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Tunneling nanotubes spread fibrillar α-synuclein fibrils by intercellular trafficking of lysosomes

Saïda Abounit, Luc Bousset, Frida Loria, Seng Zhu, Fabrice De Chaumont, Laura Pieri, Jean-Christophe Olivo-Marin, Ronald Melki, Chiara Zurzolo

Corresponding author: Chiara Zurzolo, Institut Pasteur

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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 22 December 2015

Thank you for submitting your manuscript to The EMBO Journal. Three referees have now seen your study and I am afraid that the overall opinion is not very positive.

As you can see from the comments below, while the referees find the findings interesting they also raise concerns with it that I am afraid preclude publication here. Referee #1 raises a number of different issues that should be fairly straightforward to address. However, referees #2 and 3 raise more significant ones. The main criticism raised by both referees # 2 and 3 is that further work is needed to support that TNTs can promote spreading of a-synclein between cells and that work in primary neuronal cells is needed to support such a mechanism. In vivo support for this would of course be great, but I can imagine that would be very difficult to demonstrate, but as minimum we would need support for the described mechanism in primary neuronal cells. Given this concern and as it is unclear if you would be able to address it and the other issues raised I am afraid that I can't offer to invite a revision at this stage. If you are able to add work in primary cells and extend the findings along the lines as outlined by the referees we can discuss the possibility of a resubmission.

For the present submission, I am very sorry that I can't be more positive on this occasion.

REFEREE REPORTS

Referee #1:

In this manuscript, the authors showed the evidence that TNT is involved in cell-cell transfer of α-synuclein in in vitro culture cells. The authors also showed that the transferred α-synuclein fibrils are able to seed the misfolding and aggregation of the soluble protein after transfer. These findings are a breakthrough in understanding the mechanisms underlying the progression of synucleinopathies. One of the important unanswered questions in the study is the molecular mechanisms underlying the α-synuclein fibrils-mediated induction of TNT formation, although this would be a focus of the future study in the field.

The following should be addressed for the clarification of the manuscript.
1. The last part of the Introduction (lines 72-95) likely causes misunderstanding. For example, the second sentence "We found that efficient transfer of α-synuclein fibrils between neuronal cells relied on Tunneling nanotubes (TNTs) (Abounit and Zurzolo 2012; Austefjord, Gerdes, and Wang 2014; Sun et al, 2012)" can be interpreted to; these studies (Abounit and Zurzolo 2012; Austefjord, Gerdes, and Wang 2014; Sun et al, 2012) showed that efficient transfer of α-synuclein fibrils between neuronal cells relied on Tunneling nanotubes. Furthermore, there is not description of TNTs in the introduction other than this part.
Therefore, the authors should rewrite this part by separating the TNT description (the content of lines 79-88) and a brief summary of what they found in this study (the rest). The latter part should better be more concise.

2. The authors described that "Quantifications of co-localization (ICY software, see materials and methods) revealed that almost 90% of fibrils localized within endo-lysosomal vesicles with the majority of fibrils found in lysosomes (16.7% {plus minus}6.31% in EEA1, 24.9% {plus minus}0.52% in Vamp3 and 51.8% {plus minus}3.08% in Lamp1 positive vesicles)" as shown in Figure 6B. However, the representative images in Figure 6A show that the colocalization with Vamp3 seems the most among the three organella markers. Are these images in Figure 6A stacks of confocal images? If so, the authors should show series of single focal planes for better visual recognition of colocalization.

Minor points requiring the correction:
Page 6, line 140: in the sentence "α-synuclein fibrils were shown to be pathogenic and to be able seed in the brain in vivo α-synuclein fibrils were shown to be pathogenic and to be able seed in the brain in vivo", should the expression 'to be able seed' instead be 'to be able to seed'?

Page 6, lines 146−148: the sentence "To this aim we performed..." seems grammatically incorrect.

Page 7, line 174 (and thereafter): "up-taken" seems better be "taken up". 'uptake' is a noun, and 'take up' is usually used as a verb.

Page 8, line 177: "This data show" should either be "These data show" or "This data shows".

Page 8, lines 181-185: It is not Figure EV 5 but 4 that shows the data on the effect of dynamin 1 and its dominant negative mutants

Page 11, lines 261-262: the expression "in order to be established TNTs require cells to be in relative close contact" is not clear to the reviewer, and seems grammatically incorrect.

Referee #2:

Abounit et al address an important question in the field of spreading of proteins associated with neurodegeneration, specifically parkinson's disease. They propose that fibrils of a-synuclein do not spread in the space between cells but by transfer of lysosomes between cells via tunneling nanotubules. If this is correct, then it might have importance in development of therapeutics against a-synuclein. Against these positive comments, I have one very large concern and a number of smaller ones.

Major concern:
To illustrate the conceptual limitations of the study, it should be retitled to say what is really shown,

which is that: "tunneling nanotubules spread a specific, fibrillar, form of a-synuclein through tunneling nanotubules between murine cancer cells in culture". That is to say: what is the real evidence that such a mechanism could occur in the human mature CNS and contribute to disease pathogenesis? I do not propose that the authors have to fully answer this question but they do not to provide evidence to support relevance, specifically by showing that tunneling nanotubules exist between neurons and that a-synuclein can use this mechanism for transfer. Ideally, these experiments would be performed in vivo, but primary cultures might be a reasonable substitute if mechanism were confirmed in such preparations. Additionally, to claim pathogenesis the authors need to show either of the major pathological events in Parkinsons, namely loss of specific types of neurons and the formation of Lewy bodies.

Minor concerns
- Throughout the paper, the authors say they are counting the number and size of 'fibrils' per cell. While it is true that they added fibrils to the donor cells, it is not clear that fibrils, and only fibrils, are present in the cells. Are there thioflavin positive insoluble aggregates in the cells?
- The trypsin wash step is helpful in excluding some external synuclein, but is it also possible that aggregated protein might be subject to partial proteolysis and thereby generate a small oligomer competent for seeding?
- It might be helpful to add untreated acceptor cells to figures 3E, 3F.
- What is the proposed mechanism by which a-synuclein promotes TNT formation in 4B and is it specific to fibrils of this protein?
- The distinction between tunneling nanotubules and other types of cell:cell contacts (electrical or chemical synapses, tight junctions of similar) in figure 6 is not well developed.
- I didn't find the quantification in 7B to be particularly convincing as it seems that any small amount of localization in some vesicles would lead to positive singals. Better would be to use Rho or Mander's coefficient.
- It would be helpful to show some of the controls in 7C, such as cells labelled with fibrils of lysotracker alone as colocalization is much higher for lysotracker than Lamp1.

Referee #3:

In this manuscript Abounit et al., provide evidence that alpha-synuclein fibrils are transferred between CAD cells via TNTs. The work is potentially interesting adding to a previously described mechanism for prions (Gousset et al, 2009, Nat Cell Biol) and huntingtin (Costanzo et al., 2013, J Cell Sci) and adapted for the transfer of alpha-synuclein. They make use of a non-neuronal model. Several questions are raised by this MS. The cell type used (CAD cells: which author state as neuronal cells is although a cell line derived from the CNS; are not neurons per se). Indeed, they form long processes (or TNTs). CAD cells are not neurons and some of the effects can results form the fact that the cells uptake alpha-synuclein which may promote the tubes in a non-physiological way. Actually in cultured neurons, cell-to-cell transfer happens over days whereas in the reported experiments, about 100% of the CAD cell are efficiently loaded with alpha-synuclein fibrils. The rapid transfer in CAD cells is probably true for CAD cells, but is it true for neurons? The high concentration of synuclein fibrils (1µM) will create stress, ROS increase and promote nanotube formations. Indeed there is no doubt that that cell-to-cell transfer of synuclein via TNTs happens in CAD cells under the experimental conditions, and the work can be amended. However the manuscript do not demonstrate nor address that such a mechanism is true in vivo and/or primary neuronal cultures.

On a critical note, authors correctly emphasize that cell-to-cell contact is necessary for alpha-synuclein transfer, but they ignore the fact that most of the studies (which they cite) deals with "synaptically" connected where TNTs have not been demonstrated to exist.

