EMBO
Molecular Medicine

Manuscript EMM-2011-00799

Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease

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Review timeline:

Submission date:	15 July 2011
Editorial Decision:	08 November 2011
Revision received:	28 November 2011
Editorial Decision:	23 December 2011
Revision received:	04 January 2012
Accepted:	10 January 2012

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 08 November 2011

Thank you for the submission of your manuscript "Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #1 feels that the impaired autophagic phenotype of the cells should be investigated in further detail to convincingly support your conclusions. Importantly, reviewer #3 highlights that more insight into the phenotype of reduced neurite length observed in the PD iPSC should be provided.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor

EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

In this work, Sanchez-Danes and colleagues generated a large set of PD-specific iPS cell lines either from sporadic cases or harboring LRKK2 mutations. Then, the authors examined and compared the neuronal differentiation efficiency in either patient- or control-derived iPS cells. Finally, they focused in identifying specific alterations in patient-derived neurons in morphological features and autophagy related processes.
The authors generated many iPS cell clones that were thoroughly characterized for their pluripotency features and proliferating potential. They elaborated a particular protocol for promoting DA neuronal differentiation using infection with a Lmx1a-expressing lentivirus. However, details on this protocol are not provided while referring in the text on a work to be published elsewhere. This is generating some confusion on how the authors differentiated the neurons and which steps exactly are included in this procedure. In the chart 1D the total number of TuJ1 neurons result about 30%. This is probably a mistake since the general amount of neurons should be much higher. Lacking this information about the protocol used, a series of crucial data needs to be added in the paper:

1) Are the DA neurons generated by iPS in vitro differentiation functional? Do they have neuronal activity and membrane electric potential?

2) Are these DA neurons producing effectively dopamine measurable by biochemical means?

These analyses are needed in order to ensure a correct differentiation procedure and the generation of a meaningful class of neurons.
In overall, the description of the autophagy process and its impairment in the patient-specific iPS-derived neurons is rather preliminary and stands quite superficial at least as presented now. More experiments are definitively requested in order to convincingly support the author's conclusions:

1) Authors based their analysis on the LC3 immunostaining performed with a unique antibody (cell-signaling). Although this antibody is working properly on mouse cells, its performance on human cells by means of immunofluorescence is doubtful. The authors should use this antibody in traditional immunoblottings to look at the lipidated form of microtubule-associated protein-1 light chain 3 (LC3)-II, which is generated during autophagosome formation. Only the decrease conversion of LC3-I to LC3-II will provide initial evidence for an alteration of basal autophagy.

2) A direct analysis of the autophagosome ultra-structure in the patient-derived neurons by EM analysis need to be presented.

3) Clearance of autophagosomes occurs via fusion with lysosomes.
The authors proposed that accumulation of autophagosome is due to defective clearance caused by impaired autophagosome-lysosome fusion. To test this hypothesis, they should analyze simultaneously the subcellular localization of the lysosomal marker lgp120 (LAMP1) and the autophagosomal marker LC3. These experiments should provided evidences that the extent of lgp120/LC3 co-localization is reduced in patient-specific compared to wild-type neurons, thus indicating impaired
autophagosome-lysosome fusion.

4) The authors should study the localization of the p62/SQSTM1 protein which normally accumulates in stalling autophagosomes. p62/SQSTM1 is known to be a common component of ubiquitin-positive protein aggregates in neurodegenerative
diseases, being involved in the targeting of polyubiquitinated proteins to the autophagosomes and

selectively degraded via the autophagic pathway.

5) Alterations of autophagy processes are accompanied by changes in gene expression. Authors should analyze by qPCR changes in expression levels of lysosome-protein specific transcripts like TFEB, CTSA, CTSB, ARSA, HEXA, SCPE1. This study should lead to identify significant differences in expression of these genes between patient- and control-derived neurons.

Referee #2 (Comments on Novelty/Model System):

iPS is a new and powerful in vitro system with high potential to model pathogeneic processes in disease

Referee #2 (Other Remarks):

Ramos-Sanchez et al. report on the characterization of mDA neurons derived from several iPS lines generated from control (Ctrl), idiopathic (ID-PD), and LRRK2-mutant (LRRK2-PD) PD patients. In short and long-term differentiation conditions they found differences between Ctrl and PD midbrain-like DA neurons with increased expression levels of SNCA, a rudimentary morphological phenotype, less cell viability and neurite sprouting, impaired autophagy, and vulnerability to MPP+ toxic insult in the PD DA neurons. The authors conclude that their "study provides an iPSC-based in vitro model that captures the patients' genetic complexity and allows investigating PD pathogenesis in a disease-relevant cell type".

This study is in line with an increasing number of reports on using a combination of patient-derived iPS technology with in vitro differentiation paradigms to generate model systems of disease and discover mechanisms of pathogenesis. The current study extends recent papers using this paradigm for studying PD-affected DA neurons by analyzing Ctrl, ID-PD and LRRK2-PD iPS lines in parallel and the use of long-term differentiation cultures. This is an important study. The data are solid, interesting and reveal new aspects on PD DA neuronal dysfunction in the in vitro paradigm. Moreover, they provide new insight into the feasibility of this system to study pathological processes in DA neurons derived from patients with either sporadic or mutant genetic background. This latter point should be discussed in regard of a recent paper by Soldner et al., 2011, Cell 146, 318, in which the genotype is introduced by genome editing.

Minor comments:

Fig. 1: TH and Tuj1 don't seem to overlap - NeuN staining?
Fig. S1: I am not convinced of the DAT staining in panel A. The MAB369 Chemicon antibody is against the N-terminus, however the staining looks more intracellular, similar to Girk2 or Tuj1 in the same panel.
Fig. 2 and S2: The authors should give more information on the LV.NES.LMX1A.GFP virus. Did the authors test if the Nes enhancer/TK promoter is specific to Nes+ precursors, and not leaking in other phenotypes? Is this the same construct used by the Perlmann group (Friling et al., 2009, PNAS May 5;106(18):7613-8)?
Fig. 3 and Fig. S4: If the authors have ICC and Western blot quantification for SNCA and LRRK2 that would strengthen the PCR results.
Fig. S5: I was wondering if the DAn are NeuN positive and Tuj1-negative, see comment to Fig. 1.
Discussion: The discussion is a bit technical and could benefit from some interpretation of their data regarding PD pathogenesis and what they would imply for the field of neurodegenerative diseases. In particular interesting is the similar phenotype development of the ID-PD and LRRK2-PD lines. The authors should discuss a recent paper by Soldner et al., 2011, Cell 146, 318.

