilst the authors found that a-synuclein-induced cellular toxicity does not require the formation of visible a-synuclein aggregates, the formation of aggregates in A30P and A53T a-synuclein-expressing cells does not exactly mitigate cytotoxicity. Thus, it may be premature for the authors to suggest that the formation of beta-structure is not important for neurotoxicity (p. 16).

2. Other than the experiment involving HEK cells, no information regarding the relationship between aggregation and neurotoxicity is provided in all the other models examined. The caveat is that events that favor macromolecular crowding in vivo could precipitate a-synuclein aggregation. As the authors have pointed out, increasing the concentration of all a-synuclein variants accelerated their aggregation and amyloid formation (p. 5). Moreover, TP species could seed fibril formation of the wild type protein (Fig. 2A). Differences amongst the various a-synuclein species in generating aggregates in vivo must therefore be validated and shown and not assumed.

3. Fig. 6. Besides mRNA expression as measured by quantitative PCR, it would be informative to include an anti-a-synuclein immunoblot to show the relative levels of protein expression, as well as images showing the localization of the various a-synuclein species in transfected cells. Would pharmacological inhibition of proteasome function enhance aggregate formation (or cell death) in A56P and TP-expressing cells? Is the toxicity induced by A56P and TP significantly different from that induced by A30P?

4. It is unclear why the middle and right bar graphs shown in Fig. 7A are omitted of information

pertaining to wild type and A30P synuclein.

5. In the C. elegans experiment, do the authors observed loss of TH-positive cell bodies associated with the expression of a-synuclein mutants? Is the neurite defect restricted to dopaminergic neurons in the worm if a pan-neuronal promoter is used? Similarly for the Drosophila experiment, i.e. would pan-neuronal expression of a-synuclein proline mutants direct selective dopaminergic neurodegeneration? Another caveat associated with transgenic animal overexpressing a-synuclein is that a-synuclein could regulate TH expression.

6. Show statistics for Fig. 8A. Without which, the authors cannot state that "transgenic worms expressing the A56P or TP aS variant....are more impaired...than worms expressing wt aS or the genetic variants (A30P and A53T) (p. 10).

7. All the proline mutants generated apparently could stabilize synuclein in its protofibrillar forms and generate marked neurotoxicity. What about proline mutations in other regions of the protein (other than A30P)?

Minor comments

1. Fig 1. Showing A53T and A30P fibrillization rate in Fig. 1B will allow for better correlation to be made with Fig. 1C.

2. Indicate the identity of the various a-synuclein variants on the electron micrographs shown in Fig. 3A

3. Expression of a-synuclein variants in Drosophila model is missing in Fig. S6.

Referee #2 (Remarks to the Author):

In a clever approach Karpinar et al. designed α-S mutants that should fail to form "amyloid" fibrils because of introduction of secondary structure incompatible proline residues in key regions of the protein. Indeed, the novel synthetic α-S mutants show greatly reduced propensity to form ThioT-positive material in vitro. The authors claim that instead "pre-fibrillar" species accumulate, and remarkably these mutants show cytotoxicity as do PD-associated α-S mutants without visible aggregate formation. The toxicity of the novel α-S mutants is confirmed in primary neuron cultures, and in vivo using two different invertebrate model organisms. α-S toxicity without fibril formation is indeed a very interesting finding, but the assumption of toxic "pre-fibrillar" α-S species requires more rigid demonstration. The molecular mechanism(s) of neurotoxicity remain completely unknown.

Major concerns:

1. The so-called "pre-fibrillar oligomers" are incompletely characterized. The in vitro experimental methods and figure legends are often too short to follow.

The dot blot experiment using the A11 anti-oligomer is insufficiently described. How were M and O forms of TP S separated (Fig. 2B)? How do control dot blots look for wt α-S?

The DLS experiments shown in Fig. 3B could reveal the initial formation of larger (≈100nm) species, probably oligomers. However, there appears no difference between any of the α-S variants studied. Perform DLS after >200h incubation. Does the ≈100nm species accumulate for TP S, in contrast to wt α-S, in which this peak might be consumed in favor of larger fibril sizes?

AFM would help to visualize the analyzed α-S species.

It is very confusing that after several days' incubation EM-visible fibrils do form with A56P and TP α-S (wt control missing in Fig. 4A). Identical fibril dimensions were calculated from the ssNMR experiment shown in Fig. 4B. What does that mean?? This confusing issue is not discussed at all.

The novel α-S mutants form identical fibrils as does wt α-S, only differing by reduced ThioT-labeling in vitro? If this is somehow due to decreased ß-sheet content, then this must be proven by CD spectroscopy of the aggregates, and if available FTIR spectroscopy.

2. Even more importantly, how are the aggregation characteristics of the novel α-S mutants in vivo? The HEK293T cell quantifications in Fig. 6B look impressive. However, the aggregation

results are at odds with the apparent fibril formation shown in Fig. 4. Do PD-associated mutants form thioflavin-positive aggregates in cells, whereas the synthetic mutants do not?
α-S stainings, aggregate counts and thioflavin reactivities are completely missing in all in vivo validations (Fig. 7, 8). Without exhaustive α-S aggregate characterizations in vivo, no conclusions about toxic "pre-fibrillar" α-S species are justified.

