α-Synuclein pathology and mitochondrial dysfunction
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Chapter 4

Mitochondrial trafficking impairment in dopaminergic neurons from Parkinson patient-derived iPS cells

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Abstract

While it is widely recognized that Parkinson’s disease (PD) is accompanied by mitochondrial dysfunction in dopaminergic (DA) neurons, the etiology of the disease remains elusive. Discovery of the implication of α-synuclein as the main constituent of Lewy bodies has driven research in the last decade, but the nature of the relationship between α-synuclein and mitochondrial dysfunction has not yet been elucidated. In this study, we use a patient-derived stem cell model of PD, comparing control cells with patient cells containing a triplication of or a mutation (A53T) in the SNCA gene. Using a long-term (90 days) culture approach, we analysed mitochondrial trafficking in cultured dopaminergic neurons. We demonstrated that mitochondrial trafficking is impaired in PD neurons when compared to control neurons, and mitochondria are frequently fragmented in patient-derived DA neurons. These results indicate that endogenous expression of α-synuclein in PD patient iPS-derived dopaminergic neurons results in mitochondrial pathology. Our results suggest that mitochondrial fragmentation and impaired trafficking are early events that contribute to mitochondrial dysfunction in PD.

Highlights:

- Mitochondrial trafficking is impaired in PD patient dopaminergic neurons
- PD mitochondria demonstrate fragmentation
- Alterations in mitochondrial trafficking might precede PD pathology
Introduction

Parkinson’s disease (PD) is the world’s most common movement disorder and is characterized by rigidity, slowness of movement and resting tremors. The pathophysiological hallmark of PD is the occurrence of inclusion bodies in the brain named Lewy bodies. These cellular inclusions consist of abnormal protein aggregates and their occurrence is correlated to disease progression. Neuronal degeneration is most striking in the substantia nigra pars compacta in the ventral midbrain and dopaminergic (DA) cell death in this area is thought to contribute to most of the observed symptoms.

While research has been conducted for many years, the etiology of the disease remains poorly understood and treatment of PD merely addresses the symptoms. Experimental studies with substances that have been implied in causing specific dopaminergic degeneration, such as MPTP and pesticides like rotenone have elucidated at least part of the observed cell pathology, thus linking mitochondrial dysfunction to disease progression [1, 2]. The discovery in 1997 of a dominant genetic form of PD [3], linked to the SNCA gene, implicates α-synuclein in cell pathogenesis. As one of the major components in Lewy bodies, it has been shown that monomeric and oligomeric forms of α-synuclein are able to spread in a prion-like manner, leading to spreading of disease pathology [4, 5]. The link between α-synuclein and mitochondrial dysfunction remains poorly understood, and studies ranging from linking α-synuclein to mitochondrial permeability transition pore regulation [6] to ER membrane stress have been performed [7].

Most current understanding of cell pathogenesis in PD has been acquired through cell-models, genetics, animal studies and post mortem patient material. While these studies provided important insights in various cell pathological aspects, these approaches have their limitations. The scientific revolution of induced pluripotent stem cells (iPSCs) has led to the possibility of generating previously unobtainable cell populations.
from PD patients [8]. Specifically, the generation of ventral midbrain dopaminergic neurons via iPSCs is bound to yield new understanding of PD and has already produced exciting new leads.

The link between mitochondrial dysfunction and α-synuclein has been further elucidated by assessing the vulnerability of PD iPSC-derived DA neurons to environmental toxins, showing a preferential vulnerability to maneb, paraquat and rotenone [9]. However, without using environmental toxins, hiPSC-derived DA neurons in culture conditions reveal little pathology, and have to be cultured for extended periods of time to observe pathological defects [10].

It is hypothesized that early events in the synuclein cascade might compromise intracellular trafficking, leading to altered mitochondrial dynamics and development of early mitochondrial pathology [11, 12]. While there is evidence that points in this direction, data from iPSC-derived DA neurons, with long axonal processes have not yet been presented.

