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α-synuclein assemblies sequester neuronal α3-Na+/K+-ATPase and impair Na+ gradient

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Transaction Report:

(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 29 March 2015

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been reviewed by 3 good experts in the field and their comments are provided below.

As you can see the referees find the analysis very interesting. However they also find that further work is needed in order to consider publication here. Their concerns are clearly outlined below, but the link between a-syn and a3-NKA and if this is linked to pathogenesis has to be better substantiated. Should you be able to extend the analysis and address the concerns raised then we would consider a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the raised issue at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process.

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

REFEREE REPORTS

Referee #1:

The authors find in primary neuron cultures that exogenously applied, pre-formed α-syn oligomers and fibrils form clusters all over the cell surface. Using mass spec analysis of proteins pulled down with such α-syn assemblies identifies a number of candidates, of which one is extensively validated. The α3 subunit of the Na+/K+ ATPase (NKA) co-purified with both α-syn assemblies. Quantitatively small but overall credible and mathematically significant (co-)clustering of α3-NKA with α-syn assemblies is documented in primary cell culture and in vivo. The α3-NKA is particularly involved in restoring Na+ gradients after membrane depolarization. Indeed impairments in Na+ extrusion and concomitantly enhanced intracellular Ca2+ are measured in neurons exposed to α-syn assemblies. Overall this is an important study, which identifies a novel target of α-syn neuropathology and offers new conceptual approaches to the understanding and perhaps even towards treatment of neurodegenerative α-synucleinopathies, such as Parkinson's disease.

Specific Comments

1. Description of Figure 1A,B "age" of neurons should be replaced by the more neutral term DIV throughout.

2. The proteomic data must be presented with greater care.
a. It is recurrently stated in text, figures and legends that interacting membrane proteins are detected. While the rationale and subsequent validation efforts are clear, it must be understood that α-syn assembly interacting proteins were screened in whole cell lysates. No membrane enrichment steps can be made out from the experimental procedures. Consequently, a number of non-membrane spanning proteins were detected, such as 14-3-3, BiP/HSPA5, GAPDH, TAU, etc. Do rewrite all relevant parts to make this point clear. For example titles of Table E5 should read: "proteins interacting with extracellularly applied α-syn [assemblies]".
b. Upload the proteomic raw data following the appropriate guidelines.
c. Explain the selection criteria for Table E5. What is meant by "annotation of their localization and validation with published data" (page 7)?
d. Figure E2A is dispensable, the procedure is clear enough. The criteria for the short list in Figure E2B are misunderstood or erroneous. Proteins like those mentioned in point 2a are obviously not "integral membrane proteins with an extracellular domain and/or extracellular peripheral membrane proteins" (page 7). In fact, Figure E2B should be deleted. The complete Table E5 is sufficient and much more relevant. Finally, Figure 2C can be merged into Figure E3.
e. For which of the proteins in Table E5B did the crosslink/MS approach yield confirmatory peptide masses? It is understood that the experiment 2C intended to validate α3-NKA, but a more comprehensive analysis of that experiment would be an informative additional column for Table 5B.
f. The co-IP experiment in Figure E4B is not convincing. The authors may be right that the physical interaction is weak or transient, but the extent of non-specific binding is unacceptable. Control probing for α3-NKA is missing as well. And why does fibrillar α-syn not migrate as a smear? Use of species-specific antibodies could furthermore distinguish between exogenous human α-syn assemblies and the endogenous rat α-syn protein.
In general, I find that too strong language is used to describe the direct physical interaction between α3-NKA and α-syn assemblies and their co-clustering. The evidence in Figures E4C and 5A is not impressive.
g. It was focused on α3-NKA because it "was the only one interacting with both oligomeric and fibrillar α-syn". What about AP-3 δ1 and VAP-A??

3. "...makes neurons more vulnerable to glutamate excitotoxicity" (last sentences Results, and Discussion). This is a valid speculation that needs to be declared as such. The key experiment, that α-syn assemblies do enhance glutamate-induced calcium toxicity explicitly via impaired activity of α3-NKA is not done here.

4. Page 14, line 2 correct (Figure 6Ba-b) not (Figure 6Ba, 2Bb).

Referee #2:

Misfolded (assemblies) alpha-syn has been shown to transmit from neuron to neuron, yet there are many unanswered question including the mechanism of release, uptake, propagation and toxicity. The authors focused on the effect of misfolded alpha-syn after its initial interaction with the plasma membrane. The authors suggest that α-syn assemblies form clusters within the plasma membrane of neurons. Using a proteomic-based approach they identify the α3-subunit of Na+/K+-ATPase (NKA) as a cell-surface partner of α- syn assemblies. They suggest that otherwise freely diffusing α3-NKA subunits are trapped within α-syn clusters resulting in α3-NKA re-distribution and formation of larger nanoclusters. This seems to create patches within the plasma membrane devoid of α3-NKA, thereby decreasing the efficiency of Na+ extrusion from neurons following a stimulus. Overall, I don't think this manuscript is ready for publication, it is still quite preliminary. In particular there is no demonstration that the binding of alpha-syn assemblies is involved in PD. There are no experiments that support the idea that the binding of alpha-syn oligomers or fibrils to NKA is required for alpha-syn diffusion, clustering, and change in ionic gradients (i.e mutational analysis/KO/KD experiments). There is also no demonstration that alpha-syn binding to the plasma membrane requires NKA.

Additional concerns:

The authors need to explain why oligomeric and fibrillar alpha-syn were applied at different doses. In Figure 1A/C, the authors should perform cell fractionation and western blots to support the notion that alpha-syn binds plasma membranes?

The authors should show some images or movies to support the quantification of the diffusion coefficients.

The authors need to perform knockout/knockdown of alpha3-NKA to demonstrate alpha-syn no longer binds to the plasma membraine or has reduced binding and/or that the diffusion coefficients for oligomeric/fibrillar alpha-syn are altered, as well as the knockdown-rescue experiments

Figure 5D. What are the high-resolution images shown for? It is quite difficult to see the difference. The authors need to provide quantification.

Is there any difference between the misfolded alpha-synuclein and labeled misfolded alpha-synuclein in the in vivo injection studies.

It is not clear whether there is direct binding between alpha-synuclein and alpha3-NKA?

In Figure E2 the binding of alpha-synfFibril and alpha-syn oligomer does not exhibit any saturation kinetics consistent with classic ligand receptor binding raising concerns that the interaction reported here may be non-specific. Along these lines the authors need to include knockout or knockdown studies of NKA as well as determine the affinity of alpha-syn assemblies for NKA.

The authors report that they identified 54 and 197 protein partners for oligomeric and fibrillar alpha-syn of these they narrowed down to 12 and 73 membrane proteins. It would be important to provide both the membrane proteins and the non-membrane proteins in Table E5.

Can the authors confirm that alpha-syn oligomer interacts with NKA?

Figure E4C is missing.

In the discussion the authors mention an observation for control proteomic study on pure astrocyte cultures, but these data are not presented for evaluation.

What evidence do the authors have that down regulation of NKA activity leads to increased susceptibility to glutamate excitotoxicity as suggested in the discussion.

In Figure 6, the authors need to include a set of comparisons in which alpha3-NKA is knockout or

knocked down.

In Figure 7, how do the authors know that the change in Na+ dynamics is the direct consequence of misfolded alpha-synuclein directly acting on NKA? It is not clear why the authors examined a 24 hour time point since all the other experiments on alpha-syn assemblies were performed in the 60 min time window after alpha-syn application. Is the change in calcium flux due the effects of alpha-syn on NKA?

Referee #3:

Shrivastava et al. describe in a set of well-controlled experiments their quest for putative protein interactors with recombinant oligomeric and fibrillar α-synuclein (α-syn) assemblies in primary neurons and identified, by affinity isolation and nanoLC-MS/MS analysis, neuronal α3-Na/K-ATPase (α3-NKA) as a principal binding protein. They furthermore present experimental evidence for the perturbation of Na efflux from neurons and increased glutamate induced calcium influx by α-syn assemblies. These interesting findings may greatly help to better understand molecular disease pathways in Parkinson's disease, particularly as mutations in α3-NKA confer risk of rapid-onset dystonia Parkinsonism.

Even though the large number of putative interacting proteins, including cytosolic and mitochondrial proteins clearly shows the limitations of S-protein agarose beads isolation, stringent downstream analysis and exclusion of unlikely binding partners led to a trimmed list of fourteen putative interactors. The authors subsequently focussed on α3-NKA, a protein that was identified by affinity isolation with both, oligomeric and fibrillar α-syn.

Main Comments

1. The experimental setup of this study follows the paradigm that aggregated α-syn oligomers are associated with pathogenesis, but surprisingly the authors have not investigated whether the identified protein, α3-NKA, interacts with monomeric α-syn and this leaves open the important question whether reorganisation of α3-NKA and subsequent perturbations of Na efflux and calcium influx are due to a gain of function by aggregated oligomeric species. This should be addressed by incubation of primary neurons with monomeric and oligomeric S-tag α-syn prior to chemical cross-linking (Figure 2C) and determination of α3-NKA-dependent perturbations (Figure 7).

2. The authors isolate α-syn interacting proteins in absence and presence of the chemical crosslinker DTPSS, but only report the interaction of α-syn with α3-NKA after crosslinking in Figure 2C. For completion the set of α-syn-interacting proteins in presence of crosslinking agent should be added in expanded view.

3. A method description for cell labeling with α-syn and live imaging is missing in the manuscript and should be added to Material and Methods in the main body.

4. In Figure 5 Shrivastava et al. describe the reorganisation of α3-NKA and α-syn assemblies in 21 day old striatal neurons, but apart from one area magnified in Figure 5Ab where colocalisation between a-syn and a3-NKA is clearly visible, levels of colocalisation are difficult to assess. It would thus be helpful to determine the corresponding Pearson correlation coefficients as a measure for the level of colocalisation.

5. On page 13, second paragraph the authors refer to previous reports of a prion-like propagation of α-syn and in this context present their data on synaptic clustering and association with α3-NKA following in vivo injection of α-syn. Reference to prion-like propagation of α-syn is misleading here and for clarity it should be mentioned in the results section that observations in Figure 6 do not allow the conclusion that α-syn assemblies spread in a prion-like manner.

Minor points

6. The labeling of MS/MS spectra and Western blot in Figure E4 does not correspond to the legend.

According to the legend the Western blot is Figure E4C, not E4B.