Additional comments:
3. The α-helical conformational changes of α-S upon lipid binding is curiously refractory to the insertion of single helix-breaking prolines, as in the case of the extensively studied A30P α-S. Even with such apparent structural flexibility, it is quite surprising that introduction of 3 prolines in TP α-S should have no effect at all on lipid binding. Reduced vesicle binding has been discussed as one possible pathogenic mechanism for the PD-associated A30P α-S. This aspect should be addressed with greater care for the novel mutants. α- S binding to SDS micelles is certainly most practical for NMR spectroscopy, but reflects the native situation far from perfectly (see Bussell&Eliezer, 2004; Ulmer&Bax, 2005). The experiment in Fig. S4A must be performed with acidic SUVs, or even better synaptic vesicles. Using such more relevant lipid formulations, small but significant reductions of A30P α-S binding were observed in the literature. Even in the present study using SDS micelles, one could make out in the original data reduced -helical content based on the decreased ellipticities of TP α-S (Fig. S4A).
In fact, demonstration of reduced vesicle binding might support the authors' hypothesis if the novel α-S mutants would bind less efficiently to vesicles, possibly raising the soluble pool of the hypothetical toxic α- S species.
4. In general alternative toxicity mechanisms must be considered and discussed more openly, especially in the invertebrate organisms lacking endogenous synuclein. The present study appears heavily sided towards the assumption of toxic non-fibrillar α- S species, which of course may be true, but the hypothesis would benefit from a more objective assessment.

Minor corrections
5. Page 5: Describe Fig. 1B and S1 more clearly in the Results. Lag times could only be determined for A56P α-S (yellow trace in Fig. 1B), for all other synthetic α-S mutants this seems not possible because no ThioT plateau was reached. Fibril elongation rates (probably meaning the slopes at inflection point of the time courses) again could be determined from the provided data only in the case of A56P α-S. What is not mentioned and discussed, although appears the most remarkable finding, is the reduced ThioT fluorescence (plateau level).
6. Page 5: TP α-S oligomers were able to seed fibril formation... strictly, if understood correctly, the seeding preparation contained >95% monomers and <5% oligomers. How were these numbers calculated from "quantitative analysis of the NMR signal decay" data, and what are the values for wt α-S? How significant is the increase with time from 2% to 4% of the "oligomeric fraction" for TP α-S?
The described seeding effect shown in Fig. 2A is minimal and no significance values are given. No seeding of endogenous α-S is reported for the primary neuron cultures, was this determined at all?
7. Page 6 ..."heterogeneous distribution of high molecular weight species" should read "larger species". More DLS measurements should be performed (see point 1). In addition size exclusion chromatography would be informative for this key point.
8. Page 6: Annotate and specify the missing cross-peaks in Fig. 5A.
9. Page 7: Western blot comparison of equal protein expression would be preferable to mRNA quantifications, because the effects are mediated most likely at the protein level.
10. Page 9: Mention that the α-S variants were driven by DA neuron specific promoters. Does pan-neuronal expression mediate selective DA neurodegeneration, or is the observed neurotoxicity non-selective?
Line 4 correct "visible"
11. Figures 3 and 4 should be merged. Label mutants in Fig. 3A (same as in 3B below?).
12. Page 29: Correct legend to Fig. 5 to: In (A) and (B), homonuclear...
13. Fig. 7B, y-axis: "worms lacking neurites" or "worms with neurite defects"
14. Fig. 9 can be omitted, the hypothesis is clear enough.
15. Fig. S3A there is no bottom panel (this figure has evidently been moved to Fig. S4A).

Referee #3 (Remarks to the Author):

Review on Karpinar et al.:
"Pre-fibrillar α--Synuclein Variants with Impaired ß-Structure increase Neurotoxicity in Parkinson's Disease Models"

In their study, Karpinar et al. used a structure-based rational design to generate a-synuclein mutants with reduced aggregation behaviour. To this purpose, two alanine residues located within the b-sheet rich core of a-synuclein fibrils were replaced by proline. These mutants (A56P, A76P and TP) were analyzed in comparison to wild type synuclein and the two pathogenic mutants A53T and A30P to delineate the relationship between oligomerization, fibril formation and neurotoxicity in different model systems, including HEK cells, primary neuronal cultures, C. elegans and Drosophila melanogaster.
The authors describe the aggregation behavior of the different a-synuclein variants by elegant and convincing in vitro techniques, however, the in vivo data is not conclusive and does not provide new insight into the mechanism of a-synuclein toxicity.

1. The criteria for the classification of aggregation and toxicity shown in Fig. 6 B are not clear, nor are any information's provided regarding assays used to determine toxicity, cell death/viability. For examples, how are "preapototic cells" defined? Examples of the phenotypes should be shown in addition to bar graphs. In addition, Western blots of the a-synuclein variants rather than mRNA expression levels should be shown to prove comparable protein levels (Fig. 6 A).

2. From the observation that a-synuclein mutants that display toxicity in vivo have a lower propensity to form fibrils in vitro the authors draw the conclusion that oligomers rather than fibrils are responsible for toxicity observed in different models for a-synucleinopathies. This is a plausible concept, which has been demonstrated for a variety of aggregation-prone proteins in various model systems. The authors failed to provide a direct link between in vivo structure and in vivo toxicity and they did not address some important aspects which may help to understand differences in the toxic capacity of a-synuclein variants, such as subcellular localization, turn over rates, membrane binding characteristics, clearance mechanisms, detergent solubility...

