

## Alpha-synuclein aggregates activate calcium pump SERCA leading to calcium dysregulation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

12 July 2017

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Thank you for the transfer of your manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, while referee 1 is more positive, referee 2 raises important concerns that preclude publication of the study here at this point. Both referees note that the interaction between aggregated a-synuclein and SERCA is not sufficiently convincing, and referee 1 adds that the biological significance of the findings remains unclear, in addition to several other points.

Given these substantial concerns, the amount of work required to address them, the uncertain outcome of these experiments, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from the referees, I am sorry to say that we cannot offer to publish your manuscript.

I am sorry to have to disappoint you this time. I nevertheless hope that the referee comments will be helpful in your continued work in this area, and I thank you once more for your interest in our journal. EMBO reports

### REFeree REPORTS

Referee #1:

The manuscript entitled "Alpha-synuclein aggregates activate SERCA leading to calcium

dysregulation" is an interesting and novel study. Its primary strength is the thorough biochemical and cellular demonstrations of AS-induced enhancement of SERCA function. The authors also make a strong case that this regulation has implications for neurotoxicity partial SERCA inhibition with low dose CPA alleviates the buildup of AS aggregates and subsequent neuronal damage in vitro. Apart from some minor concerns listed below that focus primarily on data presentation and analysis, I find the data generally to be very convincing. That said, while the authors make a great case for AS-mediated enhancement of SERCA, exactly what happens at later stages is left a little vague. The authors imply that inhibition of NF- $\kappa$ B under low calcium conditions could contribute to this phenotype, however, they have not actually demonstrated that CPA enhances NF- $\kappa$ B activation or that NF- $\kappa$ B activation is actually related to this latter phase of neurodegeneration. Such a demonstration could increase the impact of this study considerably.

#### Comments:

1. The data in figure 3 does reveal binding of AS and SERCA by PLA, however, at a very low efficiency relative to the degree of colocalization of SERCA and AS. The authors should comment on the efficiency of PLA and/or other potential reasons that SERCA and AS would interact with such low efficiency.
2. The authors state that CPA has no effect on SERCA-AS interaction based on PLA, however, there is no quantitation to assess this. Without quantitation, this is an overstatement and all that has been demonstrated is that CPA does not completely block this interaction. Quantitation would be appropriate here.
3. Figure 7 shows distinct patterns between GRP78 and SERCA. Since these are both ER proteins, some discussion is needed.

#### Referee #2:

In this manuscript, the authors suggest that intracellular alpha-synuclein (ASYN) aggregates bind to SERCA, acting as a positive allosteric modulator, resulting in a modest lowering of cytosolic Ca<sup>2+</sup> concentration. This conclusion is based upon an impressive array of approaches in three in vitro model systems. The authors go on to claim that this modest drop in cytosolic Ca<sup>2+</sup> concentration caused by ASYN modulation of SERCA triggers a delayed rise in cytosolic Ca<sup>2+</sup> and neurodegeneration. Although the data are of interest and potential significance, I have a number of major concerns about the biological relevance of the data and the broader significance to the pathology in Parkinson's disease.

#### Major concerns:

- The conclusions of the paper are based entirely upon over-expression of ASYN in in vitro model systems. As a consequence, it is difficult to know whether the interaction of ASYN aggregates with SERCA is biologically meaningful or not. The dose-response data shown in Figure 4, is not reassuring as 50-100  $\mu$ g/ml is a high concentration; although it is difficult to know what is physiologically meaningful, high nanomolar should be sufficient.
- The work fails to distinguish between ASYN species that have different pathogenic properties. Although the authors repeatedly state that ASYN oligomers are what is binding to SERCA, they don't distinguish between oligomers and larger species (ribbons/fibrils). As recent work has shown (Helwig et al, 2016) (Mao et al, 2016), this is an important distinction. Moreover, the authors don't distinguish between ribbons and fibrils, which recent work suggests have very different consequences for neuronal toxicity (Peelaerts et al, 2015).
- The generation of ASYN aggregates in situ is not actually characterized as a function of the staging of pathology. Are the oligomeric species generated during the early phase (lower cytosolic Ca<sup>2+</sup>) different from the species observed at later time points (higher cytosolic Ca<sup>2+</sup>)? For example, the ICC shown in Fig 3 is done at 5 days of induction, but no data is shown on the later phase.
- The connection between the positive modulation of SERCA function and the late rise in Ca<sup>2+</sup> concentration and degeneration is tenuous at best. Why should a modest lowering of cytosolic Ca<sup>2+</sup> trigger degeneration? At present, the whole interpretation of the data hinges upon the effects of CPA, as intracellular ASYN aggregates have been shown by many (including the authors) to have a wide range of effects. Because CPA specificity is not clear, an alternative approach needs to be used.

to make this point; the authors need to specifically disrupt the interaction between ASYN and SERCA, leaving other consequences of ASYN on cellular function intact. Site-directed mutagenesis of SERCA would be one strategy that could be used, but there are others. Alternatively, there are small molecular positive allosteric modulators of SERCA function that should in principal have the same effect as ASYN over-expression.

- Does ASYN modulation of SERCA elevate ER Ca<sup>2+</sup> content? The transient analysis doesn't answer this question. The authors can probably quantify the steady state Ca<sup>2+</sup> levels from the aequorin experiments already done. The two possible outcomes are that ER Ca<sup>2+</sup> is increased during the early phase, in which case the subsequent toxicity might be due to stronger Ca<sup>2+</sup> release upon stimulation; the second possible outcome is that ER Ca<sup>2+</sup> is not affected, in which case there must be compensatory mechanisms in play. If ER Ca<sup>2+</sup> loading increases during the early phase, it could trigger the opening of the recently described TMCO1 channel (Wang et al, Cell, 2016), which would subsequently increase cytosolic Ca<sup>2+</sup> levels.
- The authors should show the effect of 0.5uM CPA on SERCA Ca<sup>2+</sup> dynamics: does CPA treatment actually normalize the rate of SERCA uptake of Ca<sup>2+</sup>? (and possibly ER Ca<sup>2+</sup> levels?) Does a mild activation of SOCE contribute to the increase cytosolic Ca<sup>2+</sup> levels?
- The light level co-localization assays are essentially meaningless. These do not provide the resolution needed to make the claim that ASYN and SERCA are bound to one another. The PLA assay is fine, but again is limited by the relevance of the dosing.
- In the context of PD, the relevance of the cell models employed is very limited, since they don't recapitulate the main features of the neurons mostly affected in PD. In vulnerable neurons, the consequences of modulating SERCA activity could be very different.
- Lastly, the discussion of the relationship between ASYN pathology and neurodegeneration needs revision. First, the relationship between Lewy pathology and neurodegeneration is weak, with degeneration in the substantia nigra preceding the appearance of Lewy pathology. Moreover, many neurons with Lewy pathology appear to be unaffected for decades. This relationship has been recently reviewed (Surmeier et al. 2017). This makes the relationship between ASYN pathology and neurodegeneration much less clear than the authors would like. It is possible that oligomers, which precede the formation of ribbons/fibrils, could be toxic and work through the mechanism described. However, this needs to be worked out more clearly.
- The reliance upon parametric statistics for display and hypothesis testing is not justified.

Minor points:

- Lexical /semantic repetitions in the abstract (see first two lines: crucial/fundamental, compromised/compromised) and in the introduction (line 63-64, patients...patients...);
- Please correct the statement that says that the only pacemaking neurons in the midbrain are in substantia nigra (line 64). Or that other neurons that are vulnerable in PD are not pacemakers - many clearly are.
- How are the previously described effects of ASYN on release of Ca<sup>2+</sup> from intracellular stores related to the present results?

Author Comments

17 July 2017

We received your negative response to our paper "Alpha-synuclein aggregates activate SERCA leading to calcium dysregulation" (EMBOR-2017-44617V1) and have now considered how the decline was justified. You are of course not obliged to enter any discussion but I will like to highlight a few things that may make you reconsider the decision. Basically, I will encourage you to send the manuscript to another referee knowledgeable in cell biology instead of referee 2 that likely is a biophysicist. The evaluation by referee 1 is sound.