1st Revision - authors' response 31 May 2015

Referee #1:

The authors find in primary neuron cultures that exogenously applied, pre-formed α-syn oligomers and fibrils form clusters all over the cell surface. Using mass spec analysis of proteins pulled down with such α-syn assemblies identifies a number of candidates, of which one is extensively validated. The α3 subunit of the Na+/K+ ATPase (NKA) co-purified with both α-syn assemblies. Quantitatively small but overall credible and mathematically significant (co-)clustering of α3-NKA with α-syn assemblies is documented in primary cell culture and in vivo. The α3-NKA is particularly involved in restoring Na+ gradients after membrane depolarization. Indeed impairments in Na+ extrusion and concomitantly enhanced intracellular Ca2+ are measured in neurons exposed to α-syn assemblies. Overall this is an important study, which identifies a novel target of α-syn neuropathology and offers new conceptual approaches to the understanding and perhaps even towards treatment of neurodegenerative α-synucleinopathies, such as Parkinson's disease.

We thank the reviewer for positive assessment of our work and for the useful comments. Please find the response to individual comments below. All the changes in the text are marked in red.

Specific Comments

1. Description of Figure 1A,B "age" of neurons should be replaced by the more neutral term DIV throughout.

We have replaced “age of neurons” with “DIV” as suggested.

2. The proteomic data must be presented with greater care.

a. It is recurrently stated in text, figures and legends that interacting membrane proteins are detected. While the rationale and subsequent validation efforts are clear, it must be understood that α-syn assembly interacting proteins were screened in whole cell lysates. No membrane enrichment steps can be made out from the experimental procedures. Consequently, a number of non-membrane spanning proteins were detected, such as 14-3-3, BiP/HSPA5, GAPDH, TAU, etc. Do rewrite all relevant parts to make this point clear. For example titles of Table E5 should read: "proteins interacting with extracellularly applied α-syn [assemblies]".

We followed the reviewer’s recommendations. It is now specified “α-syn-S-tag and associated proteins were pulled down from whole cell lysates using S-protein agarose beads” (Page 7, 2nd paragraph). The tables (Appendix Table S5-7) have been labeled according to the reviewer’s suggestion “*Protein interacting with extracellularly applied α-syn in neurons/ astrocytes*”.

b. Upload the proteomic raw data following the appropriate guidelines.

We provide the full list of proteins identified in expanded view section for neurons (Appendix Tables S5-6) and astrocytes (Appendix Tables S7A-B). In addition, the whole raw dataset were deposited in the PRoteomics IDEntifications (PRIDE) repository (see Page 24) and can be accessed through the following web page: http://www.ebi.ac.uk/pride/archive/ using the login details:

Username: reviewer45645@ebi.ac.uk
Password: CREqfqfd

c. Explain the selection criteria for Table E5. What is meant by "annotation of their localization and validation with published data" (page 7)?

Table E5 is now S5 and S6 and text is in Page 8. We used NCBI (National Center for Biotechnology Information) annotation of protein localization based on Gene Ontology (GO) cell component term annotations (Ashburner, M et al. Nat. Genet. 2000, 25(1): 25-9) and experimental descriptions in a peer-reviewed publication. We have clarified and elaborated this issue on page 8 (1st paragraph).

d. Figure 2A is dispensable, the procedure is clear enough. The criteria for the short list in Figure 2B are misunderstood or erroneous. Proteins like those mentioned in point 2A are obviously not "integral membrane proteins with an extracellular domain and/or extracellular peripheral membrane proteins" (page 7). In fact, Figure 2B should be deleted. The complete Table E5 is sufficient and much more relevant. Finally, Figure 2C can be merged into Figure E3.

Figure 2 is now Figure 3. We respectfully disagree with the reviewer and find panel **3A** necessary as it visually summarizes what we did. The criteria for panel **B** were clarified "A final list of proteins reported as integral membrane proteins with an extracellular domain and/or extracellular peripheral membrane proteins consisted of 2- and 18-proteins of interest for oligomeric and fibrillar α-syn, respectively" (Page 8, 1st paragraph). We have completed/extended Table S5-6 describing the selection criteria. Finally we moved panel **C** from previous version to present "Expanded View Figure EV1" following the reviewer's recommendation.

e. For which of the proteins in Table E5B did the crosslink/MS approach yield confirmatory peptide masses? It is understood that the experiment 2C intended to validate α3-NKA, but a more comprehensive analysis of that experiment would be an informative additional column for Table 5B.

It should be highlighted that it is hazardous and extremely fastidious, if feasible, by any mean other than oriented searches, to exploit the crosslinking dataset. Cross-linking yields by definition "artifactual" interactions. Therefore, the data should be interpreted carefully. We would like to stress that the cross-linking data set was not used to demonstrate interaction between α3-NKA and α-syn but to further strengthen the pull-down and co-immunoprecipitation experiments. This oriented search not only confirmed α3-NKA and α-syn interaction but allowed us to show that α3-NKA peptides exposed at the surface of the cells are found cross-linked to α-syn. We have now added an extra column in Appendix Table S6 (column 10) to show which other proteins were also identified by cross-linking with a "+" sign.

f. The co-IP experiment in Figure E4B is not convincing. The authors may be right that the physical interaction is weak or transient, but the extent of non-specific binding is unacceptable. Control probing for α3-NKA is missing as well. And why does fibrillar α-syn not migrate as a smear? Use of species-specific antibodies could furthermore distinguish between exogenous human α-syn assemblies and the endogenous rat α-syn protein. In general, I find that too strong language is used to describe the direct physical interaction between α3-NKA and α-syn assemblies and their co-clustering. The evidence in Figures E4C and 5A is not impressive.

We have performed new co-IP experiments using a different protocol and the detergent including a pre-clearing step to reduce background. Though the background was not completely eliminated; the specific signal could be boosted. Oligomeric and monomeric α-syn assemblies were also used for IP experiments. This revealed a stronger interaction of α3-NKA with fibrillar than with oligomeric α-syn (Figure 3C) in agreement with what was found with SPT experiments (Figure 4). No interaction of α3-NKA with monomeric α-syn could be seen also in agreement with SPT (Figure EV2 A) and Na^+ imaging (Figure EV4 A-B) experiments. This point is now included in the abstract and discussion section.

We and others previously showed that fibrillar α-syn migrates as a single band at its normal molecular weight on SDS gels while migrating as a smear on native gels. See for example Figure 1 panel B in Holmqvist et al. 2014 Acta Neuropathologica 128(6): 805-20. This means that α-syn fibrils are labile in the presence of SDS (0.1% and over), as a consequence they appear as monomers on SDS-PAGE. The reviewer is perfectly right when he writes that he expects α-syn fibrils to behave as high molecular weight species and actually this is indeed the case when native-PAGE (i.e. when incubation of the fibrils with SDS is omitted). Fibrillar α-syn is under the latter conditions trapped within the gel well and yields high molecular weight species that smears while monomeric αSYN yields one band.

New 2-color STORM super-resolution imaging experiments were performed to emphasize the association between α3-NKA and α-syn clusters. Here again, fibrillar α-syn assemblies had a stronger association than oligomeric ones (Figure 6D). Thus, together with Figure 5 (binding site) and Movie EV1 (trapping of α3-NKA), these new data support and emphasize a direct interaction between α-syn and α3-NKA.

g. It was focused on α3-NKA because it "was the only one interacting with both oligomeric and fibrillar α-syn". What about AP-3 δ1 and VAP-A??

We have now modified the text as "We focused on the trans-membrane protein NKA since the α3-subunit of NKA was the only one interacting with both oligomeric and fibrillar α-syn and have an extracellular domain". Both AP-3 δ1 and VAP-A are important candidates, but they are associated with intracellular membrane domain. As the focus of the study was to identify proteins that have extracellular domain or have residues exposed to the outside of cell (Figure 3B), we excluded these candidates from the current study.

3. "...makes neurons more vulnerable to glutamate excitotoxicity" (last sentences Results, and Discussion). This is a valid speculation that needs to be declared as such. The key experiment, that α-syn assemblies do enhance glutamate-induced calcium toxicity explicitly via impaired activity of α3-NKA is not done here.

This study was performed since several lines of evidence imply that the activity of neuronal NKA may have an impact on the calcium response following activation of the glutamatergic receptors. The mechanisms behind such an effect remain to be elucidated, but may be due either to direct interaction between NKA and the NMDA receptor and/ or to reversal of the Na^+/Ca^{2+} exchanger. The latter effect could be due to the reduced capacity to restore the sodium levels in the α-syn exposed neurons (see discussion, Pg 21, 3rd paragraph)

Although studies of the mechanisms by which impaired NKA activity enhances the response to glutamate is out of scope for the current manuscript, we feel that our descriptive observation of enhanced Ca^{2+} influx in α-syn assemblies exposed adds important information to this study.

4. Page 14, line 2 correct (Figure 6Ba-b) not (Figure 6Ba, 2Bb).

This has been corrected based on new numbering of figures.

Referee #2:

Misfolded (assemblies) alpha-syn has been shown to transmit from neuron to neuron, yet there are many unanswered question including the mechanism of release, uptake, propagation and toxicity. The authors focused on the effect of misfolded alpha-syn after its initial interaction with the plasma membrane. The authors suggest that α-syn assemblies form clusters within the plasma membrane of neurons. Using a proteomic-based approach they identify the α3-subunit of Na+/K+-ATPase (NKA) as a cell-surface partner of α- syn assemblies. They suggest that otherwise freely diffusing α3-NKA subunits are trapped within α-syn clusters resulting in α3-NKA re-distribution and formation of larger nanoclusters. This seems to create patches within the plasma membrane devoid of α3-NKA, thereby decreasing the efficiency of Na+ extrusion from neurons following a stimulus. Overall, I don't think this manuscript is ready for publication, it is still quite preliminary.

We thank the reviewer for a careful evaluation of the manuscript and critical comments. We have tried to clarify most of the raised concerns. Our writing may have been ambiguous and we are now more specific about the fact that α3-NKA is not the "receptor" of α-syn. In fact, α-syn interacts with many neuronal and non-neuronal cell types. It also binds to artificial membrane. Here we show that exogenous α-syn binds to the membrane, form clusters and then capture diffusing α3-NKA. Indeed, this does not mean that α3-NKA is the only molecule that can interact with α-syn clusters as evident from the proteomic studies provided in "Appendix Tables S5-7". Unfortunately, since α3-NKA is a critical molecule in the CNS for the maintenance of ionic gradient, it cannot be knocked-out.

1. In particular there is no demonstration that the binding of alpha-syn assemblies is involved in PD.

There is now emerging evidence for a prion like propagation of α-syn (reviewed in Brettschneider et al., 2015; Nat Rev NSci). Several studies show that α-syn injection leads to PD-like deposit of α-syn in the mice brain several months following injections. We have made use of such a model (Desplats et al., 2009; Hansen et al., 2011; Volpicelli-Daley et al., 2011; Luk et al., 2012; Mougenot et al., 2012; Rey et al., 2013; Sacino et al., 2014, Holmqvist et al., 2014; etc) to approach the initial interaction of α-syn with neuronal membranes. The injected α-syn formed clusters, some of them being associated with synapses as found in our *in vitro* experiments. α3-NKA

was associated with these clusters at early time point (8-24h post injection). It is not the aim of this work to study the interaction of exogenous α-syn with the plasma membrane several months after injection, but rather to focus on initial interactions.