3. The authors need to discuss their results in context of the findings of the Lindquist group who in contrast to the present study demonstrated toxicity of wt a-synuclein in the same in vivo models (primary neurons, C. elegans, Drosophila). Can the toxicity induced by the designed a-synuclein mutants be rescued by Rab1? This question would be important to address as a lack of Rab1 rescue activity could indicate that the mechanism of toxicity might be different for the artificial a-synuclein mutants.

4. The authors mention that the pathogenic E46K a-synuclein mutant is less able to form preamyloid oligomers than wt a-synuclein. To address the question whether oligomerization of a-synuclein is indeed correlated to toxicity, it would be advisable to include the E46K mutant in their in vitro and in vivo studies.

1st Revision - authors' response 15 July 2009

Reviewer 1: Notwithstanding the utility of the various a-synuclein proline mutants used in the current study in addressing the toxicity of a-synuclein-generated protofibrils, the pathophysiological significance of these artificial mutants is debatable, as none of them are found in PD patients. Further, whilst the authors found that a-synuclein-induced cellular toxicity does not require the formation of visible a-synuclein aggregates, the formation of aggregates in A30P and A53T a-synuclein-expressing cells does not exactly mitigate cytotoxicity. Thus, it may be premature for the authors to suggest that the formation of beta-structure is not important for neurotoxicity (p. 16).

Reply: The design of the study was motivated by the fact that overexpression of wild-type, genetic mutants, phosphorylation mimics and truncation of aS did so far not provide a clear understanding of the relationship between the process of α-S aggregation and disease progression in animal

models for Parkinson's disease. Thus, we took a biophysical approach to strongly modify the aggregation process of a-synculein and study these mutants in animal models of PD. The suggestion that the formation of beta-structure is not important for neurotoxicity was based on our finding that the neurotoxic effects observed in the four model systems was inversely correlated with the amount of beta-structure observed by solid-state NMR in late-stage aggregates of the aS variants. The big problem with the genetic mutations of aS is that their aggregation and fibrillar properties are too similar to those of wt aS, making it difficult to understand the connection between aggregation and neurotoxicity. However, we agree with the reviewer that we cannot exclude that formation of beta-structure at least contributes to neurotoxicity. Therefore, we have modified the statement on page 20: "Thus formation of rigid ß-structure might not to be as important for neurotoxicity as previously thought."

Reviewer 1: Other than the experiment involving HEK cells, no information regarding the relationship between aggregation and neurotoxicity is provided in all the other models examined. The caveat is that events that favor macromolecular crowding in vivo could precipitate a-synuclein aggregation. As the authors have pointed out, increasing the concentration of all a-synuclein variants accelerated their aggregation and amyloid formation (p. 5). Moreover, TP species could seed fibril formation of the wild type protein (Fig. 2A). Differences amongst the various a-synuclein species in generating aggregates in vivo must therefore be validated and shown and not assumed.

Reply: We have now studied the aggregation of wt and TP α-S in C.elegans. The new experiments demonstrate that - by using similar concentrations of S variants fused to monomeric YFP citrine - wild type α-S forms large insoluble aggregates while TP α-S stays soluble and shows a diffuse staining throughout the muscle cytoplasm (Fig. 6). The new data are described on page 11. Thus there is a clear difference in the aggregation propensities between wild type and TP α-S in vivo, strongly supporting our main conclusion of the manuscript that the protofibrilar form of S displays increased neuronal toxicity and that molecular crowding that occurs in all cells does not alter the in-vitro observe aggregation behaviour in vivo.

Reviewer 1: Fig. 6. Besides mRNA expression as measured by quantitative PCR, it would be informative to include an anti-a-synuclein immunoblot to show the relative levels of protein expression, as well as images showing the localization of the various a-synuclein species in transfected cells. Would pharmacological inhibition of proteasome function enhance aggregate formation (or cell death) in A56P and TP-expressing cells? Is the toxicity induced by A56P and TP significantly different from that induced by A30P?

Reply: Thanks for this suggestion. Immunoblot analysis of the relative levels of protein expression in all model systems is now shown in Fig. 5, 6 and Supp. Fig. 10 showing equal expression levels of all mutants with a tendency towards higher level for the TP mutant. Immunohistochemical detection specifically of AAV-expressed human aSYN and variants demonstrates identical subcellular distribution of all variants: the proteins were found to be evenly distributed throughout the cytoplasm, and within neurites exhibited a granular staining pattern, according to a presumed localization in vesicular structures. This is now shown in Supp. Fig. 11 and mentioned in the manuscript on page 12. Proteasomal inhibition was not tested. Toxicity induced by A56P and TP is significantly different from that induced by A30P (HEK cells: Fig. 5/ primary neurons: Fig. 7 / C.elegans: Fig. 7 / Drosophila: Fig. 7).

Reviewer 1: It is unclear why the middle and right bar graphs shown in Fig. 7A are omitted of information pertaining to wild type and A30P synuclein.

Reply: Thanks for the suggestion. The data for wild type and A30P synuclein were included into Fig. 7A.

Reviewer 1: In the C. elegans experiment, do the authors observed loss of TH-positive cell bodies associated with the expression of a-synuclein mutants? Is the neurite defect restricted to dopaminergic neurons in the worm if a pan-neuronal promoter is used? Similarly for the Drosophila experiment, i.e. would pan-neuronal expression of a-synuclein proline mutants direct selective dopaminergic neurodegeneration? Another caveat associated with transgenic animal overexpressing a-synuclein is that a-synuclein could regulate TH expression.