This paper describes a novel prodegenerative cellular state characterized by reduced cytosolic calcium i) that is triggered by aggregated Alpha-synuclein, ii) likely is caused by a direct interaction between Alpha-synuclein aggregates and the ER calcium pump SERCA and iii) can be counteracted in a neuroprotective fashion by a SERCA antagonist. In the field of molecular intracellular Alpha-synuclein pathology this is "interesting and novel" as stated by ref 1.

Your key concern is "Both referees note that the interaction between aggregated Alpha-synuclein and SERCA is not sufficiently convincing". I humbly disagree with this conclusion.

Referee 1 simply ask for comments on why the proximity ligation assay (PLA) displays such a low signal as compared to the apparently large colocalization of SERCA and Alpha-synuclein epitopes revealed by conventional immunofluorescence light microscopy. We do in lines 167-172 describe that immunofluorescence microscopy cannot be used for evaluating molecular interactions as compared to PLA but we can of course elaborate further on the efficiency and quantify the PLA signals as asked for in comments 1, 2.

Apart from that referee 1 states that it is a bit vague what signalling is downstream of the reduced cytosolic calcium. This is true and reflects the biology of a reduced cytosolic calcium is novel. We can easily test the putative effect on NFkB signalling that is proposed.

Referee 2 does in fact not specifically address the interaction. Rather this person, which likely may be one of my collaborators that is a biophysicist, focuses on discrediting the work by asking for data that cannot be generated in any labs at present, and by raising concern on issues that basically are unjustified.

The main concern is regarding the kind of aggregated species generated and acting in our live-cell models, (bullets 2, 3). In the title we state "aggregates" in order not to oversimplify matters by stating any specific species. Referee 2 find it concerning we don't distinguish between oligomers and larger species like ribbons and fibrils. The term "ribbons and fibrils" in this context refers to entirely in vitro generated structures that was first described in a paper where I was co-author (Bousset et al, Nat. Comm. 2013). Later Dr. Melki's lab has generated novel preformed fibrils at different pH that hypothetically may reflect assemblies formed in different acid cellular compartments. This is just to illustrate the many potential in vitro assemblies that one could try to test for. The papers referred to addresses in vivo inter-neuronal transfer of native Alpha-synuclein (Helwig 2016) and in vitro binding of preformed Alpha-synuclein fibrils to cell cultures (Mao et al. 2016) and not toxic species acting within the cells. The cell models we use have previously been thoroughly investigated by many labs to study aggregation-dependent processes in live cells but never have insoluble fibril-like structures been identified and characterized herein. These models likely recapitulate the slow human degenerative process by modelling the early-phase soluble Alpha-synuclein species, which we designate as oligomers. We make a strong case on demonstrating the active structures of Alpha-synuclein oligomers are i) shared with insoluble preformed Alpha-synuclein fibrils, ii) rely on the C-terminus of Alpha-synuclein and not some shared amyloid type structure (lines 191-199, 355-366, 416-423).

Secondly referee 2 repeatedly states the lowering of cytosolic calcium is modest, thus implying of dubious significance. No one knows the consequences of chronically lowered cytosolic calcium, because it never has been described! Hence a lowering of cytosolic calcium of 13-17% cannot be considered modest. We clearly demonstrate it depends on Alpha-synuclein aggregation and counteracting it protects the cells so our paper opens for further studies of how this reduced calcium is involved in cytopathology.

It is asked if Alpha-synuclein affects ER calcium in our models? We did indeed study this as proposed by referee 2 but did not observe any effect. We did not show these data (but can do it) because it wasn't a surprise considering the cytosolic calcium is lowered around 20 nM and the ER concentration of calcium is around 1 mM. However, a prolonged pumping of calcium into the ER will likely at some point induce a degree of ER calcium overload with potential activation and tetramerization of TMCO1 channel (Wang et al., Cell, 2016. This we could address using chemical crosslinking.

Bullet 7: Indeed the light microscopic co-localization is meaningless as the referee points out and that is why we in line 167-168 writes "However, it is not possible using conventional immunofluorescence microscopy to determine whether the antigens actually interact". We merely demonstrate this in order to motivate the PLA technique that clearly demonstrates the aggregate-dependent interaction (see comment to referee 1). If this is confusing, as also suggested by referee 1, we can simply remove the immunofluorescence images.

Bullet 8: The statement "In the context of PD, the relevance of the cell models employed is very limited, since they don't recapitulate the main features of the neurons mostly affected in PD" is completely implausible. The degeneration described by Braak and now actively studied in the context of prion-like spreading indeed affects several neuron classes based on topology and neurotransmitters. Hence our demonstration of a basic mechanism relying on Alpha-synuclein and the ubiquitous calcium pump SERCA does indeed have potential of being involved in all affected cells.

I have not point-by-point addressed referee 2's concerns because this text already is quite lengthy but will be happy if you find it motivated. Basically I find this manuscript to be reviewed by specialists in cell biology.

Additional Correspondence

24 August 2017

Thank you for the submission of your proposed point-by-point response. I think that it looks reasonable and we would thus like to invite you to revise your manuscript with the understanding that all that the referee concerns must be fully addressed and their suggestions taken on board.

Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. I would also like to add that I can by no means predict the outcome of the peer-review process of the revised manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 8 main figures, I suggest that you layout the manuscript as a full article.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Advisor's comments (Referee #3):

I've now reviewed the paper, the original referees' reports, and the rebuttal. As you say, the two reviews are starkly contrasting.

I would favor allowing the author to provide a full revision which addresses Reviewer 1 and most of Reviewer 2's concerns. I find some of #2's concerns over the top and unnecessarily harsh, but some can be dealt with experimentally. The issue of 'dose' is an important one, and it will be worthwhile for the authors to describe the various levels of over-expression in their models. Often Tgic models

are 1.5 to 2x higher, so perhaps they are not that far off.

My own comments to add:

Most of the experiments are very carefully done and well controlled for, and the supposition that Ca<sup>2+</sup> lowering and then elevation in response to AS impact on SERCA is a novel observation that opens up new avenues for investigation and possibly therapy.

Considerations:

The last paragraph in the introduction is a big leap but reasonable to hypothesize.

The extent of over-expression in the various models should be described. Also, the amount of p25alpha overexpression required.

For the P-Serca studies can additional assays be done to confirm the impact of AS on SERCA dephosphorylation.

Fig 5- the CPA treatment does not appear to reduce AS-dependent cell loss by 40%, but more like 15%.

Fig 5EF - loss of Ag staining does not mean the cells are dead - different assays should be used for cell viability or another stain to control for that possibility.

Fig 7 - The attempt is to show some in vivo relevance to PD patients, which is reasonable. However, the figure as shown is difficult to interpret.

1st Revision - authors' response

24 November 2017

Please find our point-to-point response to referees and expert advisor in the following highlighted in **bold**.

Comments from advisor:

Most of the experiments are very carefully done and well controlled for, and the supposition that Ca<sup>2+</sup> lowering and then elevation in response to alpha-synuclein (AS) impact on SERCA is a novel observation that opens up new avenues for investigation and possibly therapy.

Considerations:

The last paragraph in the introduction is a big leap but reasonable to hypothesize.

**We will leave the paragraph. Indeed it is a big leap but reasonable to hypothesize as the advisor states. Our in vitro data support the hypothesis and it is further corroborated by the new clinical data in Fig. 7A, B demonstrating interactions between AS and SERCA in frontal cortex from patient with dementia with Lewy bodies but not in occipital cortex from controls, where no Lewy body pathology is present. Hence we believe our manuscript will form the basis for future experimental and clinical studies.**

The extent of over-expression in the various models should be described.