Referee #3:

The manuscript by Sanchez-Danes et al. generated iPSC lines from both sporadic (ID-PD) and familial PD (with LRKK2 G2019S mutation LRRK2-PD) patients. The authors report that all lines TH+ neurons can be generated from all PD iPSC lines and control lines. Interestingly, the authors

found that TH+ neurons from LRRK2-PD iPSC lines, but not from ID-PD iPSC lines exhibited a-synuclein accumulation in the cell body. On the other hand, TH+ neurons from both LRRK2- and ID- PD iPSC lines exhibited decreased neurite length and vacuole accumulation over extended culture time. The observations are interesting and suggesting that retarded neurite development/degeneration of TH+ neurons might be a common phenotype of PD, however, the data at present did not provide a causative link. Although it is difficult to test with ID-PD, how LRRK2 mutation leads to the observed defects are testable. For example, whether expression of LRRK2-G2019S in control neurons result in the same phenotype in neurite arborization and vacuole formation.

Other comments:

While the authors suggested that iPSCs were generated from 7 sporadic and 4 LRRK2 PD patients and from 4 control subjects, data only presented from 2 controls, 3 ID-PDs and 3 LRRK2-PDs in Figs. 4 and 5. In most cases, the data were pooled together or only from a single line were shown. Data not shown is not acceptable.

In Fig. 4, the quantification data does not seem to match the description. For example: The neurite length of sample type 2 cells (4B) is longer than the average length of type 1 cells (4G). In addition, it is not clear from the text and from the method section what is the criteria for selecting the cells for analysis.

1st Revision - Authors' Response 28 November 2011

Point-by-point response to the reviewer's comments:

Referee #1:

In this work, Sanchez-Danes and colleagues generated a large set of PD-specific iPS cell lines either from sporadic cases or harboring LRKK2 mutations. Then, the authors examined and compared the neuronal differentiation efficiency in either patient- or control-derived iPS cells. Finally, they focused in identifying specific alterations in patient-derived neurons in morphological features and autophagy related processes.

The authors generated many iPS cell clones that were thoroughly characterized for their pluripotency features and proliferating potential. They elaborated a particular protocol for promoting DA neuronal differentiation using infection with a Lmx1a-expressing lentivirus. However, details on this protocol are not provided while referring in the text on a work to be published elsewhere. This is generating some confusion on how the authors differentiated the neurons and which steps exactly are included in this procedure. In the chart 1D the total number of TuJ1 neurons result about 30%. This is probably a mistake since the general amount of neurons should be much higher. Lacking this information about the protocol used, a series of crucial data needs to be added in the paper:

1) Are the DA neurons generated by iPS in vitro differentiation functional? Do they have neuronal activity and membrane electric potential?

2) Are these DA neurons producing effectively dopamine measurable by biochemical means?

These analyses are needed in order to ensure a correct differentiation procedure and the generation of a meaningful class of neurons.

We appreciate the reviewer's comments regarding the lack of details on our DA neuron differentiation protocol. A manuscript detailing this protocol was under revision at the time of submitting the current manuscript, and it is now accepted for publication in *Human Gene Therapy* and already available online at http://www.liebertonline.com/doi/full/10.1089/ hum.2011.054. In this manuscript, we describe in detail our DA neuron differentiation protocol, and provide a thorough characterization of these neurons. In summary, we show that DA neurons differentiated from human pluripotent stem cells using this protocol are of ventral mesencephalic A9 subtype phenotype, display electrophysiological characteristics of DA neurons, show robust DA release

induced by a depolarizing solution of KCl, and are able to engraft and complete maturation when injected into the adult unlesioned brain of mice. These results clearly show that the DA neurons generated with our differentiation protocol are, indeed, a meaningful class of neurons for the studies currently under review. We have now updated the reference to our previous publication in the revised version of our manuscript.

In overall, the description of the autophagy process and its impairment in the patient-specific iPS-derived neurons is rather preliminary and stands quite superficial at least as presented now. More experiments are definitively requested in order to convincingly support the author's conclusions:

We agree with the reviewer that the characterization of the autophagy process in the original version of our manuscript was somewhat preliminary, and we would like to thank him/her for the constructive criticisms and experimental suggestions, which we have addressed as follows:

1) Authors based their analysis on the LC3 immunostaining performed with a unique antibody (cell-signaling). Although this antibody is working properly on mouse cells, its performance on human cells by means of immunofluorescence is doubtful. The authors should use this antibody in traditional immunoblottings to look at the lipidated form of microtubule-associated protein-1 light chain 3 (LC3)-II, which is generated during autophagosome formation. Only the decrease conversion of LC3-I to LC3-II will provide initial evidence for an alteration of basal autophagy.

We appreciate the reviewer criticism, and we have performed the requested immunoblot analyses using the same antibody in the well-established autophagic flux measurement. We have also added a second measurement in between times of inhibition to determine autophagosome formation independently of the differences in the starting basal levels. The results of these analyses clearly support the performance of the antibody and show that: 1) basal levels of LC3-II are significantly higher in DA neurons differentiated form PD-iPSC than in controls; 2) the PD-related neurons have a significantly decreased autophagic flux, calculated by the increase in the levels of LC3-II upon addition of inhibitors; 3) there are no significant differences in autophagosome formation among control and PD-related neurons (calculated as the difference between the two times of inhibition of lysosomal degradation), although we detected a trend toward increased formation in the PD group that could be a compensatory response to the reduced degradation through this pathway. In agreement with previous studies (Mizushima and Yoshimori, Autophagy 2007) and the general recommendations for measurement of autophagy (Klionsky et al., Autophagy 2008 and the 2011 version draft already circulating among authors), measurement of the LC3-I to LC3-II ratio was not as informative about the autophagic state, so we decided to include the direct flux measurements instead to avoid further confusion. We should also note that, as expected, there was some individual variability among the different samples analyzed, so we decided to test and show representative immunoblots of iPSC-derived DAn for all 15 patients included in the study. These results are included in the revised version of our manuscript as a new Fig. 5, and similar analyses of patients' fibroblasts in a new Supplementary Fig. S10.

2) A direct analysis of the autophagosome ultra-structure in the patient-derived neurons by EM analysis need to be presented.