Major Points:
1) Authors show a 100% transfer within 24h. The experiment has no controls: no time-or concentration-dependence. What is more surprising is that even tough the authors show that 100% of the donor cells were synuclein positive (Fig 1A) within 6h, they decided to perform their key experiments after 15h treatment. It is unclear why such a delay was preferred? What could be the efficiency of transfer after 1h, 6h, 10... synuclein treatment.
2) Other important controls are missing using monomers or oligomers, which have been shown to be

internalized. Indeed the authors mention that they are using disease-associated fibrils and this is the reason they see such efficient transfer and/or seeding. If this is true, smaller oligomers and monomers would be expected to be less potent in transfer and/or seeding in this model. The toxicity should have been tested as well.
3) Authors assume that fibril-size limits the transfer (Fig 1E). However, they look only at one time-period of transfer. A time-dependent characterization of the phenomenon is lacking. The release, transfer, uptake in primary cultured neurons takes place over longer periods (Volpicelli-Daley et al, 2011), demonstrating that the transfer and seeding is a slow process in neurons. If the authors want to claim about size limitation effects, they should make use of fibrils of various sizes.
4) How was the health of the cells checked after a 15h treatment? How long does it take for the recipient or donor cells before cell-death initiates? The concentration of fibrils (1µM) used in this work is very high. The donor cells being overloaded with exogenous synuclein, may simply drive transfer through TNTs resulting from the stress. Experiments should be done with lower concentrations.
5) As mentioned above CAD cells makes TNTs, however the evidence that TNTs exist between neurons is weak. The authors should provide convincing evidences that TNTs exist between real-neurons. Since primary neurons uptake fibrils, experiments using primary neurons as donor cells and CAD cells being recipient cells could be a first approach.
6) Figure 2A. A more convincing representative images and higher magnifications should be provided. The control figure (merged-image) gives a false impression of the presence of aggregates within nucleus whereas it lacks in ChFP channel. This look like and exposure issue. The authors could provide images without DAPI.
7) Exogenous synuclein induces seeding and aggregation of endogenous synuclein. Do CAD cells express synuclein? If yes, then the implication of endogenous synuclein if any in the transfer via TNTs should be evaluated eventually by using a KO donor.
8) In Figure 3A-B, it is not surprising that there is less co-localization between exogenous (green) and transferred (red) since the number of red punctas are sparse in the acceptor cells. This reduces the probability of co-localization. What needs to be quantified here is the percentage of ATTO550 spots co-localized with Alexa488 spots and not vice versa.
9) It is stated in the discussion that ROS induction leads to stress and hence lead to more tube formation. It needs to show that exogenous synuclein induces ROS generation. An increased number of tubes between cultured primary neurons treated with synuclein should also be shown.
10) A time-lapse study of the transfer would be welcome. Given the rapid and efficient transfer observed within 24h, it should be possible to capture such a mechanism by video microscopy. Is it a uni-directional transport or can some fibrils shuttle back? Do the authors also see aggregation at the TNT initiation site.
11) Lysosome transfer raises the question of the transfer of lysosomes to the TNTs? Does exogenous synuclein alters the rate of lysosome transfer via TNTs?
12) While Figure 7C-D shows nicely that donor lysosomes are involved in synuclein transfer, the reciprocal experiment shown in Fig EV5C is not convincing because the Rab-7 lysosome staining is diffuse.

Minor Points:

1. Maintain standard scientific referencing style. E.g. Baark et al., and not Heiko Baark et al. Luk et al, 2012a, b instead of Luk, Kehan , Zhang et al. 2012 or Luk, Kehan, Caroll et al.).
2. Refrain from using neuronal cells and rather use neuron-like cells. We know that these cells are far from being neurons.
3. The discussion needs to be shortened. There are too many speculations.
4. Data not shown should be provided as supplementary.

1st Revision - authors' response 04 June 2016

Referee #1

In this manuscript, the authors showed the evidence that TNT is involved in cell-cell transfer of α-synuclein in in vitro culture cells. The authors also showed that the transferred α-synuclein fibrils are able to seed the misfolding and aggregation of the soluble protein after transfer. These findings are a breakthrough in understanding the mechanisms underlying the progression of synucleinopathies. One of the important unanswered questions in the study is the molecular mechanisms underlying the α-synuclein fibrils-mediated induction of TNT formation, although this would be a focus of focus of the future study in the field.

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Therefore, the authors should rewrite this part by separating the TNT description (the content of lines 79-88) and a brief summary of what they found in this study (the rest). The latter part should better be more concise.

Response:
We followed the referee suggestions. Please see lines 73 to 84 for the introduction on TNTs and lines 85 to 94 for the findings.

2. The authors described that "Quantifications of co-localization (ICY software, see materials and methods) revealed that almost 90% of fibrils localized within endo-lysosomal vesicles with the majority of fibrils found in lysosomes (16.7% ±6.31% in EEA1, 24.9% ±0.52% in Vamp3 and 51.8% ±3.08% in Lamp1 positive vesicles)" as shown in Figure 6B. However the representative images in Figure 6A show that the colocalization with Vamp3 seems the most among the three organella markers. Are these images in Figure 6A stacks of confocal images? If so, the authors should show series of single focal planes for better visual recognition of colocalization.

Response:
*We appreciate this remark, indeed we have changed the figure (now **Figure 5A**) to show more representative pictures. However, we chose to show Z-projections to*

appreciate the distribution of α-synuclein and organelle markers within the whole cell because we believe that it is more accurate than showing only single plan images.

Minor points requiring the correction:
Page 6, line 140: in the sentence "α-synuclein fibrils were shown to be pathogenic and to be able seed in the brain in vivo α-synuclein fibrils were shown to be pathogenic and to be able seed in the brain in vivo", should the expression 'to be able seed' instead be 'to be able to seed'?

Response:
This has been rephrased (see lines 138-141).

Page 6, lines 146−148: the sentence "To this aim we performed..." seems grammatically incorrect.

Response:
This has been corrected (see line 143).

Page 7, line 174 (and thereafter): "up-taken" seems better be "taken up". 'uptake' is a noun, and 'take up' is usually used as a verb.

Response:
We thank the referee, we changed uptake to taken up.

Page 8, line 177: "This data show" should either be "These data show" or "This data shows".

Response:
This has been corrected (see line 174).

Page 8, lines 181-185: It is not Figure EV 5 but 4 that shows the data on the effect of dynamin 1 and its dominant negative mutants.

Response:
We thank the referee for this correction. The dynamin data, lines 176-182 are presented in ***Figure EV4*** *of the revised version of the manuscript.*

Page 11, lines 261-262: the expression "in order to be established TNTs require cells to be in relative close contact" is not clear to the reviewer, and seems grammatically incorrect.

Response:
This was rephrased, line 245-6.

Referee #2:

Abounit et al address an important question in the field of spreading of proteins associated with neurodegeneration, specifically Parkinson's disease. They propose that fibrils of a-synuclein do not spread in the space between cells but by transfer of lysosomes between cells via tunneling nanotubules. If this is correct, then it might have importance in development of therapeutics against a-synuclein. Against these positive comments, I have one very large concern and a number of smaller ones.

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Response:
Following this reviewer suggestions we performed experiments using primary neuronal cultures. We show that the conditions of transfer reported using CAD cells are valid for primary neurons in culture. We also provide direct evidence of TNTs between primary neurons and CAD cells as suggested by referee # 3. Because of lack of specific markers we cannot, at this stage, clearly show TNTs between primary cells, however the whole of our data support an important role for TNTs in the intercellular transfer between primary neurons in cell culture. We hope that this referee will appreciate the large amount of work we have performed following his/her specific request and will be satisfied with the combinations of the results.
As to the comment on pathogenesis, we would like to stress that this issue is not dealt within the present manuscript. Our current work analyses the cell-to-cell transfer of α-synuclein fibrils. Nonetheless the same fibrils have been shown to induce Lewy bodies-like structures when injected to model rodents in another paper (Peelaerts et al., 2015 Nature).

Minor concerns –
Throughout the paper, the authors say they are counting the number and size of 'fibrils' per cell. While it is true that they added fibrils to the donor cells, it is not clear that fibrils, and only fibrils, are present in the cells. Are there thioflavin positive insoluble aggregates in the cells?

Response:
These fibrils have been used before and proven to seed the aggregation of reporter α-synuclein in cell cultures (Bousset et al., 2013 Nature Communications). They have also been shown to induce the formation of Lewy bodies-like structures in rodents

(Peelaerts et al., 2015 Nature). We assessed the nature of the fibrils using a widely used filter retardation assay. We show that the fibrils that have been taken up, transferred and/or exported are indistinguishable from initial material. Nonetheless, we understand the referees' concerns and are very sensitive to this issue. We have therefore decided to use the terms "foci" or "puncta" instead of fibrils throughout the manuscript.

- The trypsin wash step is helpful in excluding some external synuclein, but is it also possible that aggregated protein might be subject to partial proteolysis and thereby generate a small oligomer competent for seeding?

Response:
We have washed the cells using diluted (0.1%) trypsin for 30 seconds (30s X 3) and then twice with PBS, the probability for trypsin to digest synuclein is very low within this time scale and at this concentration. Indeed after 30mn trypsin treatment WT α-synuclein fibrils are not digested (Yonetani et al, J.Biol Chem, 2009). In addition, trypsin wash has already been used to remove putative α-synuclein fibrils bound to cells in culture (Luk et al, PNAS, 2009).

- It might be helpful to add untreated acceptor cells to figures 3E, 3F.

Response:
Given that control acceptor cells have no detectable α-synuclein the quantification will give zero. This may be confusing for the reader since we want to compare α-synuclein transfer in control condition (co-culture) to filter or CM conditions. Now this is shown in ***Figure 3D, E****.*

- What is the proposed mechanism by which a-synuclein promotes TNT formation in 4B and is it specific to fibrils of this protein?