Reply: In our experiments we used a dopaminergic neuron specific dat-1 promoter to drive expression of α-S variants exclusively in the eight dopaminergic neurons of C. elegans. This was done, because the dopaminergic neurons are not required for the viability of the animal and thus their degeneration can be studied without affecting the fitness of C. elegans. Furthermore, a clear behavioral phenotype have been linked to the dopaminergic neurons allowing to assay directly their functionality in addition to the morphological changes linked to α-S expression. We have also expressed α-S under the control of a pan-neuronal promoter and observed degeneration of a large set of different neurons leading to sick animals. However, this general sickness of the animals precluded a detailed study of the neurodegenation since the cause of the neuodegeneration of a particular neuron cannot be unambiguously established. However, we do see degeneration of neurons other than the dopaminergic system. This has clearly been stated in the text now (pages 12-13). This suggests that α-S expression induces general neurodegeneration irrespective of the neuron type. This also suggests that the neuronal toxicity of α-S cannot be linked to regulation of TH expression or function. Furthermore, it has been shown that mutations in cat-2, which encodes the dopamine biosynthetic enzyme tyrosine hydroxylase in C. elegans, affect dopamine levels that in turn affect locomotory slowing in response to food. However, no morphological changes or degeneration of dopaminergic neurons has been reported in cat-2 mutants (Sawin et al., 2000, Neuron). Thus it is unlikely that the neurodegenation induced by α-S expression would be caused by TH dysregulation.

Reviewer 1: Show statistics for Fig. 8A. Without which, the authors cannot state that "transgenic worms expressing the A56P or TP aS variant....are more impaired...than worms expressing wt aS or the genetic variants (A30P and A53T) (p. 10).

Reply: We fully agree. The statistics for Fig. 8A are now shown.

Reviewer 1: All the proline mutants generated apparently could stabilize synuclein in its protofibrillar forms and generate marked neurotoxicity. What about proline mutations in other regions of the protein (other than A30P)?

Reply: We based our design on the conformational properties of the α-S monomer in solution and on the topology of S fibrils known from previous NMR measurements (Bertoncini et al., 2005; Heise et al., 2005; Vilar et al., 2008). The genetic mutation A30P is located in a region of S that is statically disordered in amyloid fibrils (Heise et al., 2005). To interfere with aggregation, we moved the single proline mutation found in the genetic A30P mutant to a position that is part of the ß-sheet rich core of α-S fibrils (Figure 1A) comprising approximately residues 38-100. From the region 38-100, we selected the alanine residues 56 and 76 as they are characterized by relatively large residual dipolar coupling values in the soluble monomer, suggestive of a rigid nature (Bertoncini et al., 2005). This is discussed on pages 4 and 16.
Thus, we expect that other proline mutations in the region 38-100 have a similar effect as the selected ones (A56P, A76P). In contrast, proline mutations in the region 1-38 are likely to have similar aggregation kinetics as A30P, i.e. quite comparable to the wild type protein, and differences in aggregation kinetics with respect to the wild type protein will strongly depend on solution conditions. Thus, as in case of the genetic variant A30P, it will be very difficult to correlate aggregation kinetics in vitro and in vivo with neurotoxicity. Similarly, proline mutations in the acidic C-terminal region (residues 101-140) are not likely to strongly influence the aggregation process, as the C-terminal region remains highly flexible in amyloid fibrils of α-S. However, without the use of the structural knowledge there would be many possibilites for placing the proline mutations, precluding a detailed characterization in the different model systems.

Reviewer 1: Fig 1. Showing A53T and A30P fibrillization rate in Fig. 1B will allow for better correlation to be made with Fig. 1C.

Reply: Thanks for this suggestion. We have now preformed aggregation assays for all aS variants at protein concentrations of 0.8 mM. The data are shown in Fig. 1D and discussed on pages 5-6.

Reviewer 1: Indicate the identity of the various a-synuclein variants on the electron micrographs shown in Fig. 3A

Reply: Done.

Reviewer 1: Expression of a-synuclein variants in Drosophila model is missing in Fig. S6.
Reply: This is shown now in Supp. Fig. 10.

Reviewer 2: The so-called "pre-fibrillar oligomers" are incompletely characterized. The in vitro experimental methods and figure legends are often too short to follow. The dot blot experiment using the A11 anti-oligomer is insufficiently described. How were M and O forms of TP aS separated (Fig. 2B)? How do control dot blots look for wt aS?

Reply: Unfortunately, the manuscript is already very data dense and we were somehow limited by the available space provided by EMBO J (55000 characters). We tried to extend the legends where possible and added many details to the experimental methods part (pages 21-26 and Supporting Material).
Monomeric and oligomeric forms of TP aS were not separated. To better reflect this, we changed in Fig. 2 the label of the fraction that contains oligomeric TP to O/M. This is now more clearly described in the Supplementary material on page 16.
The control dot blot below shows that the A11 antibody does not stain wild-type monomeric aS. However, it stains aggregated wt aS (when the ThT fluorescence signal has reached saturation), as at this stage both oligomeric and fibrillar wt aS are present. After pelleting and extensive washing of the fibrils of wt aS, the staining by A11 is no longer detectable, in agreement with the absence of oligomeric wt aS.