We appreciate the reviewer comment and, as requested, we have performed electron microscopy and morphometric analysis of the different patient-derived neurons. These studies confirmed the presence of a higher content of autophagic vacuoles in neurons differentiated from either ID-PD or LRRK2-PD iPSC than in control iPSC, and a marked increase in the relative percentage of autophagosomes when compared to autophagolysosomes, in support of problems in maturation (clearance) in the PD-cells. Interestingly, we also found a significant increase in the content of intracellular lipid droplets and the presence of a dilated ER in these cells, which have previously been described as common features of cells with compromised autophagy. We believe that these findings clearly support our point that PD patient-derived neurons, upon long-term culture, display

altered autophagy, and thank the reviewer for the useful experimental suggestion. We have included this information in the revised version of our manuscript as a new Fig. 6.

3) Clearance of autophagosomes occurs via fusion with lysosomes.

The authors proposed that accumulation of autophagosome is due to defective clearance caused by impaired autophagosome-lysosome fusion. To test this hypothesis, they should analyze simultaneously the subcellular localization of the lysosomal marker lgp120 (LAMP1) and the autophagosomal marker LC3. These experiments should provided evidences that the extent of lgp120/LC3 co-localization is reduced in patient-specific compared to wild-type neurons, thus indicating impaired autophagosome-lysosome fusion.

We agree with the reviewer's comment and, to address it, we have performed colocalization analyses of LAMP-1 and LC3 in iPSC-derived DA neurons cultured for 75 days. Due to the very rapid degradation of autophagosomes in normal neurons, the levels of visible autophagosomes (LC3-positive puncta) in control cells were too low to allow colocalization analyses (we show this in a new Supplementary Fig. S8). However, following the reviewer's reasoning, we blocked degradation of LC3 by treatment with leupeptin (which should not modify autophagosome/lysosome fusion), and were able to confirm colocalization of both proteins in control cells and a significant decrease in this colocalization in PD-derived cells. We believe that these results nicely confirm a compromised maturation of autophagosomes into autolysosomes in PD-iPSC-derived DA neurons and, again, thank the reviewer for the useful suggestion. We have included these results in the revised version of our manuscript as a new Fig. 7 and Supplementary Fig. S8.

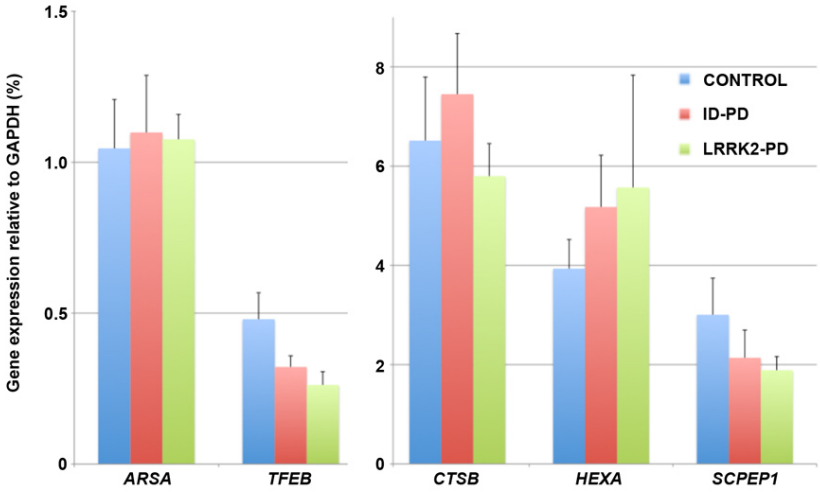
4) The authors should study the localization of the p62/SQSTM1 protein which normally accumulates in stalling autophagosomes. p62/SQSTM1 is known to be a common component of ubiquitin-positive protein aggregates in neurodegenerative diseases, being involved in the targeting of polyubiquitinated proteins to the autophagosomes and selectively degraded via the autophagic pathway.

We have performed the requested analysis of the levels of p62 and, as anticipated by the reviewer, we have detected a marked increase in the levels of this protein in a punctuate pattern compatible with its accumulation inside stalling autophagosomes. We believe that these results provide further support for a compromise in the clearance of autophagic compartments in PD-iPSC-derived DA neurons, and thank the reviewer for his/her experimental suggestion. We have included this information in the revised version of our manuscript in the new Fig. 5.

5) Alterations of autophagy processes are accompanied by changes in gene expression. Authors should analyze by qPCR changes in expression levels of lysosome-protein specific transcripts like TFEB, CTSA, CTSB, ARSA, HEXA, SCPE1. This study should lead to identify significant differences in expression of these genes between patient- and control-derived neurons.

We appreciate the reviewer's comment, and we have performed RT-qPCR analyses of the indicated lysosome-protein specific transcripts using RNA prepared from differentiated iPSC representing all 15 patients and specific primers described elsewhere (Settembre et al, Science 2011). As shown in the graph below, we did not observe differences in the levels of expression of any of these transcripts among Ctrl, ID-PD, or LRRK2-PD-derived cells. However, we should note that several cell-types other than iPSC-derived DA neurons are present in the samples analyzed. These include feeder astrocytes, but also iPSC-derived cells that are not DA neurons, which account for a variable (iPSC clone-dependent) but nonetheless considerable fraction of the cells in the sample. In the case of samples tested in bulk, such as for these RT-qPCR analyses, high transcriptional levels in cells other than DA neurons would mask actual changes in the expression levels occurring specifically in DA neurons. For this reason, in our studies we typically use immunofluorescence analyses, by which we can readily identify DA neurons (TH positive neurons) and investigate specific phenotypes in them. Owing to this limitation, the analysis of transcriptional changes occurring

specifically in DA neurons in our system would require the use of technologies that are currently well beyond the scope of the current manuscript.



Referee #2 (Comments on Novelty/Model System):

iPS is a new and powerful in vitro system with high potential to model pathogeneic processes in disease

Referee #2 (Other Remarks):

Ramos-Sanchez et al. report on the characterization of mDA neurons derived from several iPS lines generated from control (Ctrl), idiopathic (ID-PD), and LRRK2-mutant (LRRK2-PD) PD patients. In short and long-term differentiation conditions they found differences between Ctrl and PD midbrain-like DA neurons with increased expression levels of SNCA, a rudimentary morphological phenotype, less cell viability and neurite sprouting, impaired autophagy, and vulnerability to MPP+ toxic insult in the PD DA neurons. The authors conclude that their "study provides an iPSC-based in vitro model that captures the patients' genetic complexity and allows investigating PD pathogenesis in a disease-relevant cell type".