**To describe the extent of AS overexpression, we have included western blotting analysis of AS in lysates from the different cell models at specific time points used in the study and compared them to total brain lysates from a 4 months old C57BL/6 mouse. The data is included as supplemental figure S1.**

Also, the amount of p25alpha overexpression is required.

**The rational for using p25alpha expression in the OLN model is our observation that low amounts of p25alpha induced the in vitro aggregation of AS (Lindersson et al., 2004, JBC). The OLN model was described in 2009 (Kragh et al., 2009, JBC). Here we did a time course**

experiment and showed that the p25alpha level at 12 h post transfection was approx. 25 % of the level at 24 h when maximal microscopic degeneration scored as microtubule retraction was achieved. It should be pointed out that the cells only slowly starts to apoptose after 24-72 h (Kragh 2009) so the levels at 8 h and 12 h are far from being overt cytotoxic. In the present paper, we demonstrate the reduced Ca<sup>2+</sup> level already after 8 h. At this early time point an AS aggregate-dependent phenotype is activated as previously demonstrated by expression of the inhibitor of NF-κB activation iKB1a (Kragh et al., 2014, Neurobiol. Dis). The precise level of p25alpha after 8h has not been quantified but it is lower than 25 % of the maximal level achieved after 24 h.

For the P-Serca studies can additional assays be done to confirm the impact of AS on SERCA dephosphorylation.

**We have elaborated the data on the dephosphorylation rates of SERCA by showing the time resolution of dephosphorylation and including the calculated rate constants for SERCA in absence of AS, and in the presence of monomer or oligomer.**

Fig 5- the CPA treatment does not appear to reduce AS dependent cell loss by 40%, but more like 15%.

**In Fig. 5B, the AS dependent reduction in cell numbers (compared to beta-gal expressing cells) in the untreated cells is about 30 % compared to 15 % in the CPA treated cells. This was the reason we stated CPA reduced the loss by about 40 %. However, it may add to the confusion when we next in Fig. 5F states the AS dependent degeneration in primary neurons is 40 %. Here was no loss in the treated cells so the loss was in essence reduced by 100 %. In order to reduce confusion, we state that the AS dependent cell loss in the cell line model was reduced significantly and the loss in the primary neuron cultures was completely blocked.**

Fig 5EF - loss of Ag staining does not mean the cells are dead - different assays should be used for cell viability or another stain to control for that possibility.

**We agree that the absence of NeuN stain does not mean that cells are dead. Our neuronal cultures also contain glial cells so standard markers for viability assays cannot be used. Instead, we have immunostained for another neuron specific marker, MAP-2, and quantified the loss of MAP-2 positive cells in addition to the NeuN quantification (Fig S7). By NeuN quantification, we found a 40% loss of Ag positive cells, whereas the MAP-2 quantification revealed a loss of 30%. Both measures revealed that CPA treatment protects against the Ag loses. We have changed the wording in the figure from “Relative viability” to “Relative neuron loss”. When the cultures lose 30-40% of cells expressing two neuron markers then we find it justified to claim the neurons are lost.**

Fig 7 - The attempt is to show some in vivo relevance to PD patients, which is reasonable. However, the figure as shown is difficult to interpret.

**Referees 1 and 2 also raise this comment. We agree the figure was difficult to interpret. Hence we have completely redone the figure and added novel clinical data. The new Fig. 7A, B demonstrates by AS-SERCA PLA that AS and SERCA interacts in brain tissue affected by Lewy body pathology, but not in control tissue. Secondly, Fig. 7C demonstrates by differential extraction that SERCA in MSA brain tissue co-fractionate with the detergent insoluble AS aggregates in contrast to control patient where no AS and SERCA is present in the fraction. Finally to demonstrate where in AS aggregate inclusions, SERCA is present, we demonstrate the immunofluorescence microscopic analysis of isolated inclusions from PD, DLB and MSA that also was present in the former Fig. 7. Especially, the PLA analysis opens up for further clinical studies of patient cohorts, where the presence of AS-SERCA interactions may show novel brain areas being engaged prior to full blown Lewy body pathology. This is discussed in the Discussion.**

Referee #1:

The manuscript entitled "Alpha-synuclein aggregates activate SERCA leading to calcium dysregulation" is an interesting and novel study. Its primary strength is the thorough biochemical and cellular demonstrations of AS-induced enhancement of SERCA function. The authors also make

a strong case that this regulation has implications for neurotoxicity as partial SERCA inhibition with low dose CPA alleviates the buildup of AS aggregates and subsequent neuronal damage in vitro. Apart from some minor concerns listed below that focus primarily on data presentation and analysis, I find the data generally to be very convincing. That said, while the authors make a great case for AS-mediated enhancement of SERCA, exactly what happens at later stages is left a little vague.

The authors imply that inhibition of NF- $\kappa$ B under low calcium conditions could contribute to this phenotype, however, they have not actually demonstrated that CPA enhances NF- $\kappa$ B activation or that NF- $\kappa$ B activation is actually related to this latter phase of neurodegeneration. Such a demonstration could increase the impact of this study considerably.

**In order to clarify this issue, we confirm our previous finding showing that aggregation of AS induced by p25a in the OLN model leads to increased nuclear translocation of NF- $\kappa$ B, which is aggregate dependent because the aggregate inhibitor ASI-1D reduces this effect (Fig S6). Inhibition of SERCA by CPA reduces the nuclear translocation of NF- $\kappa$ B suggesting the reduced cytosolic Ca<sup>2+</sup> contributes to the NF- $\kappa$ B stress (Fig. S6). In addition, we analyzed the AS protein level (Fig 5F), and found that aggregate inhibitor ASI-1D do not change the AS level, whereas CPA reduces it by approx. 10%. If this modest reduction in total AS preferentially targets aggregated AS then it could explain the approximately 50% reduction in AS-SERCA interaction we observe by the PLA technique in CPA treated cells (Fig 5E). We have discussed this in the manuscript.**

Comments:

1. The data in figure 3 does reveal binding of AS and SERCA by PLA, however, at a very low efficiency relative to the degree of co-localization of SERCA and AS. The authors should comment on the efficiency of PLA and/or other potential reasons that SERCA and AS would interact with such low efficiency.

**Referee 1 and also referee 2 are critical for the apparently large co-localization of SERCA and AS epitopes revealed by conventional immunofluorescence microscopy and the low amount of interaction between the two as determined by PLA. We have removed the conventional fluorescence microscopy images of AS and SERCA because it indeed is inconclusive with respect to molecular interactions. We now focus on the PLA technique. The PLA signals in Fig. 3, Fig. 5, and Fig 7 are quantified as suggested.**

2. The authors state that CPA has no effect on SERCA-AS interaction based on PLA, however, there is no quantitation to assess this. Without quantitation, this is an overstatement and all that has been demonstrated is that CPA does not completely block this interaction. Quantitation would be appropriate here.

**We thank the referee for this comment. Having quantified the PLA signal (Fig. 5E) we could demonstrate CPA reduced the interaction by 50%. This formed the basis for the further investigating CPA's effect on total AS levels (Fig. 5F) that revealed a small but significant reduction. If this 10% reduction primarily affects aggregated AS then it may explain the 50% reduction in AS-SERCA interaction revealed by the PLA technique. This is discussed in the manuscript.**

3. Figure 7 shows distinct patterns between GRP78 and SERCA. Since these are both ER proteins, some discussion is needed.

**Please see above for comment to specialist advisor on Fig. 7.**

**Referee #2:**

In this manuscript, the authors suggest that intracellular alpha-synuclein (AS) aggregates bind to SERCA, acting as a positive allosteric modulator, resulting in a modest lowering of cytosolic Ca<sup>2+</sup> concentration. This conclusion is based upon an impressive array of approaches in three in vitro model systems. The authors go on to claim that this modest drop in cytosolic Ca<sup>2+</sup> concentration caused by AS modulation of SERCA triggers a delayed rise in cytosolic Ca<sup>2+</sup> and



neurodegeneration. Although the data are of interest and potential significance, I have a number of major concerns about the biological relevance of the data and the broader significance to the pathology in Parkinson's disease.