2. There are no experiments that support the idea that the binding of alpha-syn oligomers or fibrils to NKA is required for alpha-syn diffusion, clustering, and change in ionic gradients (i.e mutational analysis/KO/KD experiments).

The first section of the discussion "*Clustering of α-syn assemblies within neuronal plasma membrane and synapses*" deals with this issue. As stated above we do not believe or state that α3-NKA is required for α-syn binding to the neuronal plasma membrane. We just mean that while diffusing in the plasma membrane, α3-NKA molecules are trapped by α-syn clusters.

a) This can be seen using super-resolution microscopy (Figure 6D) emphasizing that all α-syn clusters do not necessarily co-localize with α3-NKA.
b) Mutational experiments (Figure 5), shows that α3-NKA trapping by α-syn is prevented (Figure 5D) without affecting the binding of α-syn to the plasma membrane (data not shown).
c) A movie (Movie EV1) is now provided to demonstrate the trapping of freely diffusing α3-NKA within α-syn cluster.

3. There is also no demonstration that alpha-syn binding to the plasma membrane requires NKA.

Please see point 2.

Additional concerns:

4. The authors need to explain why oligomeric and fibrillar alpha-syn were applied at different doses. In Figure 1A/C, the authors should perform cell fractionation and western blots to support the notion that alpha-syn binds plasma membranes?

The biochemical experiments were performed at the same monomeric α-syn concentration. We previously (Pieri et al., 2012, Biophys J. and Bousset et al., 2013 Nat Com) determined the concentration of α-syn particles (oligomer and fibrils) at any given monomeric α-syn concentration.

All immunocytochemistry experiments were performed using 25nM oligomeric and 0.03nM fibrillar α-syn (particle) concentration. A higher concentration of oligomeric α-syn was used because fluorescence was weaker at low concentrations (see e.g. in Appendix Figure S3). Importantly, even at higher concentrations, oligomeric α-syn exhibited weaker interaction with α3-NKA compared to fibrillar α-syn assemblies.

The binding of α-syn to the plasma membrane is shown as follows:

a) We have now replaced the confocal image with 2-color PALM/STORM super-resolution image (Figure 1C). The resolution with this approach is 20-40 nm and allows visualization of α-syn binding onto the membrane. With this approach we now show that α-syn form clusters, on the extracellular side of the neuronal plasma membrane.
b) This was further evidenced by immunodetection of α-syn clusters without permeabilization (Appendix Figure S2).
c) SPT trajectories shown in Figure 2F were as expected on the neuronal surface. The long-trajectories and high mobility (10^{-1} μm^2/sec) favors the notion that the α-syn molecules are lipid bound (also see discussion (page 19) section: "*Clustering of α-syn assemblies within neuronal plasma membrane and synapses"*).

5. The authors should show some images or movies to support the quantification of the diffusion coefficients.

We have included example of α-syn trajectories (Figure 2E and 2F) and Movie EV1 exemplifying a diffusion-trap (for trajectory shown in Figure 4A) is provided.

6. The authors need to perform knockout/knockdown of alpha3-NKA to demonstrate alpha-syn no longer binds to the plasma membrane or has reduced binding and/or that the diffusion coefficients for oligomeric/fibrillar alpha-syn are altered, as well as the knockdown-rescue experiments

Please refer to point 2 and point 4. We do not claim that α3-NKA is required for the binding of α-syn to the plasma membrane but that diffusing α3-NKA may be trapped by clustered α-

syn. This is visually illustrated by a real time movie (see point 5).

7. Figure 5D. What are the high-resolution images shown for? It is quite difficult to see the difference. The authors need to provide quantification.

5D is now Figure 7A. The text in result section has been made clear (Page 13, 2nd paragraph). In this figures we show all detected molecules. The image result from the integration of 20 000 frames. In this figure α3-NKA is more clustered following oligomer/fibril exposure than in the control condition. This phenomenon is quantified classically by counting the number of detection in clusters (Figure 7B), the distance between detected α3-NKA clusters, which reflect molecular compaction (Figure 7C) and unbiased density based clustering (Figure 7D) developed by us.

8. Is there any difference between the misfolded alpha-synuclein and labeled misfolded alpha-synuclein in the in vivo injection studies.

The labeling of fibrils/oligomers does not modify their properties or the ability of the monomers and oligomers to assemble into fibrils (Bousset et. al., Nat Comm 2013; Hansen et. al., J Clin Invest 2011; Freundt et. al., Annals Neurol 2012, etc.). The α-syn assemblies have been prepared in the exact same way as in the above-mentioned articles and appropriately characterized (see material and methods and Appendix Figure S1).

9. It is not clear whether there is direct binding between alpha-synuclein and alpha3-NKA?

Following evidences supports the notion that the binding between the two proteins is direct:

a) New 2-color STORM super-resolution imaging (Figure 6D) showing clustering of α3-NKA where α-syn aggregates is present. Fibrils beings more associated than oligomers.
b) New co-immunoprecipitation experiments (Figure 3C) showing a direct interaction of α3-NKA with fibrillar and oligomers but not monomers. Here again fibrils interacted more strongly than monomers.
c) Mass-spectrometry (Figure 3, Table ES5-6) supporting the notion that fibrils interact to a higher extent (ratio: 6.0) than oligomers (ratio: 1.6).
d) Single Particle Tracking experiments using chimeric α3/α1-NKA constructs (Figure 5) showing that the mutations of two amino acids prevented α-syn dependent trapping of NKA.
e) Movie EV5 exemplifying diffusion trap phenomenon.

10. In Figure E2 the binding of alpha-syn fibril and alpha-syn oligomer does not exhibit any saturation kinetics consistent with classic ligand receptor binding raising concerns that the interaction reported here may be non-specific. Along these lines the authors need to include knockout or knockdown studies of NKA as well as determine the affinity of alpha-syn assemblies for NKA.

In line with point 2, 3, 4 and 6, please note that α3-NKA is not the only partner of α-syn. In other words, α-syn in its oligomeric and fibrillar forms likely bind to the plasma membrane and subsequently is slowed down by a diffusion-trap mechanism. The lack of saturation kinetics (at the concentration used here) further support the notion that α-syn binds to the membrane. The experiments shown (Appendix Figure S3) were performed in order to identify a suitable concentration of α-syn to be used to decrease the background and for a good fluorescence detection of α-syn. Finally, as stated above, α3-NKA is critical for the maintenance of ionic gradient in the CNS. Therefore, it cannot be knocked-out.

11. The authors report that they identified 54 and 197 protein partners for oligomeric and fibrillar alpha-syn of these they narrowed down to 12 and 73 membrane proteins. It would be important to provide both the membrane proteins and the non-membrane proteins in Table E5.

All the proteomics data is now provided as per the journal guidelines and the section has been improved based on 1st reviewer comments (Page 8, 1st paragraph and Tables S5-S6).

12. Can the authors confirm that alpha-syn oligomer interacts with NKA?

We are now providing new co-IP (Figure 3C) and 2-color STORM (Figure 6D) results for oligomeric α-syn and α3-NKA. These data further support the interaction of α-syn oligomers and NKA. Furthermore, SPT (Figure 4), immunofluorescence (6A-C), 1-color STORM (Figure 7), *in*

vivo injection (Figure 8) and Na^+ imaging (Figure 9) indicates that oligomeric α-syn interacts with α3-NKA.

13. Figure E4C is missing.

This has been corrected.

14. In the discussion the authors mention an observation for control proteomic study on pure astrocyte cultures, but these data are not presented for evaluation.

This dataset is presented as Appendix Table 7 (7A for oligomeric α-syn, 7B for fibrillar α-syn) in the revised version of the manuscript.

15. What evidence do the authors have that down regulation of NKA activity leads to increased susceptibility to glutamate excitotoxicity as suggested in the discussion.

This study was performed since several lines of evidence imply that the activity of neuronal NKA may have an impact on the calcium response following activation of the glutamatergic receptors. The mechanisms behind such an effect remain to be elucidated, but may be due either to direct interaction between NKA and the NMDA receptor and/ or to reversal of the Na^+/Ca^{2+} exchanger. The latter effect could be due to the reduced capacity to restore the sodium levels in the α-syn exposed neurons (see discussion, Pg 21, 3rd paragraph).

Although studies of the mechanisms by which impaired NKA activity enhances the response to glutamate is out of scope for the current manuscript, we feel that our descriptive observation of enhanced Ca^{2+} influx in α-syn assemblies exposed adds important information to this study.

16. In Figure 6, the authors need to include a set of comparisons in which alpha3-NKA is knockout or knocked down.

Knocking out α3-NKA is not viable. In addition, as stated in Point 2 and 4, the clustering of α-syn is independent of its interaction with α3-NKA. Figure 8 (previously Figure 6) is intended to validate *in vivo* that α-syn formed clusters and that α3-NKA was enriched within these clusters.

17. In Figure 7, how do the authors know that the change in Na+ dynamics is the direct consequence of misfolded alpha-synuclein directly acting on NKA? It is not clear why the authors examined a 24 hour time point since all the other experiments on alpha-syn assemblies were performed in the 60 min time window after alpha-syn application. Is the change in calcium flux due the effects of alpha-syn on NKA?

Figure 7 is now Figure 9. This part of the study was designed in order to investigate the pathological consequences of alpha-synuclein assemblies exposure. Dysfunctions in neuronal NKA activity will primarily result in alterations in the control of Na^+ levels. It has been demonstrated that neurological diseases associated with mutations in α3-NKA affect Na^+ affinity and intracellular Na^+ concentration (Toustrup-Jensen et al, JBC 2014). Therefore, we analyzed Na^+ dynamics as a read-out of NKA pathology. Our protocols were designed to analyze changes in intracellular Na^+, which are specifically mediated by the NKA (0 mM K+ solution and use of the specific NKA inhibitor ouabain, see Expanded View Figure EV4). Alterations in Na^+ dynamics were examined after 24 hr in order to match the *in vivo* experiments in which α-syn assemblies were detected after several hours (Figure 8). A detailed response to the last part of point 17 can be found in point 15.