This study is in line with an increasing number of reports on using a combination of patient-derived iPS technology with in vitro differentiation paradigms to generate model systems of disease and discover mechanisms of pathogenesis. The current study extends recent papers using this paradigm for studying PD-affected DA neurons by analyzing Ctrl, ID-PD and LRRK2-PD iPS lines in parallel and the use of long-term differentiation cultures. This is an important study. The data are solid, interesting and reveal new aspects on PD DA neuronal dysfunction in the in vitro paradigm. Moreover, they provide new insight into the feasibility of this system to study pathological processes in DA neurons derived from patients with either sporadic or mutant genetic background. This latter point should be discussed in regard of a recent paper by Soldner et al., 2011, Cell 146, 318, in which the genotype is introduced by genome editing.

We are very glad that the reviewer found our study important and based in solid and interesting data. We have addressed his/her comments as follows:

Minor comments:

Fig. 1: TH and Tuj1 don't seem to overlap - NeuN staining?

We apologize for the quality of the images shown in Fig. 2A-C in the original version of our manuscript. The low magnification of the microphotographs provided, combined with image quality reduction for decreasing file size for reviewing, resulted in a confusing figure. The point raised by the reviewer is an important one, since all the TH positive cells generated in our differentiation experiments are, indeed, TUJ1 positive as well. We have now replaced these images in the new Fig. 2 of the revised version of our manuscript with clearer images, at low and high magnification, which clearly show the perfect co-localization of both markers.

Fig. S1: I am not convinced of the DAT staining in panel A. The MAB369 Chemicon antibody is against the N-terminus, however the staining looks more intracellular, similar to Girk2 or Tuj1 in the same panel.

We have not studied in detail the cellular localization of the staining pattern identified by MAB369. Indeed, with the level of resolution used in our studies, it might be very difficult to differentiate a cell membrane pattern from a cytoplasmic one. Nevertheless, several laboratories, including ours, have used this antibody extensively and always reported similar staining patterns (see, for instance Sonntag et al., Eur J Neurosci 2004; Roy et al., Nat Med 2006; Rössler et al., Neurosci 2010; Sanchez-Danes et al., Hum Gene Ther 2011; Caiazzo et al., Nature 2011).

Fig. 2 and S2: The authors should give more information on the LV.NES.LMX1A.GFP virus. Did the authors test if the Nes enhancer/TK promoter is specific to Nes+ precursors, and not leaking in other phenotypes? Is this the same construct used by the Perlmann group (Friling et al., 2009, PNAS May 5;106(18):7613-8)?

We agree with the reviewer that our manuscript does not provide a detailed description of our DA neuron differentiation protocol. A manuscript detailing this protocol was under revision at the time of submitting the current manuscript, and it is now accepted for publication in *Human Gene Therapy* and already available online at http://www.liebertonline.com/doi/full/10.1089/ hum.2011.054. In this manuscript, we describe in detail our DA neuron differentiation protocol, including the generation and characterization of the LV.NES.LMX1A.GFP virus. This lentivirus is conceptually similar to the construct used by Friling et al. (PNAS 2009) to overexpress Lmx1a in mouse ES cells, although these authors used a regular expression plasmid for this purpose, rather than a virus. In our case, since transfection of human ES cells or iPSC is notoriously difficult, we developed a lentiviral system for the robust and sustained expression of the transgene. This information is discussed in detail in the manuscript referred to above. We have now updated the reference to our previous publication in the revised version of our manuscript.

Fig. 3 and Fig. S4: If the authors have ICC and Western blot quantification for SNCA and LRRK2 that would strengthen the PCR results.

We appreciate the reviewer's experimental suggestion and have performed immunofluorescence and Western blot analyses of SNCA and LRRK2 in DA neurons differentiated from Control, ID-PD, and LRRK2-PD iPSC. Unfortunately, we could not obtain a clear signal for SNCA in immunoblots with the antibody we used, but the immunofluorescence signals are strong and specific. In the case of LRRK2, we tested a panel of antibodies developed under the auspices of the MJFF and identified one that gave strong signal in both Western blot and immunofluorescence. We have included this information in the revised version of our manuscript as a new Supplementary Fig. S5.

Fig. S5: I was wondering if the DAn are NeuN positive and Tuj1-negative, see comment to Fig. 1.

Please, see our response to the similar comment above.

Discussion: The discussion is a bit technical and could benefit from some interpretation of their data regarding PD pathogenesis and what they would imply for the field of neurodegenerative diseases. In particular interesting is the similar phenotype development of the ID-PD and LRRK2-PD lines. The authors should discuss a recent paper by Soldner et al., 2011, Cell 146, 318.

We particularly appreciate this comment from the reviewer, as we also feel that our findings may have profound implications for our understanding of PD pathogenesis. In the revised version of our manuscript, we have introduced an entire paragraph to discuss these implications in more detail. We have also added the reference to the recent paper by Soldner et al. (Cell 2011).

Referee #3:

The manuscript by Sanchez-Danes et al. generated iPSC lines from both sporadic (ID-PD) and familial PD (with LRKK2 G2019S mutation LRRK2-PD) patients. The authors report that all lines TH+ neurons can be generated from all PD iPSC lines and control lines. Interestingly, the authors found that TH+ neurons from LRRK2-PD iPSC lines, but not from ID-PD iPSC lines exhibited a-synuclein accumulation in the cell body. On the other hand, TH+ neurons from both LRRK2- and ID- PD iPSC lines exhibited decreased neurite length and vacuole accumulation over extended culture time. The observations are interesting and suggesting that retarded neurite development/degeneration of TH+ neurons might be a common phenotype of PD, however, the data at present did not provide a causative link. Although it is difficult to test with ID-PD, how LRRK2 mutation leads to the observed defects are testable. For example, whether expression of LRRK2-G2019S in control neurons result in the same phenotype in neurite arborization and vacuole formation.

We are glad that the reviewer found our studies interesting and appreciate his/her experimental suggestion. The proposed experiment was technically very challenging, as differentiated neurons are very difficult to transfect. Nevertheless, we appreciated the importance of providing a causative link for the observed phenotypes in PD-iPSC-derived DA neurons and invested considerable additional effort to prove this point. Specifically, we differentiated DA neurons from 2 different Control-iPSC lines for 65 days, and co-transfected them with plasmids encoding wild-type LRRK2 or LRRK2-G2019S and GFP. The expression of mutant LRRK2-G2019S proved to be deleterious for these cells, as we could recover very few transfected DA neurons after 10 days. However, those DA neurons that we could recover had the expected phenotype of short/few neurites. In contrast, DA neurons transfected with wild-type LRRK2 were morphologically indistinguishable from untransfected neurons. We believe that these results strengthen the notion that the phenotypes we describe in DA neurons differentiated from PD-iPSC upon long-term culture are, indeed, a consequence of the patients' genetic background, and thank the reviewer for this key experimental suggestion. We have included this information in the revised version of our manuscript in Fig. 4I-N.