Major concerns:

1. The conclusions of the paper are based entirely upon over-expression of AS in in vitro model systems. As a consequence, it is difficult to know whether the interaction of AS aggregates with SERCA is biologically meaningful or not. The dose-response data shown in Figure 4, is not reassuring as 50-100  $\mu\text{g/ml}$  is a high concentration; although it is difficult to know what is physiologically meaningful, high nanomolar should be sufficient.

**We acknowledge the problems associated with overexpression of AS but this is what is used in almost the entire literature of mechanistic studies on AS pathophysiology. More “true” models with lower expression levels have been presented recently where wild type mouse neurons are challenged with application of supramolecular levels of either in vitro preformed AS fibrils, or brain extracts from human patients, or end stage transgenic mice. In order to clarify the issue of using models of AS over-expression, we now show the level of AS in the different models used, showing that the progressive OLN model has the highest level of AS (2x AS amount compared to total brain extract from a 4 months old C57BL/6 wt mouse) (Fig S1).**

**Our biochemical experiments are merely used to demonstrate that AS aggregates acquire novel functions compared to monomers with respect to activation of SERCA. This is a procedure often used to obtain significant signals that subsequently can be studied in more detail. The paper by Shrivastava (2015 EMBO J) uses approx. 14  $\mu\text{g/ml}$  oligomeric AS on the outside of neurons to demonstrate a functional interaction with the  $\alpha 3\text{-Na}^+/\text{K}^+\text{-ATPase}$ .**

2. The work fails to distinguish between AS species that have different pathogenic properties. Although the authors repeatedly state that AS oligomers are what is binding to SERCA, they don't distinguish between oligomers and larger species (ribbons/fibrils). As recent work has shown (Helwig et al., 2016) (Mao et al., 2016), this is an important distinction. Moreover, the authors don't distinguish between ribbons and fibrils, which recent work suggests have very different consequences for neuronal toxicity (Peelaerts et al., 2015).

**The main concern is regarding the kind of aggregated species generated and acting in our live-cell models, (bullets 2, 3). In the title we state “aggregates” in order not to oversimplify matters by stating any specific species. The cell models we use have previously been thoroughly investigated by many labs to study aggregation-dependent processes in live cells, but never despite trying hard have insoluble fibril-like structures been identified and characterized herein. These models likely recapitulate the slow human degenerative process by modeling the early-phase soluble AS species, which we designate oligomers. We make a strong case on demonstrating the active structures of AS oligomers that can activate SERCA are i) shared with insoluble preformed AS fibrils, ii) rely on the C-terminus of AS and iii) not some shared property with general amyloid type structure (lines 191-199, 355-366, 416-423).**

**Referee 2 find it concerning we don't distinguish between oligomers and larger species like ribbons and fibrils. The term “ribbons and fibrils” in this context refers to entirely in vitro generated structures that was first described in a paper where I was co-author (Bousset et al., Nat. Comm. 2013). Later Dr. Melki's lab has generated novel preformed fibrils at different pH that hypothetically may reflect assemblies formed in different acid cellular compartments. This is just to illustrate the many potential in vitro assemblies that one could try to test for in cell models but as stated above we don't have evidence for any insoluble aggregates. The papers referred to address in vivo inter-neuronal transfer of native AS (Helwig et al., 2016) and in vitro binding to cell cultures of preformed AS fibrils (Mao et al. 2016). Both are extracellular events and does not address how toxic species act within cells.**

3. The generation of AS aggregates in situ is not actually characterized as a function of the staging of pathology.

**We acknowledge this point but will emphasize the manuscript isn't a clinical that will require proper patient cohorts. We consider our data as proof of concept that AS aggregates within cells can bind SERCA and this will motivate further clinical investigations using our novel AS-**

**SERCA PLA technique. We have performed the PLA on human frontal cortex from one DLB case with positive LB pathology and compared to occipital cortex of an age matched neurological healthy case with no LB pathology (Fig 7EF). We observe negligible amounts of PLA signal in the controls in contrast to robust signal in the patient tissue, supporting that the interaction between AS and SERCA is a marker for a-synucleinopathy.**

Are the oligomeric species generated during the early phase (lower cytosolic  $\text{Ca}^{2+}$ ) different from the species observed at later time points (higher cytosolic  $\text{Ca}^{2+}$ )? For example, the ICC show in Fig 3 is done at 5 days of induction, but no date is shown on the later phase.

**As described above we have removed the conventional fluorescence microscopy images, as they may confuse more than make our hypothesis clearer. We tried immunostaining with the novel aggregate specific MJF14 antibody, but encountered a problem of AS independent staining in the SH-SY5Y cell line and could therefore not detect any changes in antigen levels or subcellular locations. It should be emphasized the field is lacking tools to detect and quantify cell generated AS aggregates that isn't fibrillar in nature.**

4. The connection between the positive modulation of SERCA function and the late rise in  $\text{Ca}^{2+}$  concentration and degeneration is tenuous at best. Why should a modest lowering of cytosolic  $\text{Ca}^{2+}$  trigger degeneration?

**Referee 2 repeatedly states the lowering of cytosolic  $\text{Ca}^{2+}$  is modest, thus implying of dubious significance. No one knows yet the pathophysiology of a prolonged (approx. 20 %) reduction in cytosolic  $\text{Ca}^{2+}$ . This is why we don't want to call it modest but based on all our data prefer the term, cell biologically significant. We have now included data with the small molecule SERCA activator XCT790 (Fig. S5) and demonstrate a SERCA dependent reduction in cytosolic  $\text{Ca}^{2+}$  is followed by a later increase indicative of toxic effects of the SERCA activation. We clearly demonstrate the  $\text{Ca}^{2+}$  reduction in our models depends on AS aggregation and counteracting it by inhibiting SERCA with CPA protects the cells. So our paper opens for further studies of how this reduced  $\text{Ca}^{2+}$  is involved in cytopathology and use of SERCA activators like XCT790 may be useful in modeling aspects hereof.**

At present, the whole interpretation of the data hinges upon the effects of CPA, as intracellular AS aggregates have been shown by many (including the authors) to have a wide range of effects. Because CPA specificity is not clear, an alternative approach needs to be used to make this point; the authors need to specifically disrupt the interaction between AS and SERCA, leaving other consequences of AS on cellular function intact. Site-directed mutagenesis of SERCA would be one strategy that could be used, but there are others. Alternatively, there are small molecular positive allosteric modulators of SERCA function that should in principal have the same effect as AS over-expression.

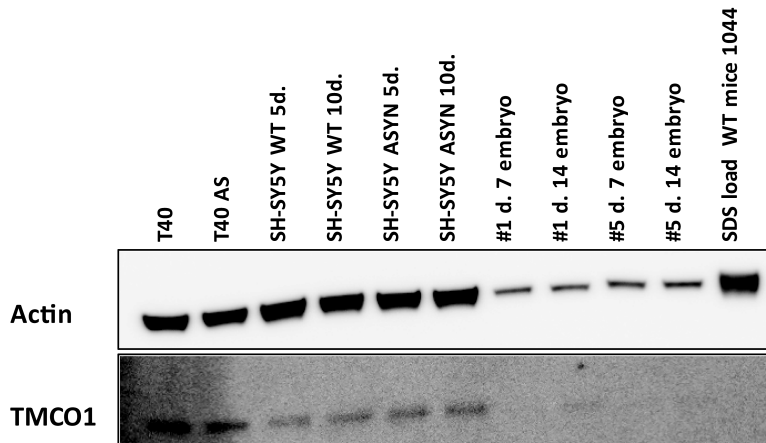
**The specificity of CPA for SERCA is well established (Seidler, JBC, 1989) and the molecular interaction has been demonstrated by co-crystallization (Monqoc, JBC, 2007). However, we agree that the late phase with elevated  $\text{Ca}^{2+}$  could be the sum of more cytopathogenic "hits" by aggregated AS than just the interaction with SERCA. Still, we clearly demonstrate the increase is dependent of AS aggregates (Fig. 1) and it can be blocked by treatment with the SERCA inhibitor CPA (Fig. 3). Inhibition of SERCA will *per se* cause an increase in cytosolic  $\text{Ca}^{2+}$  so we find it intriguing that a partial inhibition of SERCA protects against the increase in  $\text{Ca}^{2+}$  and corroborate our hypothesis that this pump is hyper-activated in the process. Mutagenesis of SERCA to block the interaction with AS aggregates will be a large novel project that will rely on solving the structure of the complex between SERCA and AS oligomers to design the relevant mutations in SERCA.**