Referee #3:

Shrivastava et al. describe in a set of well-controlled experiments their quest for putative protein interactors with recombinant oligomeric and fibrillar α-synuclein (α-syn) assemblies in primary neurons and identified, by affinity isolation and nanoLC-MS/MS analysis, neuronal α3-Na/K-ATPase (α3-NKA) as a principal binding protein. They furthermore present experimental evidence for the perturbation of Na efflux from neurons and increased glutamate induced calcium influx by α-syn assemblies. These interesting findings may greatly help to better understand molecular disease pathways in Parkinson's disease, particularly as mutations in α3-NKA confer risk of rapid-onset dystonia Parkinsonism.

Even though the large number of putative interacting proteins, including cytosolic and mitochondrial proteins clearly shows the limitations of S-protein agarose beads isolation,

stringent downstream analysis and exclusion of unlikely binding partners led to a trimmed list of fourteen putative interactors. The authors subsequently focussed on α3-NKA, a protein that was identified by affinity isolation with both, oligomeric and fibrillar α-syn.

We thank reviewer for peer reviewing this manuscript. Please see the point-by-point response as well as some information related to new data added to strengthen the manuscript. All the changes in the text are marked in red.

Main Comments

1. The experimental setup of this study follows the paradigm that aggregated α-syn oligomers are associated with pathogenesis, but surprisingly the authors have not investigated whether the identified protein, α3-NKA, interacts with monomeric α-syn and this leaves open the important question whether reorganisation of α3-NKA and subsequent perturbations of Na efflux and calcium influx are due to a gain of function by aggregated oligomeric species. This should be addressed by incubation of primary neurons with monomeric and oligomeric S-tag α-syn prior to chemical cross-linking (Figure 2C) and determination of α3-NKA-dependent perturbations (Figure 7).

Multiple evidences indicate that that monomeric α-syn do not interact and/or perturb α3-NKA.

A. New Co-IP experiments following exposure of neurons to α-syn monomers, oligomers and fibrils were performed. Fibrils showed strongest interaction, oligomers weaker one and monomers none (Figure 3C).
B. SPT experiments for α3-NKA were performed in presence of α-syn monomers (Expanded View Figure EV3 A): The diffusion of α3-NKA was not modified even at a 50 nM concentration (2x oligomer and 1666x fibrils particles concentration).
C. Na^+ imaging experiments were also performed in presence of monomeric α-syn (Expanded View Figure EV4 A-B) and the pumping activity of α3-NKA was unaffected.

2. The authors isolate α-syn interacting proteins in absence and presence of the chemical crosslinker DTPSS, but only report the interaction of α-syn with α3-NKA after crosslinking in Figure 2C. For completion the set of α-syn-interacting proteins in presence of crosslinking agent should be added in expanded view.

It should be highlighted that it is hazardous and extremely fastidious, if feasible, by any mean other than oriented searches, to exploit the crosslinking dataset. Cross-linking yields by definition "artifactual" interactions. Therefore this dataset should be carefully evaluated. We would like to stress that the cross-linking data set was not used to demonstrate interaction between α3-NKA and α-syn but to further strengthen the pull-down and co-immunoprecipitation experiments. This oriented search not only confirmed α3-NKA and α-syn interaction but allowed us to show that α3-NKA peptides exposed at the surface of the cells are found cross-linked to α-syn. We have now added an extra column in Appendix Table S6 (column 10) to show which other proteins were also identified by cross-linking with a "+" sign.

3. A method description for cell labeling with α-syn and live imaging is missing in the manuscript and should be added to Material and Methods in the main body.

We have added this to the Material and Methods section "Exposure to *α-syn, single particle tracking and analysis"*. A more detailed protocol is provided in the "Appendix" section (Page 26, Single Particle Tracking and analysis section).

4. In Figure 5 Shrivastava et al. describe the reorganisation of α3-NKA and α-syn assemblies in 21 day old striatal neurons, but apart from one area magnified in Figure 5Ab where colocalisation between a-syn and a3-NKA is clearly visible, levels of colocalisation are difficult to assess. It would thus be helpful to determine the corresponding Pearson correlation coefficients as a measure for the level of colocalisation.

To approach this issue, we previously performed an unbiased approach (Intensity Correlation Analysis), which confirmed and quantified that α3-NKA and α-syn co-localization (now Figure 6C). Furthermore, we now provide 2-color STORM (Figure 6D) of α3-NKA and α-syn. It emphasizes a stronger association and co-localization of α3-NKA with fibrillar as compared to oligomeric α-syn.

5. On page 13, second paragraph the authors refer to previous reports of a prion-like propagation of α-syn and in this context present their data on synaptic clustering and association with α3-NKA following in vivo injection of α-syn. Reference to prion-like propagation of α-syn is misleading here and for clarity it should be mentioned in the results section that observations in Figure 6 do not allow the conclusion that α-syn assemblies spread in a prion-like manner.

We have revised the last sentence of the paragraph to make things clear "*Thus this model allowed us to investigate if injected α-syn form clusters on the plasma membrane and interacts with α3-NKA in vivo at an early time-point post injection as observed in vitro*."

Minor points

6. The labeling of MS/MS spectra and Western blot in Figure E4 does not correspond to the legend. According to the legend the Western blot is Figure E4C, not E4B.

This has been now corrected. We have performed new co-IP experiments with different detergent/pre-clearing and show news set of western blots with less background (Figure 3C).

2nd Editorial Decision 18 June 2015

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the three original referees.

As you can see below, the referees appreciate the introduced changes and are supportive of the paper. However they also point out that there are some issues that still need some attention. The referees also find that the manuscript has to be written in a more careful way to accurately reflect the key findings.

Given these comments, I would like to ask you to resolve the last remaining issues in a final revision.

REFEREE REPORTS

Referee #1:

In their revision, the authors have addressed all criticisms but leave some unresolved issues. These are reiterated in the frame of my previous review below and followed by new comments (some pertaining to the revision material).

Specific Comments:

1. Description of Figure 1A,B "age" of neurons should be replaced by the more neutral term DIV throughout.

In contrast to the authors' response, culturing primary neurons for 2-3 weeks is still called "aging" repeatedly in the legend to Figure 1 and the results text. I don't think that is appropriate. It insinuates that human aging as THE risk factor of α-synucleinopathies is reflected here in a dish. In reality, keeping the cultures a couple of days longer in vitro is not organismal aging. In the present experiment, what happens is that more fibers can grow and bundle, astrocytes proliferate, etcetc. As Figure 1A lacks all cellular counterstains, it is not clear where the α-syn blobs associate to and how the underlying cultures look like. Such "observation not shown" (page 5, line 6) must be displayed. And do make sure to replace the term age-dependent with time-dependent throughout.
Also, I think the y-axis label Fig. 1B and all reference in the text is not strictly appropriate. If I understand the quantification method properly, what is measured here is the total fluorescence per microscopic field (which size what area?). Should that not be called something like "amount" of clusters? I would interpret "intensity" of clusters as fluorescence per spot (brightness).
Moreover, there are inconsistencies between Figure 1B and Figure S3C. While at 21DIV 0.03nM fibrils consistently yielded "intensities" of approximately 1.5, these values were 2.5 in Figure 1B but

25 in Figure S3C. Thus, the claimed enhanced clustering of oligomeric α-syn cannot be seen in Figure 1A as in Figure S3A. This is not only confusing but raises doubts about experimental variance.

2. The proteomic data must be presented with greater care.
a. It is recurrently stated in text, figures and legends that interacting membrane proteins are detected. While the rationale and subsequent validation efforts are clear, it must be understood that α-syn assembly interacting proteins were screened in whole cell lysates. No membrane enrichment steps can be made out from the experimental procedures. Consequently, a number of non-membrane spanning proteins were detected, such as 14-3-3, BiP/HSPA5, GAPDH, TAU, etc. Do rewrite all relevant parts to make this point clear. For example titles of Table E5 should read: "proteins interacting with extracellularly applied α-syn [assemblies]".
b. Upload the proteomic raw data following the appropriate guidelines.
c. Explain the selection criteria for Table E5. What is meant by "annotation of their localization and validation with published data" (page 7)?
d. Figure 2A is dispensable, the procedure is clear enough. The criteria for the short list in Figure 2B are misunderstood or erroneous. Proteins like those mentioned in point 2a are obviously not "integral membrane proteins with an extracellular domain and/or extracellular peripheral membrane proteins" (page 7). In fact, Figure 2B should be deleted. The complete Table E5 is sufficient and much more relevant. Finally, Figure 2C can be merged into Figure E3.

The authors' responses to criticisms c. and d. are not satisfactory. The pull-down flow (now Figure 3A) is superfluous, the method is clear enough. The criteria for the short list (now Figure 3B) remain ill defined and the figure should be deleted.
Simply looking at Table S5, it appears that GRP78 is chosen because of 4 + criteria. Note by the way that GRP78 interacts with fibrils, not oligomers in astrocytes (Table S7B). Leaving aside the fact that GRP78 is even a marker for an ER resident (chaperone) protein that has no transmembrane domain whatsoever and got the GO annotations from some cancer literature, why was α3-NKA selected getting only 3 + selection criteria? The (negative) GO term "extracellular domain" is indeed incorrect, as α3-NKA does have short extracellular stretches, comprising in part the ouabain binding site. So the authors deviated from the systematic approach manually correcting in this case but not others. Why not choose VAP-A and AP-3 δ1 (3 + criteria), or UCH-L1 (4 + criteria) or creatine kinase B? Checking uniprot.org, there is no GO term "membrane" or "plasma membrane" for CKB. Conversely, what about GAPDH, which in their own analysis scores negative in all the GO terms! And so on and so forth. As this is an irrelevant point anyway, delete this pellmell short list figure, keep the full Appendix Tables as blunt listings, and simply state that α3-NKA was deliberately picked in a hypothesis-driven approach.

e. For which of the proteins in Table E5B did the crosslink/MS approach yield confirmatory peptide masses? It is understood that the experiment 2C intended to validate α3-NKA, but a more comprehensive analysis of that experiment would be an informative additional column for Table 5B.
f. The co-IP experiment in Figure E4B is not convincing. The authors may be right that the physical interaction is weak or transient, but the extent of non-specific binding is unacceptable. Control probing for α3-NKA is missing as well. And why does fibrillar α-syn not migrate as a smear? Use of species-specific antibodies could furthermore distinguish between exogenous human α-syn assemblies and the endogenous rat α-syn protein.