Other comments:

While the authors suggested that iPSCs were generated from 7 sporadic and 4 LRRK2 PD patients and from 4 control subjects, data only presented from 2 controls, 3 ID-PDs and 3 LRRK2-PDs in Figs. 4 and 5. In most cases, the data were pooled together or only from a single line were shown. Data not shown is not acceptable.

We agree with the reviewer that the power of our analyses critically depends on our using several patients per condition. While the critical experiments were conducted in most of the patient-specific iPSC lines in the previous version of our manuscript, it is true that some of them were not. We have now extended our studies to include all the patients' and control iPSC lines in terms of DA neuron differentiation and characterization, morphological alterations of DA neurons upon long-term culture, analysis of autophagic system (basal levels of LC3-II, LC3 flux, and autophagosome formation), and morphological alterations of DA neurons upon induction of autophagy and inhibition of lysosomal proteolysis. The inclusion of these data from all the patients and controls has naturally increased the variance of our overall data (we provide results from individual iPSC-derived DA neurons in many occasions, when practically feasible, to illustrate this point), but the trends and statistic significance of the observed differences are maintained and, more importantly, the power of our analyses is greatly increased. We have included these new sets of data in the appropriate figures throughout the revised version of our manuscript.

In Fig. 4, the quantification data does not seem to match the description. For example: The neurite length of sample type 2 cells (4B) is longer than the average length of type 1 cells (4G). In addition, it is not clear from the text and from the method section what is the criteria for selecting the cells for analysis.

In the original version of our manuscript we made a mistake in the dimensions of the scale bars given for some panels. We apologize for this mistake and thank the reviewer for catching it up. We have now corrected this in the revised version of our manuscript, and have also given more details in the method section as to the criteria for selecting cells for analysis (both for morphological analyses and neurite number/length).

2nd Editorial Decision 23 December 2011

Thank you for the submission of your revised manuscript "Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease" to EMBO Molecular Medicine. We have now received the enclosed reports from the referees whom we asked to re-assess it.

As you will see, both Reviewers acknowledge that the manuscript was significantly improved during revision and Reviewer #2 indicates that it is suitable for publication. However, Reviewer #1 still raises concerns. His/her main concerns are the lack of rescue experiments and that some of the results do not fully support the conclusions.

We agree that it would be ideal to perform the suggested experiments, however, we realize that the addition of more data in this direction would be time-consuming. Should you be able to provide data addressing points 1, 2, and 4 of Reviewer #1, we would encourage you to include them into the present study. Otherwise, we would strongly encourage you to include a very brief discussion of this issue into the manuscript and to soften the corresponding conclusions. Regarding the other points, please provide quantification of the data (point 3) and higher quality images and Z-stacks (point 5).

On a more editorial note, the description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05'). Also, please provide o Table of Contents as the first page of the Supplementary Material file. Please note that EMBO Molecular Medicine does not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or supplementary figures. "Unpublished observations" may be referred to in exceptional cases, where these are data peripheral to the major message of the paper and are intended to form part of a future or separate study.

In addition, we noted that your point-by-point response contains a figure. Since this would be published in the Review Process File, please let us know whether you agree with its publication, if

you would like to remove the figure or whether you would like to opt out (more information below).

Please submit your revised manuscript within one month, unless arranged differently with the editor. I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

The revised version of the study by Sanchez-Danes and colleagues is generally improved providing some more details on the characterization of the disease-associated phenotype. The authors addressed some of my concerns raised on the previous study investigating in more details alterations in autophagy processes associated with the patient-specific cells.
However, the study lacks rigorous quantifications, convincing results and rescue experiments that might support authors' conclusions.

In particular:

1) The authors claim that alpha-synuclein staining is only rarely detectable in their control- and ID-PD iPSC-derived neurons. However, it is well known in the field that this protein is ubiquitously detectable in post-mitotic neurons both in vivo and in vitro. Therefore, it is odd that the authors do not detect robust synuclein staining in control-derived cells. In order to strength their conclusions immunoblot analysis of control and LRRK2-patient neurons should be provided in order to appreciate the difference at the population level.

2) One of the major findings of this study is the description that sporadic or LRKK2 mutated PD neurons have a tendency to undergo apoptosis when cultured for an extended time in vitro. However, this conclusion relies only on the manual counting of Caspase-3+ neurons. Due to the relevance of this message and its novelty, I strongly believe this analysis is inadequate. The authors should substantiate these results with some independent and more reliable studies. One way would be to quantify cell death in the entire culture by profiling cellular death by flow cytometry analysis using annexin-V or propidium iodine stainings. To analyze only the neuronal fraction, the authors should employ flow cytometry-graded neuronal specific antibodies. Another confounding aspect is that this long term cultures easily can induced neuronal cell death in a highly unspecific manner. Thus, it is crucial to show that this impressive cell death described herein is specifically associated to the disease. To prove this convincingly, the authors should employ a specific LRRK2 kinase inhibitor and showing that this treatment can rescue apoptosis over time. Alternatively, gene function should be reestablished and verify the rescue of the aberrant phenotype.
LRRK2 transfection studies don't help to answer this points since this procedure is highly inefficient and analysis is still manually-based.

3) The authors provided new data regarding p62 protein content in control as well as patient neurons as I requested. However, they only reported few images without any quantification analysis. It is missing how many TH+ neurons have p62 protein accumulation and how this develops with time. To provide a meaningful answer to this question it would be much preferable to perform quantitative immunoblotting assays.

4) Authors included the analysis of the LC-3 protein by western blotting as requested. Again, I see incongruence in the data presented. First of all, authors' assumption that LC-3 basal levels are increased in ID-PD-derived cells is questionable. Looking at the immunoblots 4 out of 7 ID-PD cell lines display a band of similar intensity with those of control-cells. Even in the other three cases, the difference is so tiny and the blotting so overexposed that it is not wise to produce any quantification

or draw any conclusion. Exactly the similar concern regards the LRRK2-PD derived cells.
These differences in immunoblotting are much lower respect to the representative pictures presented by the authors of LC3 and p62 immunofluorescence images on cells raising some problems in comparing data.
Even despite that, the authors did not find any significant difference in autophagosome formation after blocking lysosome function indicating a substantial comparable activity of the autophagic machinery.