**We have chosen the other approach suggested by referee 2 and have tested different positive allosteric modulators of SERCA identified by David D. Thomas' group (Cornea et al., J Biomol Screen, 2013; Gruber et al., J Biomol Screen, 2014) and observe as expected a reduction in cytosolic calcium using XCT 790. We found that prolonged incubation with XCT 790 in naïve nonmitotic SH-SY5Y cells caused biphasic  $\text{Ca}^{2+}$  response with an initial reduction followed by a later increase – as we observed when AS aggregates are formed within cells (Fig S5).**

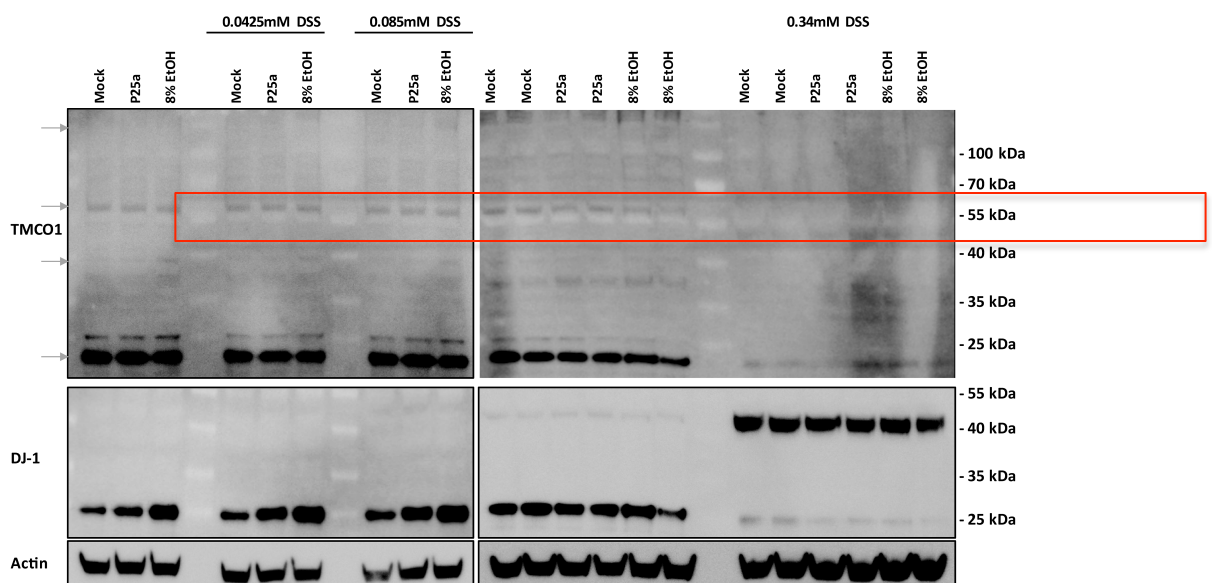
5. Does AS modulation of SERCA elevate ER  $\text{Ca}^{2+}$  content? The transient analysis doesn't answer this question. The authors can probably quantify the steady state  $\text{Ca}^{2+}$  levels from

the aequorin experiments already done. The two possible outcomes are that ER  $\text{Ca}^{2+}$  is increased during the early phase, in which case the subsequent toxicity might be due to stronger  $\text{Ca}^{2+}$  release upon stimulation; the second possible outcome is that ER  $\text{Ca}^{2+}$  is not affected, in which case there must be compensatory mechanisms in play. If ER  $\text{Ca}^{2+}$  loading increases during the early phase, it could trigger the opening of the recently described TMCO1 channel (Wang et al, Cell, 2016), which would subsequently increase cytosolic  $\text{Ca}^{2+}$  levels.

It is asked if AS affects ER calcium levels in our models? We did indeed study this in aequorin experiments as proposed by referee 2 but did not observe any significant effect. We did not show these data (but can do it) because it wasn't a surprise considering the cytosolic  $\text{Ca}^{2+}$  is lowered around 20 nM, the ER concentration of  $\text{Ca}^{2+}$  is around 1 mM and the precision for quantifying absolute levels aren't optimal in contrast to measuring rates of increases. However, a prolonged pumping of  $\text{Ca}^{2+}$  into the ER will likely at some point induce a degree of ER  $\text{Ca}^{2+}$  overload as demonstrated by mutations in the TMCO1 channel (Wang et al., Cell, 2016). The corresponding author on this paper Dr. Tie-Shan Tang has kindly advised us on how to address this question convincingly. We have analyzed if we could detect the tetramerization of TMCO1 as response of ER  $\text{Ca}^{2+}$  overload as reported in the Wang et al paper using their antibody in the OLN model since it has the highest level of TMCO1. See figure below:



Next we tried to stabilize the tetramer by crosslinking TMCO1 in t40 AS cells where AS aggregation was induced by p25alpha compared to mock negative controls and positive controls treated with 8% ethanol.



We tried 2 different concentrations of chemical cross linker (0.0425 mM and 0.085 mM DSS) that was used in the paper, but could not detect increased TMCO1 tetramerization (left blots)

neither in the p25alpha transfected cells nor ethanol treated that should have served as positive control. We also did not detect crosslinking of DJ-1 dimer that we use as internal control as it is a dimeric protein. Next we tested if higher concentration of cross linker could stabilize the TMCO1 tetramer (right blot) but still unsuccessful. We see less monomeric TMCO1 corresponding to Wang et al, when using high cross linker concentration. Though, at 0.34 mM DSS we see almost a complete stabilization of the DJ1 dimer. This shows the DSS cross linker is functional in our assay, but we cannot detect increased tetramerization of TMCO1 in our system. Hence we decided to exclude these data because we were unable to recapitulate the published TMCO1 crosslinking data even when using 8% ethanol as positive control.

The authors should show the effect of 0.5 uM CPA on SERCA Ca<sup>2+</sup> dynamics: does CPA treatment actually normalize the rate of SERCA uptake of Ca<sup>2+</sup>? (and possibly ER Ca<sup>2+</sup> levels?).

**If we in the above-described experiments could demonstrate a significant ER overload and change in ER Ca<sup>2+</sup> levels then we could also address this complex of question. However, effects were only marginal as studied by the ER-aequorin system, so with the current technology, we abstain from these studies.**

Does a mild activation of SOCE contribute to the increase cytosolic Ca<sup>2+</sup> levels?

**This may be the case, but we don't find it relevant for this manuscript. Here we focus on the significance of the early decrease in Ca<sup>2+</sup> and not the later increase. Increased Ca<sup>2+</sup> has been associated to many pathological states and dysfunctions in most homeostatic systems will ultimately cause a rise in cytosolic Ca<sup>2+</sup> because maintaining the low Ca<sup>2+</sup> is an active process working against the large transmembrane gradients between nM concentrations in cytosol and mM concentrations in ER lumen and extracellular space. It is the reduction requiring active transport, which is the intriguing phenomenon for our paper. With this said it may well be that the ultimate cell death requires the elevated Ca<sup>2+</sup> in the late phase.**

6. The light level co-localization assays are essentially meaningless. These do not provide the resolution needed to make the claim that AS and SERCA are bound to one another. The PLA assay is fine, but again is limited by the relevance of the dosing.