Response:
We propose that synuclein promotes TNT formation through oxidative stress, which has previously been shown to induce TNT formation in CAD cells. We have observed a sustained ROS increase (50% compared to control cells) following fibrils uptake which might contribute to TNT formation (discussed briefly in line 443), however although this is a very interesting question, addressing the mechanisms of TNT formation is not within the scope of this manuscript. We would like to add that we did not observe ROS increase with monomeric α-synuclein in agreement with previous reports showing no toxicity associated to 10μM monomeric α-synuclein and over (Pieri et al, 2012 Biophysical Journal; Pieri et al., 2016 Scientific Reports).

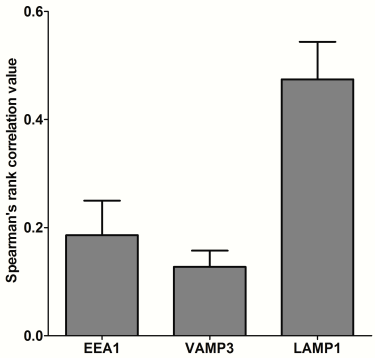
- The distinction between tunneling nanotubules and other types of cell:cell contacts (electrical or chemical synapses, tight junctions of similar) in figure 6 is not well developed.

Response

*In Figure 6, now **Figure 5B**, we have shown α-synuclein colocalizing with Lamp1 inside TNT connecting cells. We have now added details in the Figure legend to describe these structures and discriminate them from filopodia. This is now at lines 1025-32.*

- I didn't find the quantification in 7B to be particularly convincing as it seems that any small amount of localization in some vesicles would lead to positive singals. Better would be to use Rho or Mander's coefficient.

Response
*We thank the referee for this suggestion and we changed the panels to more representative pictures. Now this became **Figure 5C**. We used Spearman's (rho) rank correlation as suggested by this referee to analyse fibrils co-localization with organelles vesicles in acceptor cells. We found that the results are similar to the one we analysed with ICY method. As shown below transferred fibrils predominantly co-localized with Lamp1 positive vesicles (47.44% ±6.95%) in acceptor cells. Only a few fibrils co-localized with EEA1 or Vamp3 positive vesicles (26.26% ±6.38% and 12.74% ±3.00% for EEA1 and Vamp3, respectively). Since the data were similar we kept the ICY analyse in the paper as we used this method throughout the manuscript.*



- It would be helpful to show some of the controls in 7C, such as cells labelled with fibrils of lysotracker alone as colocalization is much higher for lysotracker than Lamp1.

Response
*We added control cells loaded with fibrils grown independently from acceptor following the referee suggestion in **Figure 5D**.*

Referee #3:

In this manuscript Abounit et al., provide evidence that alpha-synuclein fibrils are transferred between CAD cells via TNTs. The work is potentially interesting adding to a previously described mechanism for prions (Gousset et al, 2009, Nat Cell Biol) and huntingtin (Costanzo et al., 2013, J Cell Sci) and adapted for the transfer of alpha-synuclein. They make use of a non-neuronal model. Several questions are raised by this MS. The cell type used (CAD cells: which author state as neuronal cells is although a cell line derived from the CNS; are not neurons per se). Indeed, they form

long processes (or TNTs). CAD cells are not neurons and some of the effects can results form the fact that the cells uptake alpha-synuclein which may promote the tubes in a non-physiological way. Actually in cultured neurons, cell-to-cell transfer happens over days whereas in the reported experiments, about 100% of the CAD cell are efficiently loaded with alpha-synuclein fibrils.

Response
We thank the referee for this comment: we agree that CAD are not neurons "sensu stricto" but are CNS derived neuronal-like cells. Therefore we have repeated our experiments using primary cortical murine neurons. However, contrary to what the referee states we found quite rapid uptake of α-synuclein fibrils, as also previously reported in cortical neurons after 24h uptake (Freundt et al, 2012, Ann Neurol; Brahic et al, 2016, Acta Neuropathologica).

We have performed the uptake experiments in primary cortical mouse neurons (embryonic day 17) and observed that at 7div neurons internalized α-synuclein fibrils as early as 6h. After overnight, the percentage of neurons with internalized fibrils was around 75 and around 100% using respectively 0,5 and 1μM α-synuclein fibrils. Since all the previous experiments with CAD cells were performed with 1μM for 16 hours, we chose this concentration and time point for the rest of the experiments involving primary neurons. These data are included in the revised version of the manuscript in ***Figure 6A*** *and supplementary* ***Figure EV 1F****.*

The rapid transfer in CAD cells is probably true for CAD cells, but is it true for neurons?

Response
*We have co-cultured donor primary cortical neurons containing α-synuclein fibrils with acceptor naive primary cortical neurons for 3 days. Our data, now included in the revised version of the manuscript (****Figure 6B, C****), demonstrate the presence of α-synuclein puncta in acceptor neurons. This strongly suggests that the transfer is also occurring between primary cortical neurons.*

The high concentration of synuclein fibrils (1μM) will create stress, ROS increase and promote nanotube formations. Indeed there is no doubt that that cell-to-cell transfer of synuclein via TNTs happens in CAD cells under the experimental conditions, and the work can be amended. However the manuscript does not demonstrate nor address that such a mechanism is true in vivo and/or primary neuronal cultures.

Response:
Demonstrating that TNT-mediated α-synuclein fibrils transfer from neuron to neuron in vivo is beyond the scope of the manuscript. To our knowledge, this is technically unfeasible given crowding even with techniques such as expansion microscopy (Chen F. et al., 2015 Science) and given the fact that a specific TNT markers does not exist (yet). This is why additional experiments were performed with primary neurons. The additional data we present in the revised version of the manuscript show that α-synuclein fibrils transfer between donor and recipient neurons and between neurons and (as this referee suggested) neuron-like cells through TNTs. Indeed, we observed

the presence of α-synuclein puncta inside a TNT connecting a neuron and a neuron-like cell, which in addition to being in agreement with our results using CAD cells, further support a possible role of TNT as a mechanism of transfer. In any case, follow up studies when technical barriers are broken will help gain further insight and demonstrate the relevance of the mechanism in vivo.

On a critical note, authors correctly emphasize that cell-to-cell contact is necessary for alpha-synuclein transfer, but they ignore the fact that most of the studies (which they cite) deals with "synaptically" connected where TNTs have not been demonstrated to exist.

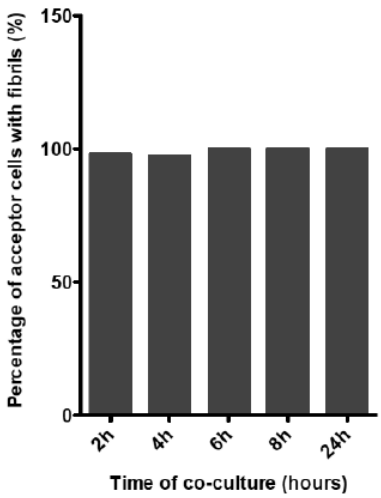
Response
While we agree with this referee on the presence of synaptic connections in the in vivo studies (although no studies have demonstrated the necessity of synaptic connections), this is not valid for all the studies that demonstrated transfer in vitro. Indeed most of the studies used cell lines that we and others found to form TNTs (i.e., PC12, SHSY5Y and HEK cells) (Desplat et al, 2009; Lee et al, 2010; Konno et al, 2012; Lee et al, 2013; Bae et al, 2014). In addition synapses-independent transfer of α-synuclein fibrils between primary neurons and second-order immature neurons has been demonstrated using microfluidic devices (Freundt et al. 2012).

In addition, in this study we used two different models to evaluate transfer in primary cortical neurons. In the first one we used donor and acceptor neurons and in the second one we used donor neurons and acceptor CAD cells. In the first case donor neurons were around one week old and the acceptor neurons were prepared from a new dissection and added on top of donor neurons, leaving them for 3 days in co-culture. Under these conditions, we ruled out a role of synaptic connections since synapses are not established within this time-frame in vitro. It is possible that synaptic connections are already established between donor neurons, however, it is very unlikely that immature acceptor neurons establish synapses with donor neurons after 72 hours. It would be interesting to try to inhibit synapses formation using this model in future studies. In the second approach we also ruled out this possibility, since transfer was evaluated after 24 hours, and undifferentiated CAD cells do not establish synaptic connections.

Major Points:
1) Authors show a 100% transfer within 24h. The experiment has no controls: no time-or concentration-dependence. What is more surprising is that even tough the authors show that 100% of the donor cells were synuclein positive (Fig 1A) within 6h, they decided to perform their key experiments after 15h treatment. It is unclear why such a delay was preferred? What could be the efficiency of transfer after 1h, 6h, 10... synuclein treatment.

Response
We have performed a time course of transfer and found that after 4hours, 100% of acceptor cells contained fibrils (see Figure below). In addition, the proportion of fibrils that are taken up by CAD cells is 2.8% compared to the total amount of added

*fibrils (1µM), see new **Figure 3C**. This proportion is reached at 16 hours. This is why we choose this time for co-culture.*



2) Other important controls are missing using monomers or oligomers, which have been shown to be internalized. Indeed the authors mention that they are using disease-associated fibrils and this is the reason they see such efficient transfer and/or seeding. If this is true, smaller oligomers and monomers would be expected to be less potent in transfer and/or seeding in this model. The toxicity should have been tested as well.