The revised co-IP looks better, includes α-syn inputs, and compares monomer, oligomers and fibrils. I am very sympathetic with attempts to specifically co-immunoprecipitate membrane proteins and sticky proteins like α-syn, which is very hard I know. Nevertheless, the experiment shown in the new Figure 3C is really only meaningful with a negative control, for example α1-NKA or another structurally related protein, such as PMCA.
While attending specificity, I find it astonishing that α-syn fibrils interact exclusively with α3-NKA. In the description of the crosslinking experiment, the authors highlight not only peptide 884-901 (loop 7-8) but also a peptide 903-928. (In fact two, should the penultimate peptide in Figure EV1A not be called 903-930?) The authors go on to state an explicit crosslink at lysine-928, which is the cytosolic boundary of TM8, quite far away from loop 7-8 and the ouabain binding pocket (cf Laursen et al. (2013) PNAS 110:10958). Together with the finding of interacting masses of β1-NKA, it indicates that the huge assemblies of α-syn clusters cover a rather large area around the juxta/membrane topology of this P-type ATPase. Given such a large interface, it is surprising that

replacing only 2 amino acid residues in loop 7-8 abolishes the "trapping" in α-syn clusters (Figure 5D). Though impressive, this experiment only tells us that these 2 amino acids are an important determinant in the context of α3-NKA. Please show biochemical confirmations of chimera expression levels, proper cellular processing and transport. Importantly, the most relevant control, namely α1-NKA itself, should be recorded as a reference. By the way, this result chapter and figure legend title should read "selective" not "direct" interaction, no?
Moreover, given the structural similarity of the plasma membrane Ca2+-ATPase, I need to see more specificity controls. Enhanced intracellular Ca2+ levels (Figure EV5) could not only be indirectly due to an inhibited Na+/K+ pump, but by analogy a direct consequence of an inhibited plasma membrane Ca2+ pump. Absence of mass spec detection is not a rigorous argument (false negatives) and as the authors argue themselves, such functional interactions do not necessarily need to be physically strong enough to be detected.

In general, I find that too strong language is used to describe the direct physical interaction between α3-NKA and α-syn assemblies and their co-clustering. The evidence in Figures E4C and 5A is not impressive.

It still find the visual evidence of α3-NKA depletion outside of α-syn clusters (or RE-distribution in the authors chapter title) not impressive. Does not by far the most α3-NKA (green) stays outside (red) α-syn clusters in Figure 6? Yes, some α3-NKA can be trapped in α-syn clusters, but should there not remain a large majority outside and thus remain functional? How can we be sure the ionic effects are due to this narrow interaction, and not a more global membrane perturbance when α-syn clumps are dumped on cells?

g. It was focused on α3-NKA because it "was the only one interacting with both oligomeric and fibrillar α-syn". What about AP-3 δ1 and VAP-A??

Additional Comments:

3. In the revised Figure 1C, it should be explained why α-syn and α3-NKA are addressed as biologically relevant clusters throughout the manuscript, but not the TMD-Dendra, which has a similar appearance at this resolution. In fact, TMD-Dendra should be evenly distributed throughout the plasma membrane. The patchy appearance is likely a result of the super-resolution image gain. Frankly, I preferred the original Figure 1C.

4. Along these lines, is STED microscopy not less prone to patchiness? Would STED microscopy as done by Blom and colleagues reveal clearer pictures, if feasible?

5. The authors argue for a lateral diffusion and insinuate some directed synaptic transport. Figure 2 does show cluster "growth" and demonstrates trapping. But what about synaptic targeting? I cannot make this out from the data. It looks as if there would be simply more clusters, and the increased co-localization with synaptic markers is simply stochastic. Importantly, the diffusion coefficients intra- and extra-synaptic are identical (Figure 2G). This should be commented.
For α3-NKA, Figure 4Dd traces do not match the quantification (Figure 4E). Stronger effects are quantified for fibrils, whereas the cumulative frequency plots show more reduction for oligomers. Also the extra-synaptic effects are stronger in Figure 4E, contrasting Figure 4D.

6. Page 9, line 6, correct "one of these peptides" not peptide.

Referee #2:

EMBOJ-2015-91397R, corr. author Dr. Triller
"α-synuclein assemblies sequester neuronal α3-Na+/K+-ATPase and impair Na+ gradient"

This is revised manuscript. The authors have performed a number of additional experiments and/or clarified the presentation of the data to minimize misinterpretations of their data. I find the abstract misleading as the authors start off by stating that "alpha-syn propagates in prion-like manner" and finish with "our results demonstrate that the reduction in alpha-3NKA activity is a determinant of alpha-syn-mediated deleterious pathway" The data presented in the current manuscript don't support

these statements. As such I strongly recommend that the first and last sentence of the abstract be eliminated. Also the last sentence of discussion is not supported by the data. A better way to state it would be as follows:
"Similarly the interaction of alphs-syn assemblies with alpha3NKA interfering with Na+ pumping of neurons may contribute to alpha-synopathies such as PD, etc." Otherwise it should be removed as well.

Referee #3:

The authors provide further experimental evidence to support the argument that oligomeric and fibrillar α-syn assemblies specifically interact with α3-NKA to perturb Na+ extrusion. Whilst additional data provided make the authors' case stronger, clarity should be provided for some open questions as specified below.

Specific responses:

Point 1: To strengthen the specificity argument, i.e. that the observed effects on α3-NKA trapping and the subsequent perturbation of Na efflux and Ca influx are due to aggregated α-syn assemblies, but not to monomeric rec. a-syn, the authors have now performed complementary experiments with monomeric α-syn.

A. A new set of co-immunoprecipitation experiments with monomeric, oligomeric and fibrillar α-syn (Figure 3C) addresses the interaction with a3-NKA. Overall, the effects are weak and do not provide unambiguous evidence that aggregated forms of a-syn, and particularly fibrillar α-syn interacts with a3-NKA. However, new data from STORM super-resolution imaging, showing colocalisation between a-syn-Alexa647 and α3-NKA-ATTO488 in Figure 6D provides further evidence for an interaction between these proteins. It is however imperative for STORM super-resolution imaging to include procedures for the correction of chromatic shifts in order to exclude the possibility that putative areas of colocalisation are due to spectra shifts, rather than to real interactions and the methods section does not specify a procedure to correct for chromatic shifts (like beads correction) and the authors should provide images corrected for chromatic offset, in case this has not been done.

B. To further strengthen the argument that monomeric α-syn does not exert the reported effects on α3-NKA trapping, the authors note that single particle tracking experiments for α3-NKA in presence of α-syn monomers in Expanded View Figure EV3 A were supportive of this view.

Figure legend EV3 describes in vivo synaptic clustering of fibrillary α-syn and association with α3-NKA and not the diffusion of α3-NKA in presence of monomeric α-syn. I am assuming that the control experiment refers to Figure EV2A. In this figure the authors report two additional controls to show that α3-NKA diffusion is unaffected by monomeric α-syn and by Abeta-oligomers. Whereas the legend reads that no slowdown in the diffusion coefficients is reported for both cases, changes are significant ($p<0.01$) for monomeric α-syn and not significant for oligomeric Abeta. This is very confusing and inconsistent with the response given.
A close examination of Figure 4D shows that traces representing extra-synaptic α3-NKA diffusion were superimposed and non-significant at 5min after incubation with oligomeric α-syn, whereas in all other cases the shifts to lower diffusion coefficients were reported as significant for extra-synaptic and synaptic diffusion. In the control experiments traces shift to the right, and this raises the question whether monomeric α-syn, but not Abeta oligomers increases α3-NKA diffusion significantly. I would like to ask the authors to comment on these inconsistencies.

C: Data provided in Figure EV4 show that α3-NKA-dependent Na+ deregulation by oligomeric, but not by monomeric α-syn.
Point 2: This reviewer accepts the argument that the current reporting of a-syn-interacting proteins in absence of crosslinking agents is favourable over that in presence of the crosslinker DTPSS.

Points 3-6: The suggested changes have been made.

Other comments:

On page 7, the title for the proteomic screen reads: "Identification of proteins that interact specifically with extracellularly applied oligomeric and fibrillar α-syn assemblies". Whereas a substantial body of work addresses the specificity of protein interactions with α-syn assemblies, no experimental evidence has been provided to attest specificity for 177 other neuronal proteins identified. The word "specifically" is therefore incorrectly used in this context and should be deleted.

2nd Revision - authors' response 09 July 2015

YELLOW: Highlights the new changes in the manuscript following 2nd revision
Blue: Reviewer's comments

Referee #1:

In their revision, the authors have addressed all criticisms but leave some unresolved issues. These are reiterated in the frame of my previous review below and followed by new comments (some pertaining to the revision material).
Specific Comments (*Italicized are comments following 1st review*):
1. Description of Figure 1A,B "age" of neurons should be replaced by the more neutral term DIV throughout.
In contrast to the authors' response, culturing primary neurons for 2-3 weeks is still called "aging" repeatedly in the legend to Figure 1 and the results text. I don't think that is appropriate. It insinuates that human aging as THE risk factor of α-synucleinopathies is reflected here in a dish. In reality, keeping the cultures a couple of days longer in vitro is not organismal aging. In the present experiment, what happens is that more fibers can grow and bundle, astrocytes proliferate, etc etc. As Figure 1A lacks all cellular counterstains, it is not clear where the α-syn blobs associate to and how the underlying cultures look like. Such "observation not shown" (page 5, line 6) must be displayed. And do make sure to replace the term age-dependent with time-dependent throughout.

We agree with the reviewer that "age" is not the appropriate term for cultures *in vitro*. The term "age-dependent" has been replaced everywhere with "culture days-dependent" including in Figure 1 and "DIV" used for days *in vitro*. "Time-dependent" has not been used as suggested since the term "time-dependent" is used for incubation time (i.e. 5 min and 60 min exposure).

The number of days neurons are cultured is a determinant of their maturity (axons, dendrites, synapses). The synapse development is shown below (**Figure A**, synapsin immunoreactivity). In our experiments, we have used Cytosine β-D-arabinofuranoside in order to reduce glial proliferation (see Appendix Methods, Page 22).