Reviewer 2: The DLS experiments shown in Fig. 3B could reveal the initial formation of larger (100nm) species, probably oligomers. However, there appears no difference between any of the aS variants studied. Perform DLS after >200h incubation. Does the 100nm species accumulate for TP aS, in contrast to wt aS, in which this peak might be consumed in favor of larger fibril sizes?

Reply: Thanks for this suggestion. For the revised version of the manuscript, we have performed DLS, AFM, EM and UV absorbance measurements of the supernatant, obtained at the end stage of aggregation (after 11 days of incubation). The new data are shown in Figure 3 and discussed on pages 6 and 7 in the paragraph "aS variants have an increased propensity to from soluble oligomers". The combined data show that A56P and in particular TP aS have an increased propensity to form soluble oligomers. However, the dynamic radii of the oligomers are similar for all mutants.

Reviewer 2: AFM would help to visualize the analyzed aS species.

Reply: We have obtained AFM images for TP aS after it had been aggregated for 11 days at a concentration of 0.8 mM (i.e. strongly increased to our previous measurements, in which the aS concentrations were mostly 0.1 mM). AFM shows oligomers and fibrils (Fig. 3B). Interestingly, many of the oligomers appear in close spatial proximity to fibrils in agreement with EM images (Fig. 3A and 3B). The new data are now discussed on pages 5-6.

Reviewer 2: It is very confusing that after several days' incubation EM-visible fibrils do form with A56P and TP aS (wt control missing in Fig. 4A). Identical fibril dimensions were calculated from the ssNMR experiment shown in Fig. 4B. What does that mean? This confusing issue is not discussed at all. The novel aS mutants form identical fibrils as does wt aS, only differing by reduced ThioT-labeling in vitro? If this is somehow due to decreased b-sheet content, then this must be proven by CD spectroscopy of the aggregates, and if available FTIR spectroscopy.

Reply: Maybe it was confusing that the ThT aggregation assay was done at 0.1 mM protein concentration and up to 2 weeks (Fig. 1B), whereas for preparation of the solid-state NMR sample TP aS was aggregated for 4 weeks at a concentration of 0.2 mM. For the revised version of the

manuscript, we have now performed the ThT aggregation assay for all aS variants at a concentration of 0.8 mM (now shown in Fig. 1D). At the end stage of this aggregation assay, the morphological properties of all aS variants were checked by EM (shown in Supp Fig. S2). In agreement, with our previous EM images of the solid-state NMR samples, all aS variants can form fibrils if the concentration is just high enough and the incubation time long enough. According to EM, the fibrils of all aS variants look similar (now shown in Supp. Fig. S2). This is also true for EM images of TP aS, although the EM images of TP aS also show a lot of oligomers that appear to be attached to the fibrils (Fig. 3A and 3B). Thus, the similar diameter of the fibrils estimated by solid-state NMR measurements (Fig. 4A) is in full agreement with the EM images.

We attribute the reduced ThioT-labeling mainly due a reduced concentration of amyloid fibrils in case of A56P and in particular in case of TP aS. This is in agreement with the observation that wt, A30P and A53T samples had a very gel-like behaviour after 6 days of aggregation, whereas the samples of A56P and TP were more liquid-like. In addition, we cannot exclude that the affinity of Thio-T to the fibrils of A56P and TP aS is lower than to the wild-type protein, potentially caused by the conformational exchange visible for the fibriallar core in the solid-state NMR spectra (Fig. 4). This is discussed in more detail in the revised version of the manuscript on page 6.

CD spectroscopy and FT-IR spectroscopy only provide an estimation of the secondary structure of all protein species in solution and are therefore most useful for globular proteins. Thus, monomeric, oligomeric and fibrillar protein contributes to the signal. In addition, these techniques do not provide residue-specific information making a distinction between ordered and disordered regions difficult. In contrast, solid-state NMR spectroscopy can select only rigid molecules by using cross-polarization. In addition, it provides residue-specific information and is therefore by far superior to CD and FT-IR.

Reviewer 2: Even more importantly, how are the aggregation characteristics of the novel aS mutants in vivo? The HEK293T cell quantifications in Fig. 6B look impressive. However, the aggregation results are at odds with the apparent fibril formation shown in Fig. 4. Do PD-associated mutants form thioflavin-positive aggregates in cells, whereas the synthetic mutants do not? α-S stainings, aggregate counts and thioflavin reactivities are completely missing in all in vivo validations (Fig. 7, 8). Without exhaustive aS aggregate characterizations in vivo, no conclusions about toxic "pre-fibrillar" α-S species are justified.

Reply: We have now studied the aggregation of wt and TP α-S in C.elegans. The new experiments demonstrate that - by using similar concentrations of S variants fused to monomeric YFP citrine - wild type α-S forms large insoluble aggregates while TP α-S stays soluble and shows a diffuse staining throughout the muscle cytoplasm (Fig. 6). The new data are described on page 11. Thus there is a clear difference in the aggregation propensities between wild type and TP α-S in vivo, strongly supporting our main conclusion of the manuscript that the protofibrilar form of S displays increased neuronal toxicity and that in vitro aggregation properties are reproduced in vivo.