**Please see above for comment to specialist advisor**

7. In the context of PD, the relevance of the cell models employed is very limited, since they don't recapitulate the main features of the neurons mostly affected in PD. In vulnerable neurons, the consequences of modulating SERCA activity could be very different.

**Please see answer to bullet 1 in response to referee 2.**

8. Lastly, the discussion of the relationship between AS pathology and neurodegeneration needs revision. First, the relationship between Lewy pathology and neurodegeneration is weak, with degeneration in the substantia nigra preceding the appearance of Lewy pathology. Moreover, many neurons with Lewy pathology appear to be unaffected for decades. This relationship has been recently reviewed (Surmeier et al. 2017). This makes the relationship between AS pathology and neurodegeneration much less clear than the authors would like. It is possible that oligomers, which precede the formation of ribbons/fibrils, could be toxic and work through the mechanism described. However, this needs to be worked out more clearly.

**We have clarified this in the discussion. We agree that Lewy body (LB) per se not represents Parkinson's disease, but they do reflect a neurons handling of abnormal AS as demonstrated by the deposition of amyloid-type filaments. Surmeier argues that it isn't the LB pathology that spreads in clinical Parkinson's disease. Rather it is cell- or region-autonomous mechanisms that dictate where LB develops. Our study does not address LB, but focuses on the functional effects of increasing concentrations of soluble oligomeric AS aggregates. These poorly characterized structures are currently the focus of large research efforts that aim of detecting and characterizing them in human brain tissue.**

9. The reliance upon parametric statistics for display and hypothesis testing is not justified.

**This point of critique is a bit unclear, since parametric statistics are used for comparing means and non-parametric statistics are used when comparing medians. Throughout our study, we use parametric tests because we compare means between groups. In order to compare one mean to another mean, we used Student's t-test, and to perform multiple comparisons, comparing several groups to one mean, we used 1-way ANOVA in combination with Sidaks post hoc test to obtain an adjusted p-value. We have made the description and rationale for choosing these tests more clear in the material and method section.**

Minor points:

10. Lexical /semantic repetitions in the abstract (see first two lines: crucial/fundamental, compromised/compromised) and in the introduction (line 63-64, patients...patients...);

**Is corrected**

11. Please correct the statement that says that the only pacemaking neurons in the midbrain are in substantia nigra (line 64). Or that other neurons that are vulnerable in PD are not pacemakers - many clearly are.

**Is corrected**

12. How are the previously described effects of AS on release of  $Ca^{2+}$  from intracellular stores related to the present results?

**We discuss this and suggest that the increased cytosolic  $Ca^{2+}$  in the late phase is caused by sensitization of ER efflux channels like ryanodine or inositol-1,4,5 tris-phosphate receptors and from extracellular space via dysregulated  $Ca^{2+}$  influx channels or exchangers.**

2nd Editorial Decision

5 January 2018

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed referee reports.

As you will see, while the referees acknowledge that the study has been improved, they also raise important concerns about the statistics, and point out that it remains unclear how SERCA promotes neurodegeneration in PD.

All of these concerns need to be addressed, and it is important that the statistics will be corrected. Please also send me a point-by-point response to these latest concerns.

I am looking forward to receiving a newly revised manuscript as soon as possible. Please let me know if you have any questions or comments.

## REFeree REPORTS

### Referee #1:

The authors have done a reasonably good job of responding to my comments and improving this manuscript. Although the ability of CPA to modulate NF- $\kappa$ B activation is now shown, whether or not this actually contributes to neurodegeneration remains undetermined. Perhaps some discussion about other work linking NF- $\kappa$ B to neurodegeneration would be helpful here.

In figure 5F, the authors showed the effect of CPA on alpha-synuclein expression. Although not formally incorrect, it is unconventional and unnecessarily confusing to show the actin band above the band-of-interest. In addition, and of greater significance, I find it difficult to accept that a 10% change in expression can lead to significance of  $p=0.0001$  by student's T test.

### Referee #2:

The authors have made a good faith effort to address my concerns. Nevertheless, I still have several concerns that have not been dealt with in the revision.

- The authors need to be more circumspect about the mechanisms driving neuronal degeneration in PD. The profound cellular specificity of cell loss in PD and the protracted time course of degeneration relative to AS aggregation and Lewy pathology is difficult to explain with the author's Ca<sup>2+</sup> dysregulation model.
- The authors need to get some guidance on the use of parametric and non-parametric statistics. These tests are used to test assumptions about sampling distributions. The nature of these distributions determines which statistics are good estimates of central tendency. This also applies to display statistics. Unless the sampling distributions are normal, parametric statistics should not be used.
- The authors should attempt to link their results to what is known about the physiology of neurons that are vulnerable in PD. In fact, Ca<sup>2+</sup> handling has been suggested to be a key determinant of vulnerability.

### Referee #3:

The authors have put considerable work into this revision with additional studies and appropriate alterations to the text.

Cross-comments from referee 1:

My comment about statistics was linked to figure 5F; I did not think that  $p=0.0001$  was likely for this data. Considered in combination with referee #2's comments, it may be that the authors have a limited understanding of statistics and support from a statistician may uncover multiple good faith errors (in a best case scenario). They should be strongly encouraged to seek out this support.

As written in my comments, the authors did a reasonable job establishing NF- $\kappa$ B as the mediator. However, they did not conceptually link this with neurodegeneration. I have no doubt that they can link it to NF $\kappa$ b through discussion of other published works.

All of Ref#2's comments are reasonable and should be addressed by the authors.

2nd Revision - authors' response

30 January 2018

Point to point addressment of reviewer comments

### Referee #1:

1. *The authors have done a reasonably good job of responding to my comments and improving this manuscript. Although the ability of CPA to modulate NF- $\kappa$ B activation is now shown, whether or not this actually contributes to neurodegeneration remains undetermined. Perhaps some discussion about other work linking NF- $\kappa$ B to neurodegeneration would be helpful here.*

We have included discussion of NF- $\kappa$ B and its relation to neurodegeneration in the discussion (line 439 - 448).

*“Their nature remains largely unknown but the stress that elicits the cytoprotective NF- $\kappa$ B AS aggregates [10] is reduced in our OLN model upon inhibition of SERCA by CPA (Fig S6). NF- $\kappa$ B is increased in PD as determined by accumulation of p65 in midbrain dopaminergic neurons and Lewy body inclusions in PD and DLB [66-70] and it is also activated by disease causing mutations in LRRK-2 when studied in iPSC-derived neurons [71]. Inhibiting NF- $\kappa$ B signalling in c-REL deficient mice induces a late onset parkinsonistic phenotype also comprising accumulation of aggregated AS [72] and the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  is increased early in anterior cingulate in PD [73] like we demonstrate in our cell model [10]. This suggests protective NF- $\kappa$ B signalling may be of particular importance for the nerve cell populations being vulnerable in PD.”.*

2. *In figure 5F, the authors showed the effect of CPA on alpha-synuclein expression. Although not formally incorrect, it is unconventional and unnecessarily confusing to show the actin band above the band-of-interest.*

We apologize for the confusion and have now placed the loading control at its appropriate position.