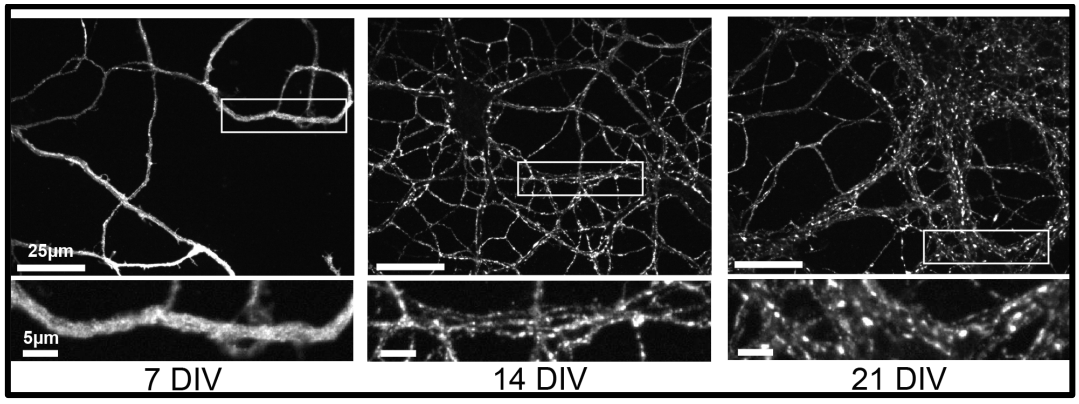
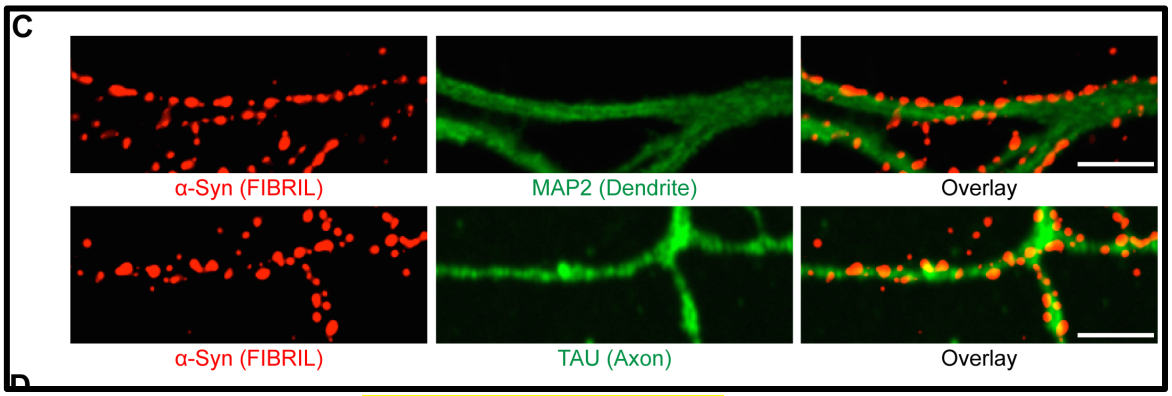


Figure A. Immunolabeling of synapsin on cultured neurons

"Observation not shown" refers to α-syn clustering on axons and dendrites, which were not shown. This is now illustrated in **Figure 1C** (see below) showing that α-syn form clusters both on axons and dendrites.



NEW panel added as Figure 1C

Also, I think the y-axis label Fig. 1B and all reference in the text is not strictly appropriate. If I understand the quantification method properly, what is measured here is the total fluorescence per microscopic field (which size what area?). Should that not be called something like "amount" of clusters? I would interpret "intensity" of clusters as fluorescence per spot (brightness).

Y-axis in Figure 1B is: "Intensity of Cluster". This corresponds to the total fluorescence intensity per cluster (bright spot). This is now explained in the methods section (Appendix methods section, page 26, paragraph 1).

We have never measured total fluorescence per field. This was not in the previous version. "No of fields" in the figure legend refer to the number of microscopic field that were quantified. As described in the methods (Appendix, page 26, paragraph 1), images were thresholded to isolate individual clusters (wavelet decomposition). Then the fluorescence intensities associated with each clusters were measured. As requested we now mention in the methods (Appendix, page 26, paragraph 1) that the spinning disk microscope field of view was 1392 x 1042 pixel.

"Amount of cluster" is not as appropriate since it signifies the absolute number of cluster. This was not measured in Figure 1B (see above).

Moreover, there are inconsistencies between Figure 1B and Figure S3C. While at 21DIV 0.03nM fibrils consistently yielded "intensities" of approximately 1.5, these values were 2.5 in Figure 1B but 25 in Figure S3C. Thus, the claimed enhanced clustering of oligomeric α-syn cannot be seen in Figure 1A as in Figure S3A. This is not only confusing but raises doubts about experimental variance.

There is no inconsistency since what is measured is different. In Figure 1B, the intensity of clusters value of ~2.5 correspond to 25nM oligomers and not to fibrils. Comparison between figures cannot take into consideration values, which are issued from various experiments and are therefore normalized independently. The value ~25 in **Figure S3C** is also for 25nM oligomers and relates to "No. of clusters" and not "Intensity of clusters".

It should be noted that the fluorescence intensity values associated with fibrils should not be compared with those obtained for oligomers, since they have specific binding properties.

2. The proteomic data must be presented with greater care.
a. It is recurrently stated in text, figures and legends that interacting membrane proteins are detected. While the rationale and subsequent validation efforts are clear, it must be understood that α-syn assembly interacting proteins were screened in whole cell lysates. No membrane enrichment steps can be made out from the experimental procedures. Consequently, a number of

non-membrane spanning proteins were detected, such as 14-3-3, BiP/HSPA5, GAPDH, TAU, etc. Do rewrite all relevant parts to make this point clear. For example titles of Table E5 should read: "proteins interacting with extracellularly applied α-syn [assemblies]".
b. Upload the proteomic raw data following the appropriate guidelines.
c. Explain the selection criteria for Table E5. What is meant by "annotation of their localization and validation with published data" (page 7)?
d. Figure 2A is dispensable, the procedure is clear enough. The criteria for the short list in Figure 2B are misunderstood or erroneous. Proteins like those mentioned in point 2a are obviously not "integral membrane proteins with an extracellular domain and/or extracellular peripheral membrane proteins" (page 7). In fact, Figure 2B should be deleted. The complete Table E5 is sufficient and much more relevant. Finally, Figure 2C can be merged into Figure E3.
The authors' responses to criticisms c. and d. are not satisfactory. The pull-down flow (now Figure 3A) is superfluous, the method is clear enough. The criteria for the short list (now Figure 3B) remain ill defined and the figure should be deleted. Simply looking at Table S5, it appears that GRP78 is chosen because of 4 + criteria. Note by the way that GRP78 interacts with fibrils, not oligomers in astrocytes (Table S7B). Leaving aside the fact that GRP78 is even a marker for an ER resident (chaperone) protein that has no transmembrane domain whatsoever and got the GO annotations from some cancer literature, why was α3-NKA selected getting only 3 + selection criteria? The (negative) GO term "extracellular domain" is indeed incorrect, as α3-NKA does have short extracellular stretches, comprising in part the ouabain binding site. So the authors deviated from the systematic approach manually correcting in this case but not others. Why not choose VAP-A and AP-3 δ1 (3 + criteria), or UCH-L1 (4 + criteria) or creatine kinase B? Checking uniprot.org, there is no GO term "membrane" or "plasma membrane" for CKB. Conversely, what about GAPDH, which in their own analysis scores negative in all the GO terms! And so on and so forth. As this is an irrelevant point anyway, delete this pellmell short list figure, keep the full Appendix Tables as blunt listings, and simply state that α3-NKA was deliberately picked in a hypothesis-driven approach.

Reviewer 1 discusses the relevance of using the GO annotations as they may either be incomplete or incorrect.

- "The (negative) GO term "extracellular domain"", e.g. proteins that do not appear as having extracellular domains, may be incorrect. Indeed, α3-NKA does have short extracellular stretches, as indicated by the literature (Laursen et al PNAS, 2013, 110:10958) but does not appear as possessing extracellular domains based on GO annotation.

- "The (negative) GO term "extracellular domain"" may appear as incorrect for GAPDH. However, GAPDH was selected because of a possible extracellular exposure reported in the literature. Indeed, Makhina T. et al (Cell Neurosci. 2009 41:206-18) have reported that GAPDH can be detected extracellularly at the cell surface of neuronal cells, using surface biotinylation and immunocytochemistry, and that addition of GAPDH antibodies to cultured cerebellar neurons inhibited L1-dependent neurite outgrowth, while application of exogenous GAPDH promoted L1-dependent neurite outgrowth.

- "The (positive) GO term "extracellular domain"" for GRP78 is again subject to debate as recent data report the possibility that GRP78 relocates at the surface of neurons (Bellani S et al, Cell Death Differ. 2014; 1:1971-83). Very interestingly, Bellani et al have recently shown, that extracellular soluble synuclein relocates GRP78 to the plasma membrane, binds to GRP78 on the cell surface of neurons and induces a clustering in microdomains of surface-exposed GRP78 necessary for the activation of the signaling cascade leading to cytoskeleton alterations.

Additionally, the reviewer points out that AP-3 delta1 and VAP-A were not picked up as "plasma membrane". These proteins while GO annotated as plasma membrane proteins were not selected as such because they are peripheral membrane proteins with an exclusive cytosolic exposure.

These negative or positive examples highlight the limits of the GO annotations and term criteria. We would like also to stress that GO term annotations may differ when using NCBI or Uniprot.

Following the reviewer comments, the justifications given above and the fact that GO annotations are sometimes incomplete or even incorrect and although this annotation is widely used, we deleted the GO term as selection criteria for our protein lists (in the text, and in the tables).

We state in the results section that "*Among the identified candidates α3-subunit of NKA was picked for further study because of the confirmatory results of a hypothesis-driven approach: the pull-down data indicate that α3-NKA is the only transmembrane protein of our list with extracellularly exposed domains and was identified both with oligomeric and fibrillar α-syn.*" (see table S5 and S6).

We have also deleted Figure 3B as recommended by the reviewer. We however would like to have Figure 3A for two main reasons:

a) Though it may be straightforward for an experienced biologist to understand our protocol; the paper should be accessible to a wide group of scientist. They may find a pictorial representation of the protocol useful and easy to understand.
b) It contributes to the maintenance of easy flow of the manuscript and resumes in an easy way the content of Appendix methods.

e. For which of the proteins in Table E5B did the crosslink/MS approach yield confirmatory peptide masses? It is understood that the experiment 2C intended to validate α3-NKA, but a more comprehensive analysis of that experiment would be an informative additional column for Table 5B.

f. The co-IP experiment in Figure E4B is not convincing. The authors may be right that the physical interaction is weak or transient, but the extent of non-specific binding is unacceptable. Control probing for α3-NKA is missing as well. And why does fibrillar α-syn not migrate as a smear? Use of species-specific antibodies could furthermore distinguish between exogenous human α-syn assemblies and the endogenous rat α-syn protein.

The revised co-IP looks better, includes α-syn inputs, and compares monomer, oligomers and fibrils. I am very sympathetic with attempts to specifically co-immunoprecipitate membrane proteins and sticky proteins like α-syn, which is very hard I know. Nevertheless, the experiment shown in the new Figure 3C is really only meaningful with a negative control, for example α1-NKA or another structurally related protein, such as PMCA.

While attending specificity, I find it astonishing that α-syn fibrils interact exclusively with α3-NKA. In the description of the crosslinking experiment, the authors highlight not only peptide 884-901 (loop 7-8) but also a peptide 903-928. (In fact two, should the penultimate peptide in Figure EV1A not be called 903-930?) The authors go on to state an explicit crosslink at lysine-928, which is the cytosolic boundary of TM8, quite far away from loop 7-8 and the ouabain binding pocket (cf Laursen et al. (2013) PNAS 110:10958). Together with the finding of interacting masses of β1-NKA, it indicates that the huge assemblies of α-syn clusters cover a rather large area around the juxta/membrane topology of this P-type ATPase.