Response
The referee is pointing out a very important issue. Indeed while studies have addressed the toxicity attributed to the different forms of synuclein (monomer, oligomers and fibrils) and resulted in controversial results, no comparative study has been done in the context of synuclein transfer. We repeatedly showed that the only α-synuclein species that is most toxic in cell culture assay and that induces disease-like phenotypes in vivo is the fibrillar form of the protein (Pieri et al., 2012; Bousset et al., 2013; Peelaerts et al., 2015; Holmqvist et al., 2014; Pieri et al., 2016). We do not consider monomeric α-synuclein as a relevant control as what we show at the end is that the fibrillar form of the protein transfers through TNTs within lysosomal vesicles. Finally, we recently showed that oligomeric α-synuclein is not a well defined species but rather a continuum of species ranging from low molecular weight species to short fibrils (Pieri et al., 2016). Thus, although it might be interesting to study the transfer of oligomers and monomers we feel that this is out of the scope of this paper.

3) Authors assume that fibril-size limits the transfer (Fig 1E). However, they look only at one time-period of transfer. A time-dependent characterization of the phenomenon is lacking. The release, transfer, uptake in primary cultured neurons takes place over longer periods (Volpicelli-Daley et al, 2011), demonstrating that the transfer and seeding is a slow process in neurons. If the authors want to claim about size limitation effects, they should make use of fibrils of various sizes.

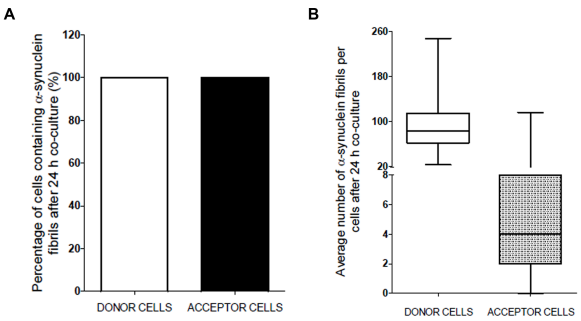
Response
We thank the referee for this comment. We evoked the size-limitation to explain our result and this was more a hypothesis than a statement however we were most probably unclear. What we see in the microscope is not single fibrils but rather bundles of fibrils. This is why we renamed these particles puncta in the revised version of the manuscript. The referees' suggestion cannot be assessed as;

1- *the fibrils are already short (0.05µm on average and made of ~4000 monomers, as shown in* ***Figure EV 1A*** *given they were slightly sonicated) and*
2- *we observed that internalization or take up is less efficient when the fibrils are longer.*

Nonetheless, we modified our hypothetical statement.
Concerning the Volpicelli-Daley et al study, they focused on intracellular transfer of fibrils and not on intercellular transfer as we do, therefore we cannot compare the kinetic of transfer.
Moreover, in another study where identical fibrils were used, transfer of fibrils between primary neurons was clearly demonstrated after 4 days in co-culture which suggests that synuclein fibrils transfer in primary neurons does not appear to be a slow process (Freundt et a, 2012).

4) How was the health of the cells checked after a 15h treatment? How long does it take for the recipient or donor cells before cell-death initiates? The concentration of fibrils (1µM) used in this work is very high. The donor cells being overloaded with exogenous synuclein may simply drive transfer through TNTs resulting from the stress. Experiments should be done with lower concentrations.

Response
We thank the reviewer for this remark and followed his/her advice. Indeed we have performed the transfer experiment with a lower concentration of fibrils (0.3µM). As expected, we found that 100% of CAD acceptor cells contained fluorescent α-synuclein (see Figure panel A below). However, the number of fibrils in acceptor cells that transferred was very low (Panel B, median number of 4). Based on our previous results that showed a higher amount of transferred fibrils in acceptor cells when donor cells are treated with 1µM (median number of 40 fibrils per acceptor cells, Figure. 1D in manuscript), we confirmed that the optimal concentration of α-synuclein to load donor cells is 1uM.



We would like also to stress that although 100% of cells contained exogenous α-synuclein puncta, the amount of fibrillar alpha-synuclein that is taken up by cells exposed to 1µM of fibrils is 28nM as now shown in ***Figure 3C****.*

Regarding the health of the cells after 15 hours exposure to a-synuclein fibrils, we performed time course experiments of LDH release in both CAD cells and primary neurons (Tran et al, 2014). No significant differences between control and treated cells after loading them with different concentrations of α-synuclein fibrils within the range used in this study were observed over time. These new data are now included in the manuscript as supplementary data; ***Figure EV 1C*** *and* ***G****.*
In our experimental models with CAD cells and primary neurons, we used donor cells after 16 hours of incubation with the fibrils, when no cytotoxicity was detected. Then, the transfer of α-synuclein was measured in acceptor cells after 24 and 72 hours, respectively, when donor cells were still healthy. Our results are in agreement with previous studies in which cell death was observed after 2 weeks of a-synuclein challenge in primary neurons (Volpicelli-Daley et al, 2011) and also with recent data suggesting that cell-to-cell passage of α-synuclein occurs between healthy neurons (Ulsoy et al., 2015). We did not measure when cell death initiated in the recipient cells, since the study of this mechanism was beyond the scope of the present study.

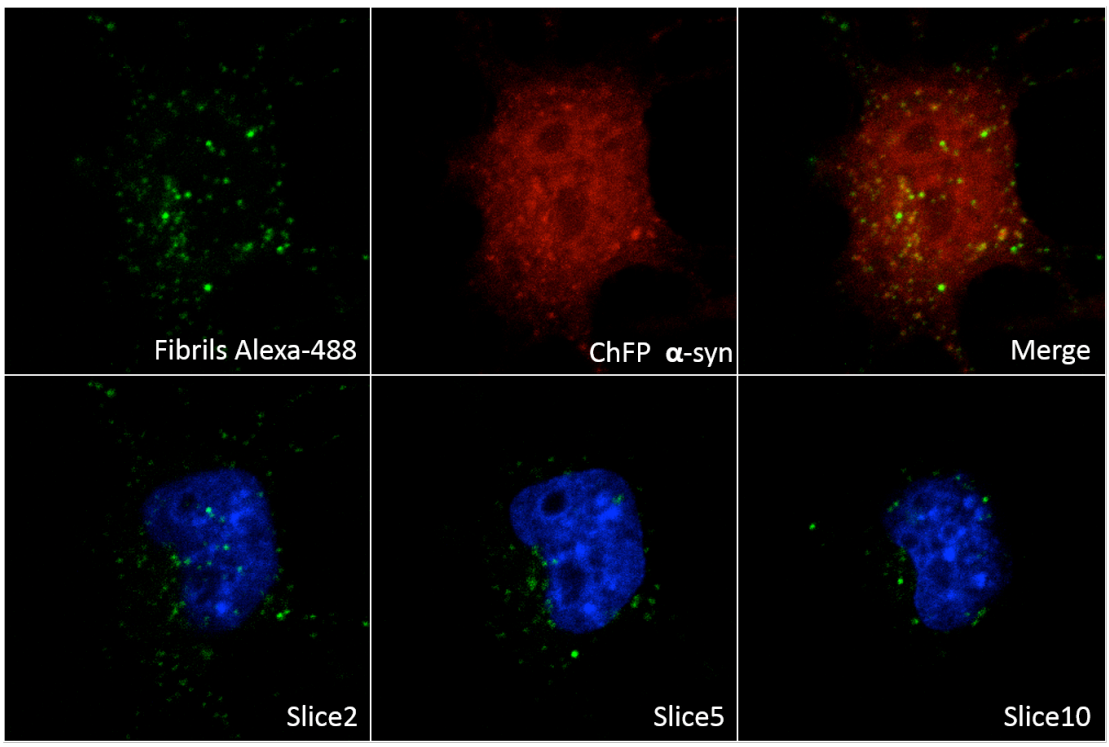
5) As mentioned above CAD cells makes TNTs, however the evidence that TNTs exist between neurons is weak. The authors should provide convincing evidences that TNTs exist between real-neurons. Since primary neurons uptake fibrils, experiments using primary neurons as donor cells and CAD cells being recipient cells could be a first approach.