*In addition, and of greater significance, I find it difficult to accept that a 10% change in expression can lead to significance of  $p=0.0001$  by student's T test.*

Due to the issue raised regarding the statistical analysis in Fig. 5F we contacted our Biostatistical department at Aarhus University. It was suggested that we analyzed the data in Fig. 5F with a non-parametric test. By this approach the difference is still significant albeit the p-value now is 0.03. The consultation with the biostatisticians gave reason to the following general change. All statistical analyses with sample size smaller than 10 are performed with non-parametric tests based on the assumption that the sample size is too small to evaluate for normal distribution. Larger samples are analysed using parametric tests. The figure legends include information on the specific test applied. We have changed the method description to the following

*“Statistical evaluation of results was performed using parametric tests comparing means between larger groups ( $\geq 10$ ) and nonparametric tests for significance in groups with smaller sample size. In order to compare one mean to another mean, Student’s t-test was conducted as parametric test and Wilcoxon signed rank test was used as nonparametric test. In order to test the null-hypothesis in multiple comparisons, when comparing several groups to one mean, 1-way ANOVA combined with Sidaks post hoc test was applied as parametric test and Kruskal-Wallis 1-way rank test with Dunns post hoc test was applied as nonparametric test to obtain an adjusted p-value using the Graphpad Prism programme (Graphpad Software)”.*

#### **Referee #2:**

*The authors have made a good faith effort to address my concerns. Nevertheless, I still have several concerns that have not been dealt with in the revision.*

1. *The authors need to be more circumspect about the mechanisms driving neuronal degeneration in PD. The profound cellular specificity of cell loss in PD and the protracted time course of degeneration relative to AS aggregation and Lewy pathology is difficult to explain with the author's  $Ca^{2+}$  dysregulation model.*
3. *The authors should attempt to link their results to what is known about the physiology of neurons that are vulnerable in PD. In fact,  $Ca^{2+}$  handling has been suggested to be a key determinant of vulnerability.*

We have in the Discussion addressed the two above points 1 and 3 in lines 539-578.

*“How may these experimental findings reconcile with previous epidemiological and genetic data on protective effects against PD by L-type  $Ca^{2+}$  channel antagonists [2, 3] and the pathoanatomical data on selective vulnerability of neuronal populations in PD recently reviewed [82]? First it should be kept in mind that both the early phase with reduced cytosolic  $Ca^{2+}$  and the following phase with increased  $Ca^{2+}$  both are inhibited by inhibiting SERCA with CPA. Hence, treatment strategies targeting the high  $Ca^{2+}$  phase may still contribute some cellular relief despite not targeting the underlying mechanism. The largest epidemiological study confirmed a protective effect of ongoing treatment with L-type  $Ca^{2+}$  channel antagonists but there was no effect of past use, which made the authors suggest the effect could represent a symptomatic relief rather than a true disease modification.*

*In our proposed paradigm of a biphasic basal  $Ca^{2+}$  change in neurons experiencing a build-up of AS aggregates, L-type  $Ca^{2+}$  channel antagonists may retard or attenuate the second phase with increased cytosolic  $Ca^{2+}$  that is associated to development of cell death. An action on the early phase can also be envisioned if expression of CaV1.3 channels partly are regulated by negative feed-back from average cytosolic calcium levels. The increased levels of CaV1.3 antigen and mRNA*



in cortical tissue with no Lewy body pathology from early PD patients [4, 5] could represent a response to soluble AS oligomers activating SERCA and reducing average  $Ca^{2+}$  in neurons not exhibiting spontaneous pace making changes in  $Ca^{2+}$  as in dopaminergic neurons of substantia nigra pars compacta. The on-going clinical study of early PD patients treated with L-type Ca channel antagonist Isradipine may inform about these issues in the near future (ClinicalTrials.gov Identifier: NCT02168842).

The selective vulnerability of neuronal populations lost in PD have allowed certain characteristics of these neurons to be proposed [82]. Areas affected by Lewy body pathology and substantial neuron loss in PD can be exemplified by substantia nigra pars compacta, locus coeruleus, median raphe nucleus. These neuronal populations display diffuse axonal projections and often possess very large numbers of presynaptic terminals [82]. AS is a protein that normally resides in presynapses in high concentrations and its aggregation process is concentration dependent. This makes presynapses likely to be the first sites to experience the build-up of AS aggregates, as supported by proteinase K blotting experiments on cortical tissue affected by dementia with Lewy bodies [83], that secondarily will activate SERCA, increase ATP consumption and reduce cytosolic calcium levels at these sites. A local aggregatory process in terminals may spread within the terminal field of individual neurons as AS readily disperses between terminals of single neurons in a process affected by neuronal activity [84]. The engagement of SERCA pumps in the large terminal fields of vulnerable neurons will increase ATP consumption putting a stress on their mitochondria and the potentially increase oxidative stress. This process be further complicated because the reduced average cytosolic  $Ca^{2+}$  may compromise activity-dependent calcium loading into mitochondria thereby compromising their oxidative phosphorylation [85]. When aggregation increases and AS aggregates are transported from terminals into axons and cell bodies then different mechanisms may come into play caused by the SERCA activation both due to lowered calcium, e.g. in the nucleus [86] and potentially also ER  $Ca^{2+}$  overload if not relieved by overload channels [87].”

2. *The authors need to get some guidance on the use of parametric and non-parametric statistics. These tests are used to test assumptions about sampling distributions. The nature of these distributions determines which statistics are good estimates of central tendency. This also applies to display statistics. Unless the sampling distributions are normal, parametric statistics should not be used.*

Please see our response to point 2 from referee 1.

### **Referee #3:**

*The authors have put considerable work into this revision with additional studies and appropriate alterations to the text.*

*Cross-comments from referee 1:*

1. *My comment about statistics was linked to figure 5F; I did not think that  $p=0.0001$  was likely for this data. Considered in combination with referee #2's comments, it may be that the authors have a limited understanding of statistics and support from a statistician may uncover multiple good faith errors (in a best case scenario). They should be strongly encouraged to seek out this support.*

Please see our response to your comment on statistic above, Referee 1 point 2.

2. *As written in my comments, the authors did a reasonable job establishing NF- $\kappa$ B as the mediator. However, they did not conceptually link this with neurodegeneration. I have no doubt that they can link it to NFKB through discussion of other published works.*

Please see our response to your comment on statistic above, Referee 1 point 1.

*All of Ref#2's comments are reasonable and should be addressed by the authors.*



Thank you for the submission of your newly revised manuscript. We have now received the enclosed report from referee 2 who was asked to assess it. Referee 2 still has a few more suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript.

A few other changes are also needed:

Figures S4B and S5 state that only 2 experiments were performed, but error bars and p-values are shown, which is not possible. Please either repeat the experiment at least one more time or remove the error bars and p-values. If  $n=2$ , all data points from both experiments can be shown along with their mean.

The 7 supplemental figures need to be converted into either EV figures (we can offer a maximum of 5 EV figures) that are embedded in the main manuscript online and expand when clicked, or need to be included in an Appendix file with table of content and page numbers. You can find more information about our file types in our guide to authors online. Since some of the 7 supplemental figures are small, you might want to combine some to reduce the total number to 5. EV figures need to be uploaded as individual files.

The reference format needs to be corrected. The numbered EMBO reports style is part of EndNote. Up to 10 author names need to be listed before "et al".

Please send us a running title, up to 5 keywords, an author contribution statement and a conflict of interest statement.

I would like to suggest to add "SERCA" to the title, and please also explain what SERCA is in the abstract. Please consider re-writing of the abstract. Abstracts usually do not start with a hypothesis but with background information that is important to understand your hypothesis and the advance reported by your study. Please describe the findings of your study using present tense in the abstract.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible. When you upload a new manuscript version, you can bring forward all the old files and then only replace the files that need to be replaced.

I also attach a word file here with tracked changes of the figure legends and some queries. Please correct the text and send us back the revised word file.

## REFeree REPORT

### Referee #2:

" The author's employment of non-parametric statistical tests for small sample sizes is an improvement. But this is not enough. First, for larger samples, the normality assumption of the parametric tests needs to be run. Second, the reliance upon parametric statistics (means, SEMs) for DISPLAY purposes is not justified unless the conditions necessary for using parametric statistics for hypothesis testing are justified.

" Again, the addition to the Discussion section is an improvement but the historical perspective is not quite right. First, many groups have shown that the most vulnerable neuronal population - SNc dopaminergic neurons - have large fluctuations in cytosolic calcium concentration attributable to opening of Cav1 channels during pacemaking. Inhibiting these channels and fluctuations diminished the sensitivity of these neurons to toxins associated with PD. It was this observation - reported in a Nature paper by Chan et al. in 2007 and the suggestion that dihydropyridines might be

neuroprotective that led to the epidemiological work, not the other way around. Subsequent work has shown that these calcium oscillations drive mitochondrial oxidant stress; this calcium driven stress is undoubtedly predicated upon release of calcium from the ER, as many studies have shown this is the route of calcium into the mitochondrion (one of which is later cited by the authors). The potential interaction between SERCA activation, CICR and mitochondrial respiration should be more explicitly discussed in light of this background.