Given such a large interface, it is surprising that replacing only 2 amino acid residues in loop 7-8 abolishes the "trapping" in α-syn clusters (Figure 5D). Though impressive, this experiment only tells us that these 2 amino acids are an important determinant in the context of α3-NKA. Please show biochemical confirmations of chimera expression levels, proper cellular processing and transport. Importantly, the most relevant control, namely α1-NKA itself, should be recorded as a reference. By the way, this result chapter and figure legend title should read "selective" not "direct" interaction, no? Moreover, given the structural similarity of the plasma membrane Ca2+-ATPase, I need to see more specificity controls. Enhanced intracellular Ca2+ levels (Figure EV5) could not only be indirectly due to an inhibited Na+/K+ pump, but by analogy a direct consequence of an inhibited plasma membrane Ca2+ pump. Absence of mass spec detection is not a rigorous argument (false negatives) and as the authors argue themselves, such functional interactions do not necessarily need to be physically strong enough to be detected.

Following the 1st revision, we did new Co-IP experiments with a modified protocol in order to increase the signal/background ratio. The original experiments were performed using fibrillar α-syn assemblies. In order to provide "internal" controls, we included oligomeric and monomeric α-

syn assemblies. As expected, interactions with α3-NKA were absent with monomers and weak with oligomers. The stronger interaction between α3-NKA and fibrillar α-syn was confirmed with 2-color dSTORM imaging (Figure 6D), SPT (Figure 4), and Na+ imaging (Figure 9). Monomeric α-syn (the negative control) showed no interaction/effect in these experiments. Furthermore, as additional controls, mass-spec experiments (provided following 1st revision and based on reviewer's suggestion), did not reveal the presence of NKA-α2 or β1 subunits in astrocytes, which normally expresses them. These are very strong control experiments indicating that both oligomeric and fibrillar assemblies of α-syn DOES NOT INTERACT with the latter proteins.

The reviewer is right, the "absence of mass spec detection is not a rigorous argument of the absence of a protein". We would like nonetheless to stress the following: for α3-NKA, ATP1a3 is composed of 1013 amino acids, 809 of which are non-trans-membrane amino acids. We detected overall 11 peptides of the 44 expected theoretical tryptic peptides in the mass range of 800 to 4000 Da. For Ca^{2+}-ATPase, Atp2b3 is composed of more than 1200 amino-acids, 1000 of which are non trans-membranous. None of the 50 expected theoretical tryptic peptides in the mass range of 800 to 4000 Da were detected. While this is not an absolute "proof of absence", this finding is nonetheless compelling and suggests that if α-syn interacts with Ca^{2+}-ATPase the complex must be much less abundant than that with α3-NKA (ATP1a3) otherwise one if not more peptide(s) would have been detected in the same dynamic range. Thus trying to co-IP α-syn with proteins that were not identified in several independent mass-spectrometry experiments (neurons and astrocytes) will not add to the specificity of the manuscript.

Reviewer's suggestion that "enhanced intracellular Ca^{2+} level following α-syn exposure could be a direct consequence of an inhibited plasma membrane Ca^{2+} pump" is way off. PMCA is a low capacity Ca^{2+} transporter, and therefore the difference in Ca^{2+} response between control and α-syn exposed neurons should have been progressive rather than instantaneous.

Experiments in **Figure 9** were specially designed to test the function of the α3 isoform. The α3-NKA has a much lower Na^+ affinity than α1-NKA and the increase in intracellular Na^+ that accompanies the exposure to a K^+ free solution will by far exceed the V_{max} for α1 subunit. Rat α3 has also a much higher ouabain sensitivity than rat α1. In Figure EV4 ouabain at 1 μM concentration was used, which should completely inhibit α3, but have no effect on α1. Thus we clearly demonstrate that it is the α3-NKA physiology that is affected by α-syn and not that of α1-NKA.

Furthermore, mutation of 2-residues (see Figure 5) prevented/reduced the slow-down mediated by fibrillar α-syn. 4-different constructs of α3-NKA (wild-type and chimeras) were used and all of them had similar diffusion properties (Figure 5) and membrane expression (**Figure B**, below). We do not claim that the 2-amino acids are the only binding site and the text in the manuscript states: "*Thus amino acid Leu (L)878 and Asn(N)879 in the loop between TM7-TM8 of α3-subunit of NKA (Figure 5A) are likely to play a key role in α3-NKA interaction with α-syn assemblies."* – Page 11, 1st paragraph.

All SPT experiments were performed using pHluorin-expressing NKA wild type or α3/α1-NKA constructs. All constructs exhibited a similar level of surface expression **Figure B** below.

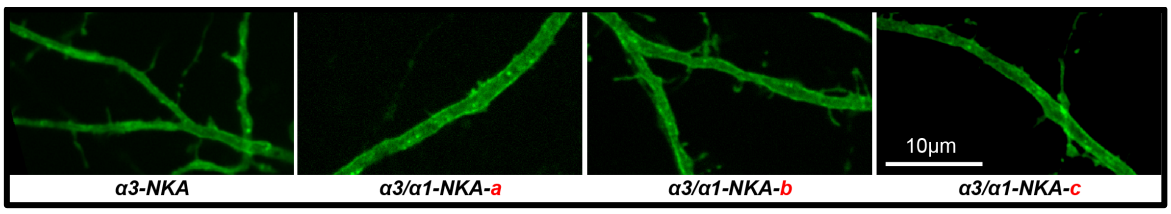


Figure B. Expression of Wild-type and chimeric constructs

Reviewer 1 is right that the penultimate peptide (with a mass of 3337.66135) in Figure EV1A is the 903-930 peptide and not the 903-928. This has been corrected. The reviewer 1 is wrong when he/she claims that we "state an explicit crosslink at lysine-928". In the figure legend and in the text page 9, we clearly indicate that the cross-linked residue might be either at Serine 915 or at

Lysine 928. The MS/MS spectrum did not allow determining precisely which of these two residues was modified by the DTSSP.

We have removed the term "Direct" and replace it by "Selective" as suggested by the reviewer.

In general, I find that too strong language is used to describe the direct physical interaction between α3-NKA and α-syn assemblies and their co-clustering. The evidence in Figures E4C and 5A is not impressive.

It still find the visual evidence of α3-NKA depletion outside of α-syn clusters (or Re-distribution in the authors chapter title) not impressive. Does not by far the most α3-NKA (green) stays outside (red) α-syn clusters in Figure 6? Yes, some α3-NKA can be trapped in α-syn clusters, but should there not remain a large majority outside and thus remain functional? How can we be sure the ionic effects are due to this narrow interaction, and not a more global membrane perturbance when α-syn clumps are dumped on cells?

We performed an unbiased analysis (Figure 7D) to estimate molecular density (see Appendix Methods). With STORM super-resolution, we found that at a given density of 5000 detection events, nearly 40% of them were clustered. While in presence of α-syn, 50-60% of them were within clusters. This led to a 10-20% reduction of the freely available population and thus will have physiological consequences. As stated above, **Figure 9** was to test the function of the α3 isoform and distinguish it from α1 isoform. This clearly showed that the physiological effect on Na^+ perturbation is a direct consequence of loss-of-function of α3-NKA. Here again we do not claim that the re-distribution of α3-NKA is the sole mechanism: in the discussion section, we write that Page 21, 1st paragraph, we state that: "*In addition, the binding of α-syn to the extracellular loop of α3-NKA may interfere with its turnover and transition between E1 and E2 forms, thus reducing pumping activity*".

g. It was focused on α3-NKA because it "was the only one interacting with both oligomeric and fibrillar α-syn". What about AP-3 δ1 and VAP-A??

Please refer to the response to points a-d above.

Additional Comments:

3. In the revised Figure 1C, it should be explained why α-syn and α3-NKA are addressed as biologically relevant clusters throughout the manuscript, but not the TMD-Dendra, which has a similar appearance at this resolution. In fact, TMD-Dendra should be evenly distributed throughout the plasma membrane. The patchy appearance is likely a result of the super-resolution image gain. Frankly, I preferred the original Figure 1C.

TMD-dendra data were acquired for 10000 frames (and not until saturation), as we were interested in identifying the membrane silhouette and not in the whole TMD-Dendra distribution. The confocal figure was replaced by super-resolution to address some of the concerns raised by reviewer 2. Actually this new figure is much more informative since it contains quantitative information.

4. Along these lines, is STED microscopy not less prone to patchiness? Would STED microscopy as done by Blom and colleagues reveal clearer pictures, if feasible?

Both STED and STORM are complementary approaches, and it is not expected to have differences in the distribution of a protein when looked with these 2 different techniques. We perform studies on several other membrane proteins and the patchiness has nothing to do with PALM/STORM, and reflects actual molecular distribution.

5. The authors argue for a lateral diffusion and insinuate some directed synaptic transport. Figure 2 does show cluster "growth" and demonstrates trapping. But what about synaptic targeting? I cannot make this out from the data. It looks as if there would be simply more clusters, and the increased co-localization with synaptic markers is simply stochastic.

Importantly, the diffusion coefficients intra- and extra-synaptic are identical (Figure 2G). This should be commented.

Reviewer is correct that there is no evidence for direct synaptic targeting and co-localization may result from stochastic diffusion process and trapping. We have modified the discussion accordingly as follows (Page 19, 1st paragraph): "*Unlike Aβ oligomers, which are primarily enriched at excitatory synapse (Renner et al, 2010), α-syn molecules clustered both at excitatory and inhibitory synapses and may also form clusters on the extra-synaptic membrane including axonal and dendritic membrane. Since α-syn diffusion was not significantly different in- and out of synapse, it favors the notion that α-syn confinement and clustering is not dependent on the compartment where it occurs.*"

Both *in vitro* and *in vivo* data suggests that ~50% α-syn clusters are at synapses. In addition, Figure 6B (and Figure 8E) show that in presence of α-syn there is a higher synaptic accumulation of α3-NKA. The time-dependent synaptic accumulation of α3-NKA is due to lateral diffusion. Actually it was stated in the discussion section: "*Given that α3-NKA is more enriched at synapses, the effect will be important on synaptic ion concentration*" (Page 22, 1st paragraph).

For α3-NKA, Figure 4Dd traces do not match the quantification (Figure 4E). Stronger effects are quantified for fibrils, whereas the cumulative frequency plots show more reduction for oligomers. Also the extra-synaptic effects are stronger in Figure 4E, contrasting Figure 4D.