Response
Following the suggestion of this referee we performed additional experiments using primary neuron co-cultures and co-cultures of primary neurons and CAD cells. Our data with primary cortical neurons indicates that; ***i)*** *α-synuclein fibrils are internalized and localized in lysosomes (after 16h and up to 72h),* ***ii)*** *when co-cultured with an acceptor population of primary neurons (div 0), donor neurons are able to transfer α-synuclein to acceptor neurons, after 3 days in co-culture,* ***iii)*** *the transferred α-synuclein puncta are also mainly located in lysosomes of acceptor cells, but are present in lower number and smaller size than those found in donor cells. In addition, we also found that transfer of α-synuclein is significantly diminished when* ***a)*** *acceptor cells are cultured for 72 hours with conditioned medium (CM) from donor cells and* ***b)*** *donor and acceptor neurons are plated in different coverslips and maintained in the same dish for 3 days in culture (sharing medium but not in contact). All these data are now included in the revised version of the manuscript in* ***Figure 6*** *and* ***Figure*** *7.*
Since the existence of TNTs between mature neurons is a very challenging task, not only because of the high density of dendrites and axons present in our co-culture system, but also due to a lack of a specific TNT marker, we followed the reviewer's suggestion. We therefore performed experiments in which donor neurons (previously loaded with 1 μM of α-synuclein fibrils for 16 hours) were co-culture with GFP-transfected-acceptor CAD cells for 24 hours. By using this set up we observed that; ***i)*** *donor neurons are able to transfer α-synuclein to CAD cells in a very efficient way, and* ***ii)*** *the number and size of the transferred puncta are very similar to those detected in acceptor CAD cells, in the CAD-CAD transfer model. Interestingly, by using this model we could also detect that some TNTs containing α-synuclein puncta*

were connecting CAD cells and neurons. All these data are now included in the revised version of the manuscript, ***Figure 8A-C****.*

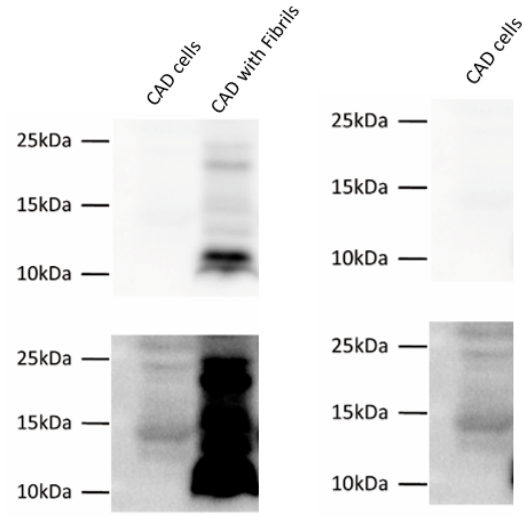
6) Figure 2A. A more convincing representative images and higher magnifications should be provided. The control figure (merged-image) gives a false impression of the presence of aggregates within nucleus whereas it lacks in ChFP channel. This look like and exposure issue. The authors could provide images without DAPI.

Response
We kept the image in ***Figure 2A*** *because we do not believe that this is an issue. If a-synuclein was in the nucleus we believe that we would have seen a colocalization signal, which we do not see. Below are shown the single slices of the reconstructed image clearly showing that in the middle of the nucleus (slice 5) there is no aggregate. In the top panels we show the figure without DAPI. In the case the referee would prefer this image we could use it in* ***Figure 2A****.*



7) Exogenous synuclein induces seeding and aggregation of endogenous synuclein. Do CAD cells express synuclein? If yes, then the implication of endogenous synuclein if any in the transfer via TNTs should be evaluated eventually by using a KO donor.

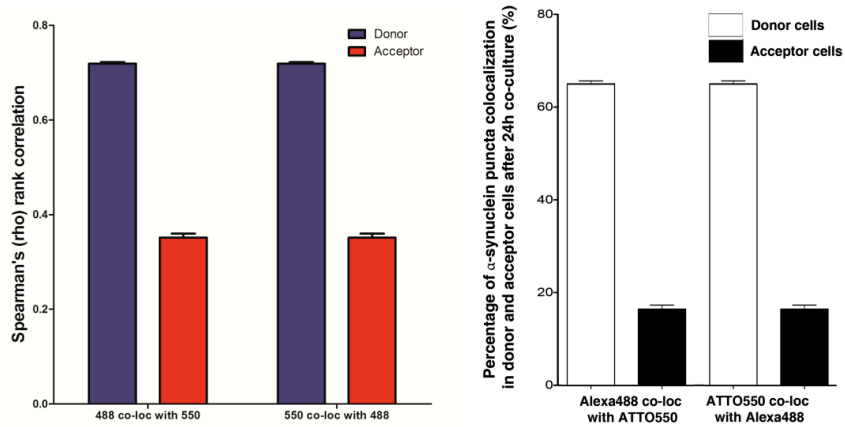
Response
We have performed several immunoblots and found no detectable amounts of α-synuclein in control CAD cells. We can barely detect α-synuclein in control CAD cells on highly overexposed blots (see blots below). This suggests that CAD cells express very low levels of a-synuclein. We therefore respectfully disagree with the reviewer and do not believe this experiment is important.



Of note, blots showed are either overexposed (bottom) or normally exposure (top) in control CAD and CAD loaded with α-synuclein fibrils.

8) In Figure 3A-B, it is not surprising that there is less co-localization between exogenous (green) and transferred (red) since the number of red punctas are sparse in the acceptor cells. This reduces the probability of co-localization. What needs to be quantified here is the percentage of ATTO550 spots co-localized with Alexa488 spots and not vice versa.

We thank this reviewer for this comment. On one hand, the referee is right and we should have focused on characterizing the co-localization of the transferred fibrils (red) with the newly taken-up ones (green). To this end, we re-analysed all the data (about 155 cells from 3 different experiments) using two different co-localization methods: 1) ICY which is based on Object-based method: Spot detector methodology and 2) Spearman: Pixel based method to determine the relationship between fluorophores intensity. With both these methods we found similar results, however we kept ICY in the paper, as this is the software we mainly used, but added below for this referee the Spearman's correlation data analysis. The graphs below show colocalization analysis done with the Spearman's method (left panel) or with the spot detection method using ICY (right panel). We also realized that the picture was deceiving as in the acceptor cells only few red dots were visible therefore we changed this to a more representative image.



On the other hand, we believe that the fact that red fibrils are present in a lower amount in cells compared to the green ones doesn't bias the percentage of colocalisation (red with green). Indeed, as we can see in the schematic presented below, when the amount of red fibrils is lower than the green ones, the probability of red fibrils to colocalize with green fibrils is highly increased (as seen on the right panel (2)) and this is because red fibrils have more chance to be found at the same localization (at the vicinity) of green fibrils, which are more and well distributed throughout the cells.

Overall, after following the suggestion of this reviewer and having used two different methods, we believe that the low percentage of red fibrils colocalizing with the green fibrils (found using 2 methods) in acceptor cells (compared to donor cells) is accurate.

9) It is stated in the discussion that ROS induction leads to stress and hence lead to more tube formation. It needs to show that exogenous synuclein induces ROS generation. An increased number of tubes between cultured primary neurons treated with synuclein should also be shown.

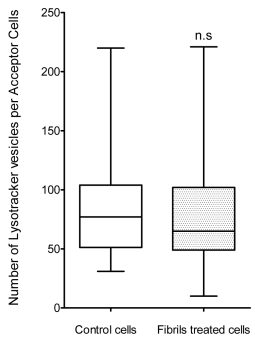
Response
*After the reviewer's comment, we determined whether exposure of cells to α-synuclein fibrils was inducing/increasing intracellular ROS production in CAD cells. ROS production was measured with the CellROX Green reagent in cells loaded with 1 μM of α-synuclein fibrils for 1 to 9h, and there was a consistent increase (50% more than control) of ROS production in α-synuclein loaded cells. These results are now included in the manuscript (**Figure EV 1D-E**), and the discussion was changed accordingly. We feel nonetheless that understanding this process at the molecular level is beyond the scope of this study.*
For the reasons stated above we cannot show veritable TNT connections in primary neurons until we find a specific marker

10) A time-lapse study of the transfer would be welcome. Given the rapid and efficient transfer observed within 24h, it should be possible to capture such a mechanism by video microscopy. Is it a uni-directional transport or can some fibrils shuttle back? Do the authors also see aggregation at the TNT initiation site.

Response
As the referee, we think that tracking the transfer in real-time of fibrils inside the TNT would be very interesting. However we think that addressing this "dynamics" question is beyond the scope of this manuscript. Moreover time-lapse TNTs imaging is very difficult since these structures are fragile and transient. Therefore to set up such methods in the time frame of a reply is not possible. We hope to be able to characterize this in the next few years.

11) Lysosome transfer raises the question of the transfer of lysosomes to the TNTs? Does exogenous synuclein alters the rate of lysosome transfer via TNTs?

Reponse
We have done the experiment that the referee suggested and found that treating donor cells with α-synuclein fibrils do not alter the number of lysosomes transfer between CADs after 24 hours co-culture (77 and 65 lysotracker vesicles per acceptor cells respectively in control and fibrils-treated cells) (see Figure below).



12) While Figure 7C-D shows nicely that donor lysosomes are involved in synuclein transfer, the reciprocal experiment shown in Fig EV5C is not convincing because the Rab-7 lysosome staining is diffuse.

Response
We agree with the reviewer. This is why we used lysotracker (see ***Figure 5D, E****) to confirm the Rab7 experiment outcomes.*

Minor Points:
1. Maintain standard scientific referencing style. E.g. Baark et al., and not Heiko Baark et al. Luk et al, 2012a, b instead of Luk, Kehan , Zhang et al. 2012 or Luk, Kehan, Caroll et al.).

Response
We followed the reviewers' suggestion.

2. Refrain from using neuronal cells and rather use neuron-like cells. We know that these cells are far from being neurons.

Response
We followed the reviewers' suggestion.