Reviewer 2: The a-helical conformational changes of aS upon lipid binding is curiously refractory to the insertion of single helix-breaking prolines, as in the case of the extensively studied A30P aS. Even with such apparent structural flexibility, it is quite surprising that introduction of 3 prolines in TP aS should have no effect at all on lipid binding. Reduced vesicle binding has been discussed as one possible pathogenic mechanism for the PD-associated A30P aS. This aspect should be addressed with greater care for the novel mutants. aS binding to SDS micelles is certainly most practical for NMR spectroscopy, but reflects the native situation far from perfectly (see Bussell&Eliezer, 2004; Ulmer&Bax, 2005). The experiment in Fig. S4A must be performed with acidic SUVs, or even better synaptic vesicles. Using such more relevant lipid formulations, small but significant reductions of A30P aS binding were observed in the literature. Even in the present study using SDS micelles, one could make out in the original data reduced a-helical content based on the decreased ellipticities of TP aS (Fig. S4A). In fact, demonstration of reduced vesicle binding might support the authors' hypothesis if the novel aS mutants would bind less efficiently to vesicles, possibly raising the soluble pool of the hypothetical toxic α-S species.

Reply: Thanks for this suggestion. We have now quantified the amount of aS bound to phospholipid vesicles using gel filtration and CD spectroscopy. The new data are presented in Table 1, Supp. Fig. 8 and Supp. Table S1, and discussed in the paragraph "Membrane binding characteristic of aS variants" on pages 8-9. The experiments demonstrate that A30P and TP aS have a similar affinity for phospholipid vesicles as A30P aS. Indeed, the vesicle-affinity of A56P aS is slightly higher than

that of A30P aS, suggesting that the increased neurotoxicity of A56P and TP aS is not due to a change in membrane binding.

Reviewer 2: In general alternative toxicity mechanisms must be considered and discussed more openly, especially in the invertebrate organisms lacking endogenous synuclein. The present study appears heavily sided towards the assumption of toxic non-fibrillar aS species, which of course may be true, but the hypothesis would benefit from a more objective assessment.

Reply: We agree with the reviewer that we cannot exclude, that the design mutants interfere with some other mechanism aS is involved in. Indeed, this was discussed in the discussion section of the original version of the manuscript. In this discussion, we had made some comments to two potential mechanisms, notably membrane binding and protein-protein interactions. Based on the new experiments, which show that A56P and TP have a similar binding affinity for phospholipid vesicles as A30P aS (Table S1 and Supp. Fig. S8), we have now extended this discussion (pages 18-19). A56P aS has even a slightly higher affinity than A30P aS to membranes, suggesting that the increased neurotoxicity of the design mutants is not due to a modified membrane affinity. We feel that a solid discussion of other potential toxic mechanisms of aS is probably more appropriate for a review article.

Reviewer 2: Page 5: Describe Fig. 1B and S1 more clearly in the Results. Lag times could only be determined for A56P aS (yellow trace in Fig. 1B), for all other synthetic aS mutants this seems not possible because no ThioT plateau was reached. Fibril elongation rates (probably meaning the slopes at inflection point of the time courses) again could be determined from the provided data only in the case of A56P aS. What is not mentioned and discussed, although appears the most remarkable finding, is the reduced ThioT fluorescence (plateau level).

Reply: Thanks for pointing this out. We have now preformed aggregation assays for all aS variants, which were also tested in animal models for PD, at protein concentrations of 0.8 mM. The data are shown in Fig. 1D and discussed in detail on pages 5-6. In addition, we have performed additional DLS, EM, AFM and UV absorbance measurements (as suggested by the reviewer). The new measurements show that A56P and in particular TP aS from a larger amount of oligomers (please see above and Figure 3).

Reviewer 2: TP aS oligomers were able to seed fibril formation... strictly, if understood correctly, the seeding preparation contained >95% monomers and <5% oligomers. How were these numbers calculated from "quantitative analysis of the NMR signal decay" data, and what are the values for wt aS? How significant is the increase with time from 2% to 4% of the "oligomeric fraction" for TP aS? The described seeding effect shown in Fig. 2A is minimal and no significance values are given.

Reply: The NMR signal decay for all aS variants including error estimates based on independent repeat measurements are shown in Fig. 1C. Similarly, the data shown for seeding of wt aS by oligomers of TP aS were obtained from three to four independent experiments and the standard deviations are shown as error bars. In addition, we describe the procedure now in more detail in the methods section of the Supporting Material (page 16): "To follow the decrease in concentration of monomeric S during aggregation, 0.5 ml samples containing 0.1 mM S in 50 mM Na-phosphate, 100 mM NaCl, 0.1% NaN3, pH 7.4 and 90 % H2O / 10 % D2O were incubated at 37 ∞C and stirred by 5x2 mm stirring bars inside a standard NMR tube. At appropriate time intervals, 1D 1H spectra were measured and the decay in signal intensity was plotted as a function of time. The drop in signal intensity during aggregation is due to formation of higher molecular weight aggregates not detectable by solution-state NMR. Thus, the NMR signal intensity remaining during the course of the aggregation allows estimation of the concentration of monomeric protein. Simultaneously performed EM measurements, which were performed in the early stages of the aggregation, only showed small oligomeric species and no amyloid fibrils. In addition, no increase in ThioT signal compared to the monomeric protein was detected during the lag phase. Thus, the reduction of NMR signal intensity during the lag phase of fibril formation allows estimation of the concentration of soluble oligomers. In case of the NMR aggregation assay performed for TP S at a concentration of 0.1 mM (Figure 1C), no amyloid fibrils were detected during the complete time course of the experiment, indicating that the reduction in signal intensity is solely due to formation of soluble oligomers. Errors in the estimation of the oligomer concentration depend on the basis of the signal-to-noise ratio in the NMR spectra and are determined from the variation observed in three

independently performed aggregation assays. In case of TP S, they were ±2%."