There is no contradiction but some explanation is needed. Actually, the diffusion coefficient and the area explored are different parameters encompassing different concepts. However, the variation of the median diffusion coefficient (mobility) can be compared with that of the variation of the median explored area (confinement) values (now shown in Table S9). This comparison can give information on the type of confinement. This is why, following the reviewer comment, we have now added a column in **Table S9** (see below): it shows the "percentage reduction" of the diffusion coefficient and that of the surface area explored following α-syn exposure.

In order to be clear, we also show the actual values (not percentage) and to this aim replaced **Figure 4E** by a new version (see below). This new version shows the distribution of "Area Explored" values. This new figure emphasizes that α-syn assemblies also significantly affect extra-synaptic α3-NKA molecules. This is related to the fact (reported above) that α-syn assemblies also form clusters outside of synapses where the diffusion is slowed down and confined.

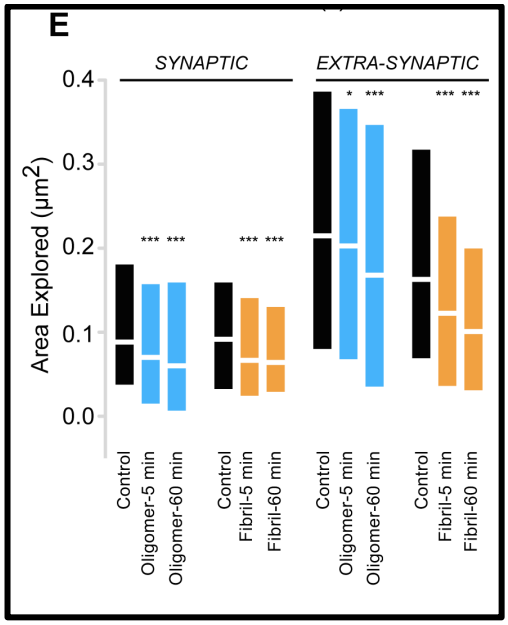
Modified Appendix Table S9

(In red: Percentage difference from median value of control)

Oligomeric α-syn (SYNAPTIC)					
	No of QDs	**Median Diffusion Coefficient ($\mu m^2/s$)**		**Median Area Explored (μm^2)**	
Control	268	0.0551		0.0884	
Oligomer-5 min	308	0.0440 (**)	-20%	0.0702 (***)	-21%
Oligomer-60 min	306	0.0385 (***)	-30%	0.0602 (***)	-32%
Oligomeric α-syn (EXTRA-SYNAPTIC)					
Control	1273	0.1021		0.2148	
Oligomer-5 min	1277	0.0968 (ns)	-5%	0.2028 (*)	-6%
Oligomer-60 min	1360	0.0823 (***)	-19%	0.1679 (***)	-22%

Fibrillar α-syn (SYNAPTIC)					
Control	373	0.0603		0.0917	
Fibril-5 min	528	0.0546 (*)	-9%	0.0668 (***)	-27%
Fibril-60 min	466	0.0476 (***)	-21%	0.0641 (***)	-30%
Fibrillar α-syn (EXTRA-SYNAPTIC)					
Control	1234	0.0925		0.1631	
Fibril-5 min	1484	0.0694 (***)	-25%	0.1228 (***)	-24%
Fibril-60 min	1520	0.0583 (***)	-36%	0.1010 (***)	-38%

Modified Figure 4E



6. Page 9, line 6, correct "one of these peptides" not peptide.

Corrected.

Referee #2:

EMBOJ-2015-91397R, corr. author Dr. Triller
"α-synuclein assemblies sequester neuronal α3-Na+/K+-ATPase and impair Na+ gradient"
This is revised manuscript. The authors have performed a number of additional experiments and/or clarified the presentation of the data to minimize misinterpretations of their data. I find the abstract misleading as the authors start off by stating that "alpha-syn propagates in prion-like manner" and finish with "our results demonstrate that the reduction in alpha-3NKA activity is a determinant of alpha-syn-mediated deleterious pathway" The data presented in the current manuscript don't support these statements. As such I strongly recommend that the first and last sentence of the abstract be eliminated. Also the last sentence of discussion is not supported by the data. A better way to state it would be as follows:
"Similarly the interaction of alphs-syn assemblies with alpha3NKA interfering with Na+ pumping of neurons may contribute to alpha-synopathies such as PD, etc." Otherwise it

should be removed as well.

We thank the reviewer for accepting our changes following first revision. As suggested, the two sentences from abstract have been removed and the last sentence of the discussion has been modified (blue text).

Referee #3:

The authors provide further experimental evidence to support the argument that oligomeric and fibrillar α-syn assemblies specifically interact with α3-NKA to perturb Na+ extrusion. Whilst additional data provided make the authors' case stronger, clarity should be provided for some open questions as specified below.

Specific responses:

Point 1: To strengthen the specificity argument, i.e. that the observed effects on α3-NKA trapping and the subsequent perturbation of Na efflux and Ca influx are due to aggregated α-syn assemblies, but not to monomeric rec. a-syn, the authors have now performed complementary experiments with monomeric α-syn.

A. A new set of co-immunoprecipitation experiments with monomeric, oligomeric and fibrillar α-syn (Figure 3C) addresses the interaction with a3-NKA. Overall, the effects are weak and do not provide unambiguous evidence that aggregated forms of a-syn, and particularly fibrillar α-syn interacts with a3-NKA. However, new data from STORM super-resolution imaging, showing colocalisation between a-syn-Alexa647 and α3-NKA-ATTO488 in Figure 6D provides further evidence for an interaction between these proteins. It is however imperative for STORM super-resolution imaging to include procedures for the correction of chromatic shifts in order to exclude the possibility that putative areas of colocalisation are due to spectra shifts, rather than to real interactions and the methods section does not specify a procedure to correct for chromatic shifts (like beads correction) and the authors should provide images corrected for chromatic offset, in case this has not been done.

We thank the reviewer for his input. A new paragraph has been added in the Appendix methods section (Page 28, 3rd paragraph) explaining the correction method. We use beads for estimating and correcting both stage drift correction and color-alignment in STORM imaging. 2-color alignment was done by simultaneously aligning 3-4 beads. The images presented in Figure 1C and 6D are following stage drift-correction as well as beads alignment.

B. To further strengthen the argument that monomeric α-syn does not exert the reported effects on α3-NKA trapping, the authors note that single particle tracking experiments for α3-NKA in presence of α-syn monomers in Expanded View Figure EV3 A were supportive of this view. Figure legend EV3 describes in vivo synaptic clustering of fibrillary α-syn and association with α3-NKA and not the diffusion of α3-NKA in presence of monomeric α-syn. I am assuming that the control experiment refers to Figure EV2A. In this figure the authors report two additional controls to show that α3-NKA diffusion is unaffected by monomeric α-syn and by Abeta-oligomers. Whereas the legend reads that no slowdown in the diffusion coefficients is reported for both cases, changes are significant ($p<0.01$) for monomeric α-syn and not significant for oligomeric Abeta. This is very confusing and inconsistent with the response given. A close examination of Figure 4D shows that traces representing extra-synaptic α3-NKA diffusion were superimposed and non-significant at 5min after incubation with oligomeric α-syn, whereas in all other cases the shifts to lower diffusion coefficients were reported as significant for extra-synaptic and synaptic diffusion. In the control experiments traces shift to the right, and this raises the question whether monomeric α-syn, but not Abeta oligomers increases α3-NKA diffusion significantly. I would like to ask the authors to comment on these inconsistencies.

The absolute diffusion coefficient may vary from one experiment another. Cumulative

distribution was used in order to allow a good visualization of the effects. An effect is considered genuine, if independent experiments show a similar trend (e.g. decrease or increase or no change). This is an important approach in order to avoid bias resulting from a single experiment (on a single culture). For all the experiments (except Figure 5D), we consistently observed a diffusion slow-down of NKA following α-syn oligomers and fibrils exposure. No slowdown in diffusion of NKA could be seen with monomeric α-syn or Aβ oligomers (Figure EV2) but rather a slight acceleration, which was seen only in one experiment. Therefore the slight increase in diffusion coefficient is unlikely a real, reproducible, phenomenon.

The median diffusion coefficient values for Fig 4D-E is shown in Table S9. We have added new column (red text) to show the percentage reduction compared to control. In figure 4Db, there was no significant change in diffusion after a treatment (5min) with oligomeric α-syn application. This indicates that short time incubation with α-syn oligomers does not slow-down NKA. Oligomers form few small sized clusters in 5 min while several large sized clusters are seen after 60 min. However fibrils could slow down and capture α3-NKA more efficiently, as early as 5 min (refer Table S9).

C: Data provided in Figure EV4 show that α3-NKA-dependent Na+ deregulation by oligomeric, but not by monomeric α-syn.

Point 2: This reviewer accepts the argument that the current reporting of a-syn-interacting proteins in absence of crosslinking agents is favourable over that in presence of the crosslinker DTPSS.

Points 3-6: The suggested changes have been made.

Other comments:

On page 7, the title for the proteomic screen reads: "Identification of proteins that interact specifically with extracellularly applied oligomeric and fibrillar α-syn assemblies". Whereas a substantial body of work addresses the specificity of protein interactions with α-syn assemblies, no experimental evidence has been provided to attest specificity for 177 other neuronal proteins identified. The word "specifically" is therefore inorrectly used in this context and should be deleted.

We have removed the word 'specifically' as suggested.

3rd Editorial Decision 19 July 2015

Thank you for sending us your revised manuscript. I asked referee #1 to review the revision and I have now heard back from the referee. As you can see below the referee appreciate the introduced changes and supports publication here.

I am therefore very pleased to accept the manuscript for publication here.

Before I send you the formal acceptance letter, I just to discuss a point made by the referee. The referee is suggesting moving figure B from the point-by-point response into the main text. That is fine with me, but I will leave it up to you. Can you let me know what you prefer to do? If you would like to add the figure to the main manuscript then I will send the manuscript back to you so that you can upload it.

REFEREE REPORT

Referee #1

The authors have responded and discussed the criticisms very well. Thank you very much for the clarifications. Not being a high-resolution imaging expert, I clearly need more guidance in that

direction as opposed to Fig. 3A, which is perfectly straightforward to me ;-)

The only thing that the authors may want to do is to work the control Figure B of the rebuttal letter into the main Figure 5.

Finally, as the novelty and importance of this report is very high, I recommend this manuscript to be highlighted.

3rd Revision - authors' response 13 July 2015

We thank you and the 1st reviewer for providing final feedback on the manuscript. Based on reviewer's suggestion, we have now added a new supplementary figure (Appendix Figure S5). This figure shows that all the 4 plasmids used in Figure 5 are properly targeted to the plasma membrane. The accompanying text is highlighted in yellow (Main manuscript, Page 11).