3. The discussion needs to be shortened. There are too many speculations.
Response
We shortened the discussion.

4. Data not shown should be provided as supplementary.

Response
We followed the reviewers' suggestion.

2nd Editorial Decision 07 July 2016

Thanks for submitting your revised manuscript to The EMBO Journal. The resubmission has now been seen by the original three referees. As you can see below, the referees appreciate that the analysis has been improved by the added neuronal data. However, there are two issues that still need to be resolved.

1) As indicated by referee #2 you need to provide a more balanced description of the findings and what the data shows. For example we don't know if the a-synuclein that is transferred via nanotubes is pathogenic and we also don't know if this process is related to disease pathology. It is fair enough to discuss this in the discussion, but take a look at the title, abstract and result section and make sure that this is consistent with what is shown in the manuscript. Regarding referee #3's point about using "young neurons" I am OK with that approach as long as this is clearly indicated in the text and the potential caveats are discussed.

2) Referee #3 is concerned that the used imaging approach doesn't conclusively support that you are seeing neuron-to-neuron transfer. I would like to hear your response to this issue. Do you have any further data to alleviate this concern?

I am happy to discuss the last issues further.

REFEREE REPORTS

Referee #1:

The authors appropriately revised the manuscript according to the reviewer's comments and suggestions.

Referee #2:

The paper by Abounit et al is now improved by the additional of data in neurons. While not as extensive as the CAD cell data it adds just about enough to support the overall conclusions. However, there is one small aspect that needs to be addressed.

The authors have not, neither here nor in the other papers they quote, established that these fibrils are pathogenic in human disease. While that has been claimed it is not proven. Because the authors are working in mouse cell culture, they cannot make a claim about human pathogenesis narrowly here. Therefore, and as previously stated, the title must be changed to say 'fibrillar' a-synuclein. The same concern is true for the abstract - neither these authors nor anyone in the literature have proven that synuclein spread contributes to pathogenesis, even less for PD progression. The abstract needs to be toned down.

Referee #3:

In their rebuttal, Abounit et al., performed new experiments to strengthen the claimed existence of TNTs in mediating the transfer of synuclein. Albeit their effort, the new version adds no new information than before and the questions remain unanswered if TNTs do exist in primary neurons and in vivo. The overall message remains same as new data adds to no information. As stated during the first revision that CAD cells are not neurons and they do not form synapses and there is no doubt that they do not form TNTs. Indeed, they are easily form TNTs in this paper and previous works by the same group. The experiments performed on neurons (with several experimental caveats; see next paragraph) show that cell-to-cell contact is necessary for synuclein transfer does not really demonstrate that the transfer requires TNT. If they insist on physical contact, they should show that the physical plasma membrane barrier is compromised (or TNT evolves) follwoing synuclein uptake.

All the neuron experiments were performed on very young, immature neurons, when synapses are not developed and they do not show that neurons form TNTs. This is illogical considering the authors are studying age related disorder. New acceptor neurons were added on top of ~8 div donor neurons and transfer monitored after 3 days. This means the acceptor neurons are only 3days old. This unconventional way of plating a 2nd neuron culture on top of an existing culture is a good approach, but often not preferred for neurons as they are quiet fragile and accounts for high cell death. Conditioned medium experiments were performed on 10DIV acceptor neurons, which should not be compared to 3DIV neurons. The secretion of prion-like proteins such as synuclein, tau, etc depends a lot of neuronal activity, which makes immature neurons not the ideal model. Authors should be aware that in a primary culture model, it is quiet tricky to distinguish between processes arising from different neurons. Therefore, with the current imaging approaches allowed for neuron-to-neuron transfer, it is impossible to state whether the transfer really occurred between neurons i.e. the fluoresce puncta seen is within the acceptor neurons or belong to axon/dendrite originating from a donor neuron. For all experiments, axonal labeling should be also be performed and better imaging approaches (and images), such as EM or super-resolution be applied.

Authors should refrain in discussion from assertions, such as their demonstration of 100% transfer because of superior quality of material when the material from same laboratory has been used in many other studies with lower level of transfer. Authors did not perform "advanced in-depth analysis" and several clustering algorithms are routinely used in laboratories throughout the world.

2nd Revision - authors' response 20 July 2016

Referee #1:

The authors appropriately revised the manuscript according to the reviewer's comments and suggestions.

Response
-We are very happy that this referee is satisfied with our additional experiments.

Referee #2:

The paper by Abounit et al is now improved by the additional of data in neurons. While not as extensive as the CAD cell data it adds just about enough to support the overall conclusions. However, there is one small aspect that needs to be addressed.

The authors have not, neither here nor in the other papers they quote, established that these fibrils are pathogenic in human disease. While that has been claimed it is not proven. Because the authors are working in mouse cell culture, they cannot make a claim about human pathogenesis narrowly here. Therefore, and as previously stated, the title must be changed to say 'fibrillar' a-synuclein. The same concern is true for the abstract - neither these authors nor anyone in the literature have proven that synuclein spread contributes to pathogenesis, even less for PD progression. The abstract needs to be toned down.

Response
-We thank this referee for acknowledging our work. We have further followed his/her suggestions by changing the title and by removing the word "pathogenic" to characterize the fibrils. We also toned down the abstract.

Referee #3:

In their rebuttal, Abounit et al., performed new experiments to strengthen the claimed existence of TNTs in mediating the transfer of synuclein. Albeit their effort, the new version adds no new information than before and the questions remain unanswered if TNTs do exist in primary neurons and in vivo. The overall message remains same as new data adds to no information. As stated during

the first revision that CAD cells are not neurons and they do not form synapses and there is no doubt that they do not form TNTs. Indeed, they are easily form TNTs in this paper and previous works by the same group. The experiments performed on neurons (with several experimental caveats; see next paragraph) show that cell-to-cell contact is necessary for synuclein transfer does not really demonstrate that the transfer requires TNT.

Response
We thank the referee for his/her new comments but we disagree with his/her statement that our new evidences did not add any weight to the manuscript.
We agree with this referee that the experiments performed in primary neurons show that cell to cell contact enhances α-synuclein fibrils intercellular transfer, as we have stated in the revised manuscript, and does not demonstrate conclusively that primary neurons form TNTs, but we believe that we provided strong evidence to this case, within the current technical limitation.
TNT-"like" structures have been shown in primary cultures before (especially between neurons-astrocytes and astrocytes-astrocytes) by others and us (Wang et al, 2011 and Victoria et al., 2016). However in the absence of specific markers for TNTs no one at the current state of the art knowledge can fully demonstrate the existence of TNTs in primary culture (or in vivo), and this was not our claim in this paper.
Indeed to reply to this referee`s original comments on this issue we performed exactly the experiments that he/she suggested (eg; co-culture and transfer of α-synuclein from primary neurons to CAD cells). In this case we observed high transfer which was cell-to-cell contact dependent. Most importantly, under these conditions we could identify TNTs between CAD cells and the soma or neurites of primary cortical neurons, and some containing α-synuclein puncta (Fig 8).
For the record we paste below the previous comment of this reviewer and our reply:

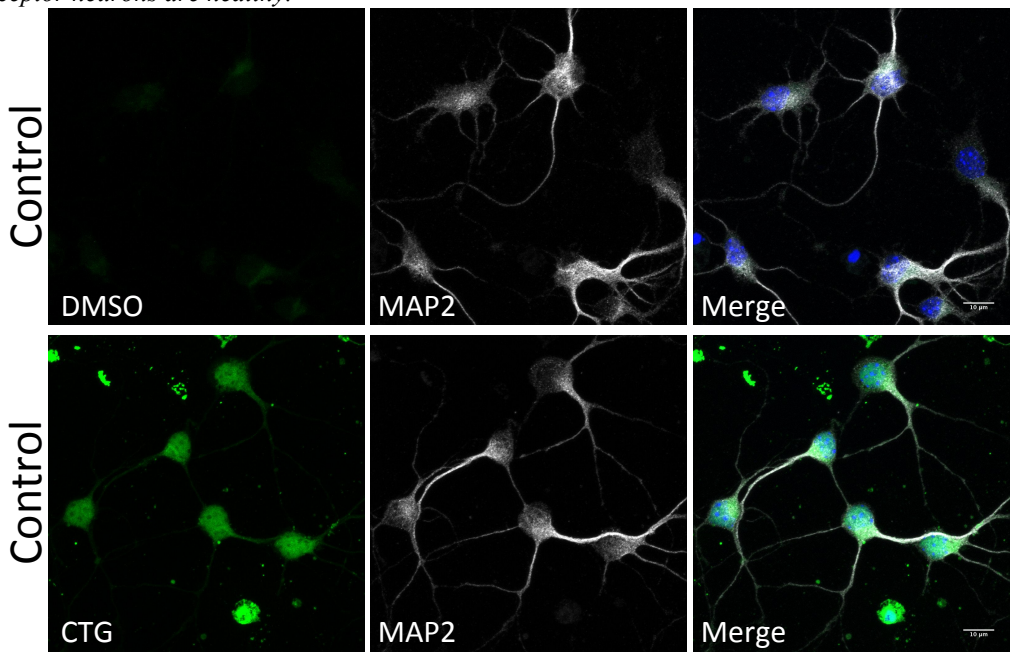
As mentioned above CAD cells makes TNTs, however the evidence that TNTs exist between neurons is weak. The authors should provide convincing evidences that TNTs exist between real-neurons. Since primary neurons uptake fibrils, experiments using primary neurons as donor cells and CAD cells being recipient cells could be a first approach.
Response
Following the suggestion of this referee we performed additional experiments using primary neuron co-cultures and co-cultures of primary neurons and CAD cells. Our data with primary cortical neurons indicates that; i) a-synuclein fibrils are internalized and localized in lysosomes (after 16h and up to 72h), ii) when cocultured with an acceptor population of primary neurons (div 0), donor neurons are able to transfer a-synuclein to acceptor neurons, after 3 days in co-culture, iii) the transferred a-synuclein puncta are also mainly located in lysosomes of acceptor cells, but are present in lower number and smaller size than those found in donor cells. In addition, we also found that transfer of a-synuclein is significantly diminished when a) acceptor cells are cultured for 72 hours with conditioned medium (CM) from donor cells and b) donor and acceptor neurons are plated in different coverslips and maintained in the same dish for 3 days in culture (sharing medium but not in contact). All these data are now included in the revised version of the manuscript in Figure 6 and Figure 7.
Since the existence of TNTs between mature neurons is a very challenging task, not only because of the high density of dendrites and axons present in our co-culture system, but also due to a lack of a specific TNT marker, we followed the reviewer's suggestion. We therefore performed experiments in which donor neurons (previously loaded with 1 μM of a-synuclein fibrils for 16 hours) were co-culture with GFPtransfected-acceptor CAD cells for 24 hours. By using this set up we observed that; i) donor neurons are able to transfer a-synuclein to CAD cells in a very efficient way, and ii) the number and size of the transferred puncta are very similar to those detected in acceptor CAD cells, in the CAD-CAD transfer model. Interestingly, by using this model we could also detect that some TNTs containing a-synuclein puncta were connecting CAD cells and neurons. All these data are now included in the revised version of the manuscript, Figure 8A-C.

If they insist on physical contact, they should show that the physical plasma membrane barrier is compromised (or TNT evolves) following synuclein uptake.

Response
-We are not sure we understand what the referee means by "physical plasma membrane barrier is compromised". Maybe this is just a misunderstanding. Indeed when TNT form the plasma membrane is still intact and not compromised. We do not believe and not claim here that the cell-to-cell contact dependent transfer of α-synuclein fibrils is dependent on the plasma membrane "being compromised". In addition, we have used in our transfer experiment settings (for CAD cells) trypsin wash to remove all external α-synuclein fibrils that might be bound on the outside of the cell donor plasma membrane which exclude transfer mediated by plasma membrane associated materials.
In addition, our new LDH experiment in the presence of intracellular α-synuclein fibrils provides an indication that the plasma membrane is not compromised at the time points evaluated. We hope we have alleviated the concern of the referee.

All the neuron experiments were performed on very young, immature neurons, when synapses are not developed and they do not show that neurons form TNTs. This is illogical considering the authors are studying age related disorder. New acceptor neurons were added on top of ~8 div donor neurons and transfer monitored after 3 days. This means the acceptor neurons are only 3days old. This unconventional way of plating a 2nd neuron culture on top of an existing culture is a good approach, but often not preferred for neurons as they are quiet fragile and accounts for high cell death.

Response
-We thank the referee to acknowledge that this is a common method of neuronal co-culture and we agree that young neurons are more fragile. Primary neurons in general are very difficult to transfect efficiently and once they are seeded is not possible to detach and/or pass them, thus we cannot use in our co-culture experiments older acceptor neurons or neurons of the same age as donors for technical limitation. We agree with the referee that younger neurons can be more fragile and indeed have checked that in this co-culture conditions there is no high cell death. In every experiment performed, along with the CellTracker green (CTG)-labelled acceptor neurons plated on top of donor cells, we included the three following controls; 1) DMSO-treated neurons plated alone (for control of CTG- vehicle), 2) CTG-labelled neurons plated alone, and 3) CTG-labelled neurons plated on top of ~8 div wild type neurons. In conditions 1) and 2) we observed higher cell death compared to what we usually have on routine experiments with wild type neurons, however as it can be observed in the images presented below, after 3 div, cells are healthy and viable in all conditions (Figure 1 below). In addition, we detected better growth of acceptor neurons in the co-culture condition (condition 3) compared to neurons plated alone (1 and 2), so we are confident that the acceptor neurons are healthy.



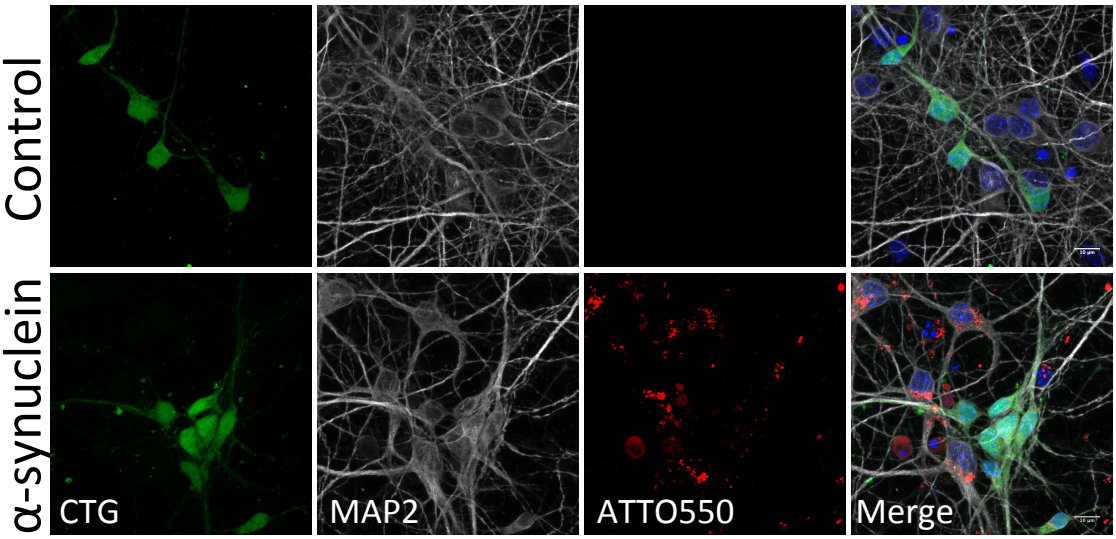


Figure 1: The image shows healthy neurons after 3 div in culture alone or in co-culture.

Conditioned medium experiments were performed on 10DIV acceptor neurons, which should not be compared to 3DIV neurons. The secretion of prion-like proteins such as synuclein, tau, etc depends a lot of neuronal activity, which makes immature neurons not the ideal model

Response
-We agree that the age of neurons is important, however in our hands this does not seem to affect α-synuclein internalization in culture. Indeed we have performed the internalization experiments with 3 , 6 and 9 div neurons and observed that they take up similar amounts of α-synuclein fibrils (see Figure 2 below). However, for the same concerns expressed by this referee, we decided to use mature neurons (10 div) as donors that would allow a full secretory pathway to be established.

The "conditioned medium" and the "no contact" experiments were control experiments designed to evaluate the contribution of secretion after 72h (same time as transfer). They were performed in parallel with the transfer experiments using the neurons coming from the same dissection.
Therefore, in the case of CM experiments we took the 72h conditioned medium from donor neurons (so they will be 10 div) and added it to same age neurons plated on different coverslips (for 3 days), while at the same time we added freshly dissected neurons on top of donor neurons from the same dissection plated in parallel to the ones used to take CM (so 7 div neurons) for 3 days.
Technically we could use younger neurons as acceptor of the CM, but considering the referees comments and also the results obtained with the separated coverslips experiment described below (for which we used the two age neurons 7 and 3) we do not see much point in repeating this experiment.
In the case of the separated coverslips (No contact condition) we have used same age neurons (~8 div) as donor and plated fresh neurons on the second coverslip as acceptor and kept them for 3 days in the same plate. In addition for this experiment (and maybe this would alleviate the concerns of this referee) we also used same age neurons as donor and acceptor (8div) plated on different coverslips. In these experiments we observed no or very low α-synuclein transfer to the acceptor neurons independently of the age of the neurons (3 or 10 div at the end of the experiment).