Reviewer 2: No seeding of endogenous aS is reported for the primary neuron cultures, was this determined at all?

Reply: This was not determined. We agree with the reviewer that there are many more interesting experiments that can be done with the design mutants in the future.

Reviewer 2: Page 6 ..."heterogeneous distribution of high molecular weight species" should read "larger species". More DLS measurements should be performed (see point 1).

Reply: Corrected. Regarding the additional DLS measurements, please see our comments above (new Fig. 3 and additional text on page 7 in revised version of the manuscript).

Reviewer 2: In addition size exclusion chromatography would be informative for this key point.

Reply: This was actually one of the first things we had done, but the early-stage oligomers of A56P and TP aS were not stable on the column.

Reviewer 2: Annotate and specify the missing cross-peaks in Fig. 5A.

Reply: We agree with the reviewer and explicitly state in the revised paper which cross peaks are missing in the spectrum of the A56P mutant. We additionally detect changes in chemical shifts. Both aspects are now included in the revised discussion on page 8.

Reviewer 2: Page 7: Western blot comparison of equal protein expression would be preferable to mRNA quantifications, because the effects are mediated most likely at the protein level.

Reply: Thanks for the suggestion. Western blot comparison of equal protein expression in all model systems are now shown in Figs. 5, 6 and Supp. Fig. 10.

Reviewer 2: Page 9: Mention that the aS variants were driven by DA neuron specific promoters. Does pan-neuronal expression mediate selective DA neurodegeneration, or is the observed neurotoxicity non-selective?

Reply: In our experiments we used a dopaminergic neuron specific dat-1 promoter to drive expression of α-S variants exclusively in the eight dopaminergic neurons of C. elegans. This was done, because the dopaminergic neurons are not required for the viability of the animal and thus their degeneration can be studied without affecting the fitness of C. elegans. Furthermore, a clear behavioral phenotype have been linked to the dopaminergic neurons allowing to assay directly their functionality in addition to the morphological changes linked to α-S expression. We have also expressed α-S under the control of a pan-neuronal promoter and observed degeneration of a large set of different neurons leading to sick animals. However, this general sickness of the animals precluded a detailed study of the neurodegeneration since the cause of the neuodegeneration of a particular neuron cannot be unambiguously established. However, we do see degeneration of neurons other than the dopaminergic system. This has clearly been stated in the text now (pages 12-13). This suggests that α-S expression induces general neurodegeneration irrespective of the neuron type.

Reviewer 2: Line 4 correct "visible"
Figures 3 and 4 should be merged. Label mutants in Fig. 3A (same as in 3B below?).
Page 29: Correct legend to Fig. 5 to: In (A) and (B), homonuclear...
Fig. 7B, y-axis: "worms lacking neurites" or "worms with neurite defects"
Fig. 9 can be omitted, the hypothesis is clear enough.
Fig. S3A there is no bottom panel (this figure has evidently been moved to Fig. S4A).

Reply: Thanks for spotting these mistakes. The corrections were made, except for merging of Fig. 3

and 4, as Fig. 4 is more connected to Fig. 5, but Fig. 5 is already very big.

Reviewer 3: The criteria for the classification of aggregation and toxicity shown in Fig. 6 B are not clear, nor are any information's provided regarding assays used to determine toxicity, cell death/viability. For examples, how are "preapototic cells" defined? Examples of the phenotypes should be shown in addition to bar graphs. In addition, Western blots of the a-synuclein variants rather than mRNA expression levels should be shown to prove comparable protein levels (Fig. 6 A).

Reply: As suggested by the referee, expression levels of alpha-synuclein were now quantified with an anti-a-synuclein immunoblot in addition to qPCR (Fig. 5). Examples of cells with aggregates and preapoptotic cells are now shown in Fig. 5D. Preapoptotic cells are characterized by rounding and contained large, amorphous aggregates of EGFP. Imaging showed that they had lost stress fibers and focal adhesions, but maintained membrane integrity. Time-lapse imaging showed that this appearance was followed by the formation of apoptotic bodies (Opazo et al. 2008). The results section was rephrased (page 10) and the method section (pages 22-23) expanded to include a detailed description of the way aggregates and toxicity were determined in HEK293 cells:
" To visualize α-S variants in living cells, we have recently established and validated a method that labels α-S variants with EGFP through the specific interaction between a PDZ binding motif and its PDZ domain (Opazo et al., 2008). The advantage of this method is that only a 6 amino acid PDZ binding motif is added to the α-S C-terminus and not the entire EGFP protein. In our hands, the appearance of cells transfected with the same construct varied greatly, making it difficult to determine a "typical" appearance and compare α-S variants based on this. We therefore chose to manually classify cells into four broad groups: "homogenous", "with a single aggresome", "with many aggregates" or "preapoptotic", and compare the relative frequencies of these appearances. With this approach, we have previously investigated the differences between WT, A30P, A53T, a C-terminally deleted α-S, the effects of HSP70 coexpression, inhibition of proteasome, autophagy and lysosomal degradation (Opazo et al., 2008). Preapoptotic cells are characterized by rounding and contained large, amorphous aggregates of EGFP. Imaging showed that they had lost stress fibers and focal adhesions, but maintained membrane integrity. Time-lapse imaging showed that this appearance was followed by the formation of apoptotic bodies (Opazo et al., 2008). Cells "with a single aggresome" contained one clearly visible, round aggregate of EGFP but appeared otherwise healthy. Staining showed a basket of vimentin and gamma-tubulin around the aggregate, characterizing it as an aggresome. Time-lapse imaging showed that small, peripheral aggregates were often transported towards the aggresome (Opazo et al., 2008)."