5) The authors also performed LC3/LAMP1 double staining and concluded that PD-related cells have a lower co-localization signal indicating a compromised autophagy.
This conclusion is again unclear since the pictures presented do not allow drawing this conclusion. It is even not clear whether the images are in a confocal axis and if so, it is not presented the Z-stacking view which would be the only way to ensure this conclusion. Again, to be confident about the association between this possible defect and the disease, the authors should use a LRRK2 kinase inhibitor and show the rescue of the autophagy defects.

In conclusion, the authors presented a new set of experiments attempting to answer to my concerns. Unfortunately, the results are not robust enough and leave unaddressed the understanding of the phenotype and even more to substantiate in any means authors' conclusions. More rigorous experiments need to be performed in order to clarify phenotypic differences between patient and control derived cells. Importantly, rescue experiments are totally missing in order to ensure a direct link between the LRRK2 mutation and the hypothetical phenotypic defect.

Minor change:

Page 9, third last line: autophagosome should be replaced with lysosome

Referee #2 (Comments on Novelty/Model System):

The authors have sufficiently addressed my concerns and improved their manuscript. This is an interesting and well-conducted study on some of the mechanisms involved in PD pathogenesis using iPS technology. The data not only provide further insight into the pathophysiology of PD dopamine neurons, they also demonstrate the usefulness of iPS technology as a model system for understanding disease processes. This is important information for the scientific community.

Referee #2 (Other Remarks):

The authors have sufficiently addressed my concerns and improved their manuscript. This is an interesting and well-conducted study on some of the mechanisms involved in PD pathogenesis using iPS technology. The data not only provide further insight into the pathophysiology of PD dopamine neurons, they also demonstrate the usefulness of iPS technology as a model system for understanding disease processes. This is important information for the scientific community.

2nd Revision - Authors' Response 04 January 2012

Point-by-point response to the reviewer's comments:

Referee #1:

The revised version of the study by Sanchez-Danes and colleagues is generally improved providing some more details on the characterization of the disease-associated phenotype. The authors addressed some of my concerns raised on the previous study investigating in more details alterations in autophagy processes associated with the patient-specific cells.

However, the study lacks rigorous quantifications, convincing results and rescue experiments that might support authors' conclusions.

In particular:

1) The authors claim that alpha-synuclein staining is only rarely detectable in their control- and ID-PD iPSC-derived neurons. However, it is well known in the field that this protein is ubiquitously detectable in post-mitotic neurons both in vivo and in vitro. Therefore, it is odd that the authors do not detect robust synuclein staining in control-derived cells.

We respectfully disagree with the reviewer's comment. While alpha-synuclein is ubiquitously expressed, its immunohistochemical detection in healthy brains (from either mice, monkeys or humans) has been predominantly associated with neuronal fibers rather than neuronal cytoplasm (see, for instance: Vila et al., 2000; Braak et al., 2004; Purisai et al., 2005; Chu and Kordower, 2007; Beach et al., 2008). Therefore, the absence of robust alpha-synuclein immunostaining in the cytosol of our control iPSC-derived dopaminergic neurons fully concurs with these observations. In contrast, increased levels of cytoplasmic alpha-synuclein have been repeatedly shown to be pathogenic, both in vitro and in vivo. For instance, (i) progressive cytosolic accumulation of alpha-synuclein occurs in dying substantia nigra dopaminergic neurons from MPTP-intoxicated mice and monkeys (Vila et al., 2000; Purisai et al., 2005); (ii) the number of substantia nigra dopaminergic neurons exhibiting cytoplasmic alpha-synuclein staining increases dramatically with age in monkeys and humans, which correlates with substantial decreases in tyrosine hydroxylase (TH) immunoreactivity in these neurons (Chu and Kordower, 2007); (iii) viral vector-mediated overexpression of alpha-synuclein in cell bodies of substantia nigra dopaminergic neurons causes progressive nigrostriatal denervation in rats and monkeys, being therefore used to experimentally model PD in these species (Kirik et al., 2002; Kirik et al., 2003); (iv) duplications and triplications in the alpha-synuclein gene, which is associated with increased alpha-synuclein protein levels, causes familial forms of PD (Singleton et al., 2003; Ibanez et al., 2004). Consistent with a potential pathogenic role for increased cytoplasmic alpha-synuclein levels, we report here that most iPSC-derived dopaminergic cells from LRRK2 patients exhibit diffuse cytoplasmic accumulations of alpha-synuclein, compared to control cells or to those derived from sporadic patients. These findings are in agreement with results from animal studies reporting post-translational regulatory interactions of mutant LRRK2 and alpha-synuclein (Cookson, 2010) and with a recently published study in which alpha-synuclein levels were also shown to be increased in iPSC-derived dopaminergic neurons from LRRK2 patients, compared to control cells (Nguyen et al., 2011).

In order to strength their conclusions immunoblot analysis of control and LRRK2-patient neurons should be provided in order to appreciate the difference at the population level.

While we agree with the reviewer that immunoblot analyses of alpha-synuclein would normally be helpful to quantitatively determine the levels of alpha-synuclein, and we attempted to perform these analyses in the previous round of revision at the request of Reviewer #2, our antibodies did not give a clear signal in blots. We did not pursue this further (e.g. by trying different antibodies) since we reasoned that this type of analyses would actually not be very informative in our case, as dopaminergic neurons represent only a small fraction of the total number of cells present in the heterogeneous iPSC differentiation cultures used here, and also because Reviewer #2, the only one to initially suggest this experiment, was satisfied with our response.

In any case, to further clarify the point raised by the reviewer, we have modified the text in the revised version of our manuscript as follows:

- In page 7, the sentence "...the majority of DAn differentiated from Ctrl-iPSC and ID-PD iPSC were negative for α-synuclein...", now reads "the majority of DAn differentiated from Ctrl-iPSC and ID-PD exhibited barely detectable levels of endogenous alpha-synuclein in their cytoplasm…"
- In page 29, the legend of Figure 3D now reads: "Quantitative analyses of DA neurons showing cytoplasmic accumulation of alpha-synuclein."
- In panel D of figure 3, y-axis label has been changed to "DAn showing cytoplasmic alpha-synuclein accumulation (%)"

2) One of the major findings of this study is the description that sporadic or LRKK2 mutated PD neurons have a tendency to undergo apoptosis when cultured for an extended time in vitro. However, this conclusion relies only on the manual counting of Caspase-3+ neurons. Due to the relevance of this message and its novelty, I strongly believe this analysis is inadequate. The authors should substantiate these results with some independent and more reliable studies. One way would be to quantify cell death in the entire culture by profiling cellular death by flow cytometry analysis using annexin-V or propidium iodine stainings. To analyze only the neuronal fraction, the authors should employ flow cytometry-graded neuronal specific antibodies.