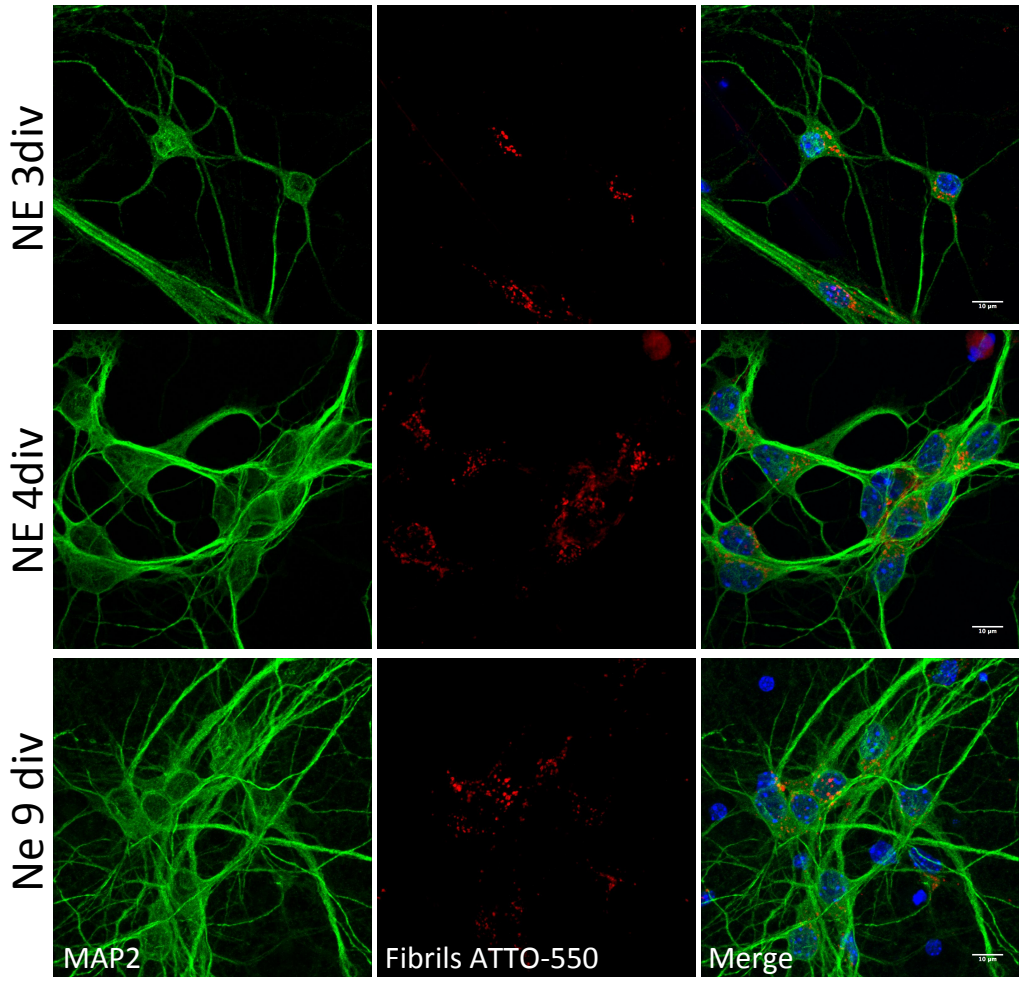


Figure 2: The images are representative of the α-synuclein uptake of different age neurons.

Authors should be aware that in a primary culture model, it is quiet tricky to distinguish between processes arising from different neurons. Therefore, with the current imaging approaches allowed for neuron-to-neuron transfer, it is impossible to state whether the transfer really occurred between neurons i.e. the fluoresce puncta seen is within the acceptor neurons or belong to axon/dendrite originating from a donor neuron. For all experiments, axonal labeling should be also be performed and better imaging approaches (and images), such as EM or super-resolution be applied.

Response
-We agree with the comment that it is tricky to discern in a crowded neuronal network in primary culture the protrusions coming from two different cell populations of the same age. We had the same concern and this is why we performed our co-culture experiments by optimizing the number of donor neurons and acceptor neurons added on top so to obtain a co-culture in which we could accurately detect both populations. In addition, the acceptor neurons were readily recognizable because they were pre-labelled with CellTracker green. Therefore with our imaging system, there was absolutely no confusion between donor and acceptor neurons. As can been seen in the bottom panel in Fig. 1 (refer to panel α-synuclein) and in Fig 3 below, both figures showing a larger area of the co-culture, the two donor and acceptor populations are easily distinguishable in the co-culture conditions used throughout the paper.

Furthermore, as mentioned in the manuscript (Appendix; materials and methods), the image analysis was made using the ICY software and the plugin developed by Fabrice de Chaumont. Since image acquisition was made by tacking Z-stack images going from the bottom of the coverslip to the end of the upper visible acceptor cells, we selected the slices covering the cell volume of the green acceptor population, for example, and performed the analysis by segmenting and quantifying α-synuclein puncta, only in the green neurons, and mainly around the cell body (which is why we

preferred MAP-2 instead of an axonal marker for the donor population). In order to avoid overlapping with the donor neurons, cells from different populations that were in close proximity were excluded from the analysis. To clarify this point, we included below images showing an example of detection of α-synuclein puncta and segmentation of the acceptor neurons (previously labelled with cell tracker green) (Figure 3).

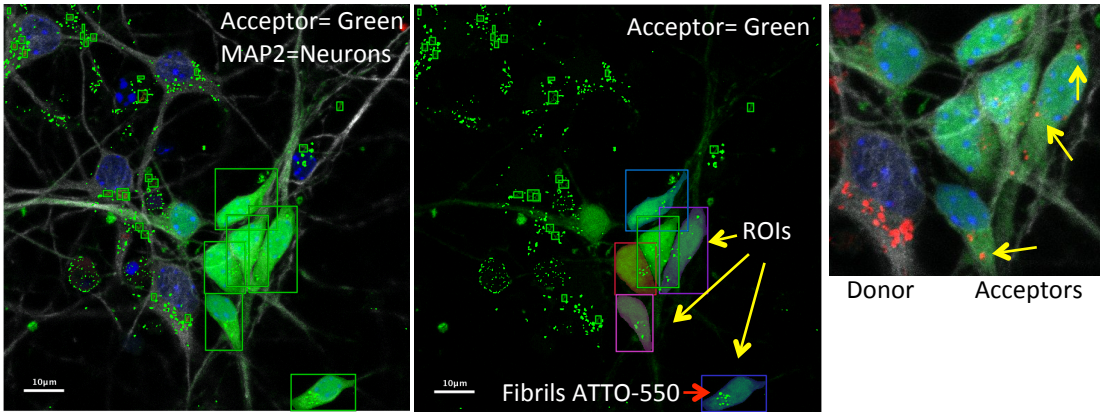


Figure 3: Image analysis to quantify the amount of α-synuclein puncta in acceptor neurons (labelled in green).

Authors should refrain in discussion from assertions, such as their demonstration of 100% transfer because of superior quality of material when the material from same laboratory has been used in many other studies with lower level of transfer. Authors did not perform "advanced in-depth analysis" and several clustering algorithms are routinely used in laboratories throughout the world.

Response
-For the advanced in-depth analysis, we referred to the software plugin that was specifically generated for identification and quantification of α-synuclein fibrils which to our knowledge has not been done before, not even with the same fibrillar material coming from the Melki's lab. We agree that several algorithms are routinely used for quantitative imaging, however we provided for the first time the number and the size of α-synuclein fibrils in the frame of transfer using dedicated software as detailed in the appendix: materials and methods and exemplified above in Fig. 3.

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Corresponding Author Name: Chiara Zurzolo
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2015-93411

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All experiments were repeated at least three times. We analyzed for each experiment at least 100 cells in order to have statiscally relevant analysis and to identfy trends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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5. For every figure, are statistical tests justified as appropriate?	The number of independent experiments performed, the type of statistical tests and definitions of "center values" used are specified in the figure legend for every figure; pages 39-52 of the manuscript.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis are described in the Materials and methods section of the manuscript, on page 27. We used Student's t-test when the distribution was normal . The statistical relevance was obtained by calculated the p value using the paired two-tailed Student's t-test (bar graphs). We represented the bar graphs showed in the figures as mean ± s.e.m. We chose to use a nonparametric test Mann-Whitney to compare medians (in that case the normal assuption is not required). The statistical relevance of the box-and-whisker plots was calculated with the Mann-Whitney test. All column graphs, plots and statistical analyses were done using GraphPad Prism version 5 software.
Is there an estimate of variation within each group of data?	For each group, we have shown the standard error of the mean.

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Is the variance similar between the groups that are being statistically compared?	Statistical analysis was performed using the GraphPad Prism software. For Student`s t-test the software calculates the homogeneity of variances by means of the F-test. If the F-test for homogeneity of variances was significant a non-parametric test was used instead.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Main antibodies used are described in Appendix; Materials and methods: Lamp1, BD Biosciences, rat, Cat. no. 553792, page 5. Mouse anti-α-synuclein, BD Biosciences, Cat. no. 610787, page 7. Mouse anti-α-tubulin, Sigma Aldrich, Cat. no. T9026, page 7. MAP-2, Merck Millipore, mouse, Cat. no. MAB3418, page 9. CellTracker Green (CTG) probe, Thermo Fisher Scientific, Cat. no. C2925, page 3.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	CAD cells were a gift from Hubert Laude (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) and were tested for mycoplasma contamination.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We did't use any animal models for the study. However, we worked with primary neurons isolated from wild type mouse embryos. This is described in the Appendix; Materials and methods; Animals and Primary neuronal cultures, page 2.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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