" The authors miss the relationship between calcium and oligomerization of aSYN, which is well documented. This should create a negative feedback mechanism for oligomerization in the authors model.

3rd Revision - authors' response

13 February 2018

Thank you for the thorough review and detailed instructions for the final revision. Regarding referee #2 that appears as a strong opinion leader in the debate of Parkinsons disease being due to "prion-like" pathology or selective vulnerability. We have, as suggested, elaborated the discussion about the calcium fluctuations in SNc dopaminergic neurons and inserted the reference mentioned. However being rather agnostic with respect to such debates and in order to balance the discussion I have also inserted references demonstrating that a frank overexpression of normal alpha-synuclein, as demonstrated in rare families with gene multiplications, can present as dementia with Lewy bodies where parkinsonism also is present. That simply to say the alpha-synuclein dependent pathology not necessarily specifically affects SNc dopaminergic neurons but disturbs mechanisms of importance for broader populations of neurons.

*Response to referee 2*

1. *" The author's employment of non-parametric statistical tests for small sample sizes is an improvement. But this is not enough. First, for larger samples, the normality assumption of the parametric tests needs to be run. Second, the reliance upon parametric statistics (means, sems) for DISPLAY purposes is not justified unless the conditions necessary for using parametric statistics for hypothesis testing are justified.*

With respect to our assumptions of normal distribution of our observation in the larger samples we have now, as requested, tested this assumptions and found them valid. This has been described in line 821-824.

"Statistical evaluation of results was performed using parametric tests for data sets following normal distribution by comparing means  $\pm$  SD. For smaller data sets where normal distribution could not be proven, statistical evaluation was conducted by nonparametric tests and data presented as geometric mean  $\pm$  95% confidence interval."

2. *"Again, the addition to the Discussion section is an improvement but the historical perspective is not quite right. First, many groups have shown that the most vulnerable neuronal population - SNc dopaminergic neurons - have large fluctuations in cytosolic calcium concentration attributable to opening of Cav1 channels during pacemaking. Inhibiting these channels and fluctuations diminished the sensitivity of these neurons to toxins associated with PD. It was this observation - reported in a Nature paper by Chan et al. in 2007 and the suggestion that dihydropyridines might be neuroprotective that led to the epidemiological work, not the other way around. Subsequent work has shown that these calcium oscillations drive mitochondrial oxidant stress; this calcium driven stress is undoubtedly predicated upon release of calcium from the ER, as many studies have shown this is the route of calcium into the mitochondrion (one of which is later cited by the authors). The potential interaction between SERCA activation, CICR and mitochondrial respiration should be more explicitly discussed in light of this background.*

We have included the suggested reference and elaborated the discussion with line 596-608.

"How these mechanisms driven by a reduced average cytosolic Ca<sup>2+</sup> interacts with cell type specific effects in vulnerable substantia nigra dopaminergic neurons will have to be tested? They may affect the Ca<sup>2+</sup>-induced Ca<sup>2+</sup>-release from ER facilitated by the oscillating in Ca<sup>2+</sup> driven by the L-type Ca<sup>2+</sup> channels and thereby the well described filling of Ca<sup>2+</sup> into mitochondria [91]. However the recently described ER overload channel TMCO1 may well add another layer of complexity in conditions where SERCA activation will cause Ca<sup>2+</sup> overload in ER [90]. However, irrespective of

vulnerable areas in PD it should be kept in mind that AS aggregate pathology has potential to affect brain cells on a broader scale. This has been demonstrated by the clinical studies of families with triplications in the SNCA gene that express increased levels of normal AS protein as reviewed [92]. These families often presented with parkinsonism caused by degeneration of the vulnerable substantia nigra pars compacta but often displayed dementia and other non-motor symptoms at the time of diagnosis or shortly after. This was due to wide spread accumulation of aggregated AS in neurons but also glial cells.”

3. *"The authors miss the relationship between calcium and oligomerization of  $\alpha$ SYN, which is well documented. This should create a negative feedback mechanism for oligomerization in the authors model.*

We have expanded the discussion in line 462 – 466 on this issue

“Several investigations have studied direct effects of  $\text{Ca}^{2+}$  on AS aggregation in vitro. They demonstrate a proaggregatory effect of increased  $\text{Ca}^{2+}$  levels but often with fluorescently labelled AS proteins [74] and those using non-modified proteins used high  $\text{Ca}^{2+}$  concentrations [23] because the C-terminal  $\text{Ca}^{2+}$ -binding site in AS has an  $\text{IC}_{50}$  around  $200 \mu\text{M}$ ) [75].”

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Cristine Betzer

Journal Submitted to: EMBO Reports

Manuscript Number: EMBOR-2017-44617V2-Q

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

**A- Figures****1. Data****The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if  $n < 5$ , the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

**2. Captions****Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/ varied/ perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple  $\chi^2$  tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

**B- Statistics and general methods**

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Neurons isolated from females were excluded, page 24 line 522-52: "Neurons isolated from male pups were only used, due to transgene insertion into x-chromosome and to random x-chromosome inactivation in females.". The criteria was preestablished.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Genotype of neurons and treatment of neurons and cells were randomized and evaluated blinded (p 26). Handling and staining of human material was also done blinded (p28).
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	1-way ANOVA assumes that the data is normal distributed, that all the populations have the same standard deviation, and thus the same variance tested with Browne-Forsythe test and Bartlett's test, and that data is unmatched.
Is there an estimate of variation within each group of data?	All data is shown as mean with standard deviation to show the variation
Is the variance similar between the groups that are being statistically compared?	yes

**C- Reagents****USEFUL LINKS FOR COMPLETING THIS FORM**<http://www.antibodypedia.com><http://1degreebio.org><http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo><http://grants.nih.gov/grants/olaw/olaw.htm><http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm><http://ClinicalTrials.gov><http://www.consort-statement.org><http://www.consort-statement.org/checklists/view/32-consort/66-title><http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun><http://datadryad.org><http://figshare.com><http://www.ncbi.nlm.nih.gov/gap><http://www.ebi.ac.uk/ega><http://biomodels.net/><http://biomodels.net/miriam/><http://ijb.biochem.sun.ac.za>[http://oba.od.nih.gov/biosecurity/biosecurity\\_documents.html](http://oba.od.nih.gov/biosecurity/biosecurity_documents.html)<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All commercially available antibodies used in this study are denoted with catalog number in material and method section. Antibodies not commercially available are cited.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were routinely checked for mycoplasma contamination using PCR Mycoplasma Test Kit i/C from Promokine (p 23 line 497).

\* for all hyperlinks, please see the table at the top right of the document

#### D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

#### E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The human cerebellum dentate nuclei for used for sequential extraction (3 MSA cases and 3 neurological healthy cases) were donated from the Brain Bank at Bispebjerg-Frederiksberg University Hospital (University Hospital of Copenhagen, DNK; approved by the Danish Data Protection Agency, j.no. BBH-2010-06, I-suite 00971). The use of the brain tissue was approved by the regional ethical committee of Region Hovedstaden (DNK), journal no. H-16025210 (p30). The human tissue used for proximity ligation assay (1 DLB case and 1 neurological intact case) was from brain donors recruited with informed consent through a regional brain donor program with ethics approval from the Human Research Ethics Committees of South Eastern Sydney Local Health District and the Universities of Sydney and New South Wales (Sydney) which complies with the statement of human experimentation issued by the National Health and Medical Research Council of Australia (p28).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	p 29 In 635-637 "Human tissue was obtained with informed consent and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report"
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

#### F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.  Examples: <b>Primary Data</b> Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 <b>Referenced Data</b> Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4Q26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

#### G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA
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