We respectfully disagree with the reviewer's point that one of our major findings is that patient-derived neurons have increased tendency to undergo apoptosis upon long-term culture, since this would represent a vast oversimplification of our findings. We provide evidence that patient-derived neurons, over long-time culture, undergo a neurodegenerative process characterized by morphological alterations (Fig. 4A-C, Suppl. Fig. 7), significant reduction in the number (Fig. 4G) and length (Fig. 4H) of neurites, and increased number of apoptotic cells (Fig. 4D-E). Apoptotic cells can be easily detected in immunofluorescence analyses by their fragmented nuclei and positive staining for cleaved caspase-3. Compared to other procedures, such as PI or annexin-V staining mentioned by the reviewer, our analyses are much more time consuming, but offer the great advantage of providing direct visualization of cells, and therefore of their morphology and identity (by co-labeling with TH antibodies in our case). For these reasons, we analyzed the presence of apoptotic cells by immunofluoresce, and propose this as a useful assay that can provide a quantifiable readout for drug screening. However, in our studies, neuronal apoptosis *per* se, is not analyzed in further detail, nor are the causes or mechanisms leading to it. We agree with the reviewer that using alternative assays to confirm the numbers of apoptotic cells in our experiments would probably be a nice corroboration (as it would be the case for any other piece of data in our study), but at this point we do not consider this a priority for the following reasons: 1) The relatively minor impact of this result in our overall description of the increased susceptibility of patient-derived neurons to undergo neurodegeneration upon long-term culture (assessed, as explained above, by different experimental means); 2) We are convinced that our results in these experiments are sound, since cleaved caspase-3 staining is a widely used assay for detecting apoptosis and, in our hands, worked very nicely and gave strong, unambiguous signals, and 3) None of the reviewers (including Reviewer #1) raised any concern in this respect in the first round of revision. Had Reviewer #1 requested these confirmatory assays in the first round of revision, we could easily have carried them out along with the other experiments, but, at this point, we do not consider that the time, effort, and resources necessary to run these assays on their own (representing a new series of expansion/ differentiation/long-term culture of 15 iPSC lines for over 4 additional months) are justified, and that the publication of our manuscript should be delayed for this reason.

Another confounding aspect is that this long term cultures easily can induced neuronal cell death in a highly unspecific manner. Thus, it is crucial to show that this impressive cell death described herein is specifically associated to the disease.

The long-term culture conditions used in our experiments represent a poorly-defined experimental environment where, indeed, many pathogenic mechanisms might be at work. It is in

this sense that we agree with the reviewer that such conditions "can induce neuronal cell death in a highly unspecific manner"; that is, rather than through a specific pathogenic mechanism (e.g. oxidative stress, mitochondrial dysfunction, …). Such an unspecific mechanism (or best put, unspecified combination of mechanisms) could be likened to the process of aging. For this reason, and since culture time was the only variable we acted upon (by increasing it) to induce the phenotypes, we refer to these conditions in the revised version of our manuscript as:

Page 15: "which may have induced culture-related stress conditions mimicking in vivo aging in PD patients".

We should note, however, that having culture conditions that act in an unspecific (or, rather, unspecified) manner, does not have any bearing on whether increased apoptosis (or any other phenotype we describe) is specifically associated or not to the disease. To address this issue, we compared the appearance of the different phenotypes (altered DAn morphology -Fig. 4A-C, Suppl. Fig. 7-; number of neurites -Fig. 4G-, length of neurites -Fig. 4H-, and number of apoptotic DAn -Fig. 4D-E) in neurons derived from 4 healthy individuals and from 11 PD patients (7 from sporadic PD and 4 from LRRK2 mutants). As clearly shown in our results, the appearance of all these phenotypes is specifically associated to the disease.

To prove this convincingly, the authors should employ a specific LRRK2 kinase inhibitor and showing that this treatment can rescue apoptosis over time. Alternatively, gene function should be reestablished and verify the rescue of the aberrant phenotype.

LRRK2 transfection studies don't help to answer this points since this procedure is highly inefficient and analysis is still manually-based.

We appreciate the reviewer's experimental suggestion, but strongly disagree with the usefulness or feasibility of such approach. Since the reviewer now requests using similar "rescue experiments" at different points in his/her comments, we will discuss the issue in more detail here.

Generally speaking, the ability to perform "rescue experiments" in any context essentially depends on the knowledge of the cause (genetic or otherwise) of the phenotype to be rescued. Unfortunately, our understanding of PD pathogenesis is far from complete, but we do know that it is highly complex and does not lend itself easily to "rescue experiments". This is acknowledged by the reviewer in the case of sporadic PD, where no suggestions for "rescue experiments" are proposed. However, in the case of PD associated to LRRK2 mutations, the reviewer now requests that we repeat our experiments using LRRK2 kinase inhibitors in parallel, as these should rescue the phenotypes. We do not believe that these experiments are justified, for the following reasons:

1) The reviewer assumes that the kinase activity of LRRK2 kinase is the responsible for PD pathogenesis, but this is incorrect, as no formal proof for this link has been provided (Cookson 2010) and, in turn, several lines of evidence prove otherwise. For instance, it has been shown that transgenic mice overexpressing LRRK2 or a kinase-dead mutant form of LRRK2 showed similar Golgi fragmentation, impairment of microtubule dynamics, and alpha-synuclein-mediated neuropathology, indicating that the kinase domain of LRRK2 is not critical for these PD-related phenotypes (Lin et al., 2009). Similarly, wild-type LRRK2 and G2019S-mutated LRRK2 had comparable effects on reporter protein levels and ubiquitin levels, which could not be rescued by LRRK2 kinase inhibitors, again indicating that LRRK2 kinase activity is not a major determinant of its ubiquitin-proteasome system-inhibitory phenotype (Lichtenberg et al, 2011). Furthermore, the finding that homo- and heterozygous carriers of LRRK2 mutations are clinically indistinguishable (Ishihara et al., 2006), further supports a kinase-independent mechanism for PD pathogenesis.
2) In the previous round of revision, Reviewer #3 suggested overexpressing mutant LRRK2-G2019S in control neurons, in order confirm a causative link for the observed phenotypes in PD-

iPSC-derived DA neurons. We invested considerable additional effort to prove this point and included this information in the revised version of our manuscript (Fig. 4I-N). We believe that this experimental approach nicely complements the one now suggested by the reviewer, and that the positive results obtained clearly demonstrate its feasibility. (We do not understand how the fact that these experiments were inherently inefficient and time-consuming would detract from the value of the positive results obtained).