Reviewer 3: From the observation that a-synuclein mutants that display toxicity in vivo have a lower propensity to form fibrils in vitro the authors draw the conclusion that oligomers rather than fibrils are responsible for toxicity observed in different models for a-synucleinopathies. This is a plausible concept, which has been demonstrated for a variety of aggregation-prone proteins in various model systems. The authors failed to provide a direct link between in vivo structure and in vivo toxicity and they did not address some important aspects which may help to understand differences in the toxic capacity of a-synuclein variants, such as subcellular localization, turn over rates, membrane binding characteristics, clearance mechanisms, detergent solubility...

Reply: We have now studied the aggregation of wt and TP α-S in C.elegans. The new experiments demonstrate that - by using similar concentrations of α-S variants fused to monomeric YFP citrine - wild type α-S forms large insoluble aggregates while TP α-S stays soluble and shows a diffuse staining throughout the muscle cytoplasm (Fig. 6). The new data are described on page 11. Thus there is a clear difference in the aggregation propensities between wild type and TP α-S in vivo, strongly supporting our main conclusion of the manuscript that the protofibrilar form of S displays increased neuronal toxicity. In addition, we show now by dynamic light scattering, atomic force microscopy, electron microscopy and UV absorbance that the design mutants cause strongly increased accumulation of soluble oligomers at later stages of aggregation (Fig. 3 and page 7). Furthermore we have determined the membrane binding characteristics of all aS variants. Finally, immunohistochemical detection specifically of AAV-expressed human aS and variants demonstrates identical subcellular distribution of all variants: the proteins were found to be evenly distributed through cytoplasm, and within neurites demonstrated a granular staining pattern, according to a

presumed localization in vesicular structures.

Reviewer 3: The authors need to discuss their results in context of the findings of the Lindquist group who in contrast to the present study demonstrated toxicity of wt a-synuclein in the same in vivo models (primary neurons, C. elegans, Drosophila). Can the toxicity induced by the designed a-synuclein mutants be rescued by Rab1? This question would be important to address as a lack of Rab1 rescue activity could indicate that the mechanism of toxicity might be different for the artificial a-synuclein mutants.

Reply: Our starting hypothesis was that α-S, which is only able to form protofibrils is more toxic for neurons than α-S that is able to form fibrils. In order to be able to compare and detect an increased toxicity we used α-S expression levels for our experiments at which wild type α-S does not cause strong neurodegeneration. This is cearly stated now in the Material and Methods section (page 24). However, in our own experiments we also detected similar strong degeneration of dopaminergic neurons by increasing wild type α-S expression levels. However, our main objective was to compare the degeneration induced by different α-S variants at a given concentration.
As far as the rescue of the α-S induced neurodegeneration by Rab1 expression is concerned, we do not think that this experiment would contribute to our analysis of the different α-S variants. It would clearly go beyond the scope of the paper especially since the exact function of Rab1 in C. elegans and Drosophila has not been studied so far. Although, we agree that it would be exciting to determine the exact mechanism leading to α-S induced toxicity we strongly feel tat this is beyond the scope of this paper which links S protofibrils to neurodegeneration and thus represents a first step towards a mechanistic characterization process. We therefore think that the TP α-S might a valuable tool for the scientific community to address the exact mechanism leading to α-S induced neurodegeneration in the future.

Reviewer 3: The authors mention that the pathogenic E46K a-synuclein mutant is less able to form preamyloid oligomers than wt a-synuclein. To address the question whether oligomerization of a-synuclein is indeed correlated to toxicity, it would be advisable to include the E46K mutant in their in vitro and in vivo studies.

Reply: This is beyond the scope of this study. It has previously been demonstrated that the E46K a-synuclein mutant has similar aggregation kinetics as wt, A30P and A53T a-synuclein, i.e. much faster aggregation than our design mutants. Investigation of E46K a-synuclein in the four model systems is definitely interesting, but is a study by itself.

Decision letter 30 July 2009

Thank you for submitting your revised manuscript for our consideration. We have now heard back from our three referees, and I am happy to inform you that all of them consider the manuscript adequately improved in response to their original comments, and have no further objections to its publication (remaining comments of referee 1 are attached below for your information only). We shall therefore be pleased to accept your manuscript for publication in The EMBO Journal!

You will receive a formal acceptance letter shortly.

Yours sincerely,

Editor
The EMBO Journal

Referee 1 (comments to authors):

In the revised version, the authors have added key experimental results in support of their main finding that protofibrillar forms of a-synuclein represent the major toxic principle underlying a-synuclein-mediated neurotoxicity. They have also modified their text, annontate their figures better, and expanded on their discussion in response to the suggestions that I have made. On the whole, the authors have done a satisfactory job in addressing my previous concerns. In my opinion, the revised manuscript represents a significantly improved version of the original. I am content with the revisions and have no further comments.