3) We would like to emphasize that our manuscript scope is the implementation of a patient-specific iPSC-based system that, for the first time, recapitulates salient features of both sporadic and genetic forms of PD. We do not claim to have solved the pathogenesis of PD (and, therefore, cannot rescue PD-related phenotypes) but we are convinced that this system will open new ways for investigating specific pathogenic mechanisms in a genuinely human context, as we have done in the case of autophagy.

3) The authors provided new data regarding p62 protein content in control as well as patient neurons as I requested. However, they only reported few images without any quantification analysis. It is missing how many TH+ neurons have p62 protein accumulation and how this develops with time. To provide a meaningful answer to this question it would be much preferable to perform quantitative immunoblotting assays.

In the previous round of revision, the reviewer requested "the authors should study the localization of the p62/SQSTM1 protein", and we have done so (even though the field of autophagy is avoiding each time more the use of p62 as a molecular marker of this process), because we agreed with the reviewer that analyzing p62 localization (by immunofluorescence, how else?) could be helpful to confirm alterations in protein homeostasis and altered clearance of proteins. The reviewer now asks these experiments to be repeated and analyzed by immunoblotting. We strongly disagree with the reviewer's comment and believe that including the immunoblot analysis of p62 requested in this new revision would not be informative and, if anything, may result confusing for the overall interpretation of the work. The field of autophagy has become very cautious in the use of p62 levels as autophagic readout because:

1) p62 is also degraded by the ubiquitin/proteasome system, and in conditions such as the ones in our study, in which there is a severe compromise of quality control, it would be difficult to attribute an increase in p62 levels solely to problems with the autophagic system;

2) p62 has been shown to be transcriptionally upregulated in conditions of stress such as those resulting from chronic oxidative stress and, consequently, an increase in the levels of p62 in our system although could be easily interpreted as problems in autophagy, could also be consequence of increase oxidative stress in these cells, which we expect to occur as result of the problems with organelle and protein turnover;

3) Recent studies presented in two recent autophagy conferences and already accepted for publication, show now that p62 transcriptional regulation is also modulated by autophagy (through changes in the availability of free amino acids in the cell) and that, although p62 levels may have some value in the analysis of autophagy under normal conditions and during the first 4 hours of starvation, any measurement at later time points or in conditions in which autophagy is severely compromised (as it is the case in our study) cannot be used as read out of autophagic function.

4) Authors included the analysis of the LC-3 protein by western blotting as requested. Again, I see incongruence in the data presented. First of all, authors' assumption that LC-3 basal levels are increased in ID-PD-derived cells is questionable. Looking at the immunoblots 4 out of 7 ID-PD cell lines display a band of similar intensity with those of control-cells. Even in the other three cases, the difference is so tiny and the blotting so overexposed that it is not wise to produce any quantification or draw any conclusion. Exactly the similar concern regards the LRRK2-PD derived cells.

These differences in immunoblotting are much lower respect to the representative pictures presented by the authors of LC3 and p62 immunofluorescence images on cells raising some problems in comparing data.

Even despite that, the authors did not find any significant difference in autophagosome formation after blocking lysosome function indicating a substantial comparable activity of the autophagic machinery.

We performed immunoblot analyses for LC3 as requested by this reviewer, and tried to point out in the text the limitations that this type of analysis has, compared to the image analysis, in our experimental model. The reviewer is probably more used to work with homogeneous cultures in which we completely agree that measurement of LC3 flux by immunoblot is the gold-standard in the field, and we also use it routinely in our analyses. However, due to the specific characteristics of iPSC differentiation cultures, these are heterogeneous cell populations in which neuronal cells only contribute a small percentage of the total number of cells present in the culture. Image analysis is ideal in this case because we can separately analyze neurons and, consequently the data presented in the immunofluorescence for LC3 and in the morphometric analysis by electron microscopy, which focus solely in neurons, reflect very dramatic changes in this system. In contrast, when using immunoblot, only a fraction of the LC3 present in the sample actually comes from neurons. As a matter of fact, we were very pleased that, even with these limitations, we were able to detect significant changes in basal LC3 levels and in the autophagic flux by immunoblot (much higher than what we expected to see considering that autophagy in the surrounding supporting cells was unaltered). The fact that we can detect the changes by immunoblot further increases our confidence on the serious nature of the autophagic compromise in the neurons in our system, and provides additional support to the striking changes that we present in the image-based procedures, where the analysis is restricted to DA neurons, main focus of this manuscript.

In any case, to further clarify the point raised by the reviewer, we have included the following sentence in the revised version of our manuscript:

Page 9: “To directly analyze autophagic clearance we compared the rates of degradation of LC3-II in the different cells by immunoblot (LC3-flux assay), even though we were aware that neural-specific changes could be underestimated in this type of analysis, as DAn represent only a fraction of the cells present in whole-culture lysates”.

5) The authors also performed LC3/LAMP1 double staining and concluded that PD-related cells have a lower co-localization signal indicating a compromised autophagy.

This conclusion is again unclear since the pictures presented do not allow drawing this conclusion. It is even not clear whether the images are in a confocal axis and if so, it is not presented the Z-stacking view which would be the only way to ensure this conclusion. Again, to be confident about the association between this possible defect and the disease, the authors should use a LRRK2 kinase inhibitor and show the rescue of the autophagy defects.

We have now included higher magnification views and Z-stacks for the images in Fig. 7A-C, which clearly illustrate the co-localization (of absence of it) of LC3 and LAMP1 in the different samples.

Regarding the use of LRRK2 kinase inhibitors, please, see our response to point 2 above.

In conclusion, the authors presented a new set of experiments attempting to answer to my concerns. Unfortunately, the results are not robust enough and leave unaddressed the understanding of the phenotype and even more to substantiate in any means authors' conclusions. More rigorous experiments need to be performed in order to clarify phenotypic differences between patient and control derived cells. Importantly, rescue experiments are totally missing in order to ensure a direct link between the LRRK2 mutation and the hypothetical phenotypic defect.

We respectfully disagree with the reviewer's conclusion and would like to draw his/her attention to the considerable effort we made to address the helpful suggestions made by the reviewer in the previous round of revision, as well as to the positive results obtained.

Regarding the "rescue experiments", please, see our response to point 2 above.

Minor change:

Page 9, third last line: autophagosome should be replaced with lysosome

Done

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