In this study, we have used dermal fibroblast-derived iPSCs from 2 autosomal dominant familial PD patients and a healthy gender and age-matched control to generate DA neurons. Our PD4 iPSC lines have a triplication of the SNCA locus (Parkinson Disease 4), and our PD1 iPSC lines have a reported mutation in the SNCA gene causing the missense variant A53T α-synuclein (Parkinson Disease 1). We show that (mutated) α-synuclein impairs mitochondrial trafficking in PD patient-derived iPSC-DA neurons cultured for 90 days in comparison to iPSC-DA neurons from healthy controls. Our findings provide evidence for a link between α-synuclein and early mitochondrial pathology in PD.

Materials and methods
Generation of hiPSCs

Human iPSCs were generated using a slightly modified protocol of Okita et al. (2011). Briefly, one million cells were nucleofected using an Amaxa nucleofector 1 using 0.85 μg pCXLE-hMLN, 0.85 μg pCXLE-
hOCT3/4 and 1.3 µg pCXLE-hSK (Addgene #27079, #27076, #27078). Individual clones were picked and expanded, and checked for expression of pluripotency factors on regular basis (OCT3/4, SOX2, TRA1-60, NANOG, live alkaline phosphatase). Embryoid bodies (EBs) were allowed to spontaneously differentiate in order to assess the pluripotent differentiation potential, followed by immunostaining for ectoderm (TUJ1), endoderm (GATA4) and mesoderm (DESMIN) (See supplemental figure S1). Geltrex® coated plates and Essential 8™ medium (Thermo Fisher) were used to maintain cells, while passaging was done using ReLeSR™ (Stemcell Technologies). All cells were maintained in a humidified incubator at 37°C with 5%CO2 and used for experiments between passage 25 and 35.

Dopaminergic differentiation

Dopaminergic differentiation was achieved using a slightly adapted protocol from Kriks et al. [13]. Briefly, cells were maintained to over 50% confluency after which medium was changed to 50% DMEM/F12 and 50% KSR medium (containing KnockOut™ DMEM and 15% KnockOut™ Serum Replacement, Thermo Fisher). Medium composition was gradually shifted to DMEM/F12 supplemented with N1 (Sigma) over 7 days, while the following small molecules were added; 10 µM SB-431542 (Stemcell Technologies, day 1-5), 100nM LDN-193189 (Stemcell Technologies, day 1-11), 3 µM Purmorphamine (Stemcell Technologies, day 3-7), 500 nM Smoothened agonist (Stemcell Technologies, day 3-7), 100 ng/ml SHH (Peprotech, day 3-8), 50 ng/ml FGF8 (Peprotech, day 3-8) and 2 µM CHIR99021 (Stemcell Technologies, day 5-13). At day 12, the medium was switched to Neurobasal A™ (NBA) medium (supplemented with SM1-Vit.A 50x (Stemcell Technologies), N1 250x (Sigma), BDNF (20 ng/ml, Peprotech), Ascorbic Acid (200 µM, Sigma), GDNF (20 ng/ml, Peprotech), dibutyryl cAMP (0.5 mM, Sigma), DAPT (10 µM, Stemcell Technologies), TGF-β3 (1 ng/ml, Peprotech) (BAGCDT) and 0,1% Pen/Strep (Lonza)) to start
terminal neuronal induction. At day 20 of differentiation, cells were dissociated with Accutase (Sigma) and plated on Geltrex® coated tissue culture treated plates. To purify cultures, cultures were exposed to glucose deprivation and lactate (5mM) supplementation (GDLS) at 26DIV for 6 days (manuscript submitted). The resulting purified cultures were recovered in NBA for 6 days, dissociated with Accutase and passaged to either Geltrex® coated Nunc™ Lab-Tek™ II chamber slides™ or to Geltrex® coated borosilicate glass coverslips. Cells were maintained to 60 DIV, after which medium was changed to supplemented NBA without Pen/Strep until 90 DIV.

**Calcium imaging recordings**

Nunc™ Lab-Tek™ II chamber slides™ cultured neurons were incubated with 0.5 µM Fluo4-AM (Thermo Fisher, Cat No. F14217) and placed in an imaging chamber at 37°C with 5% CO2 and 95% humidity at 90 DIV. Image sequences were recorded using a Deltavision Elite live cell imaging system (GE Healthcare) equipped with PLAPON 60x oil, NA 1.42, WD 0.15 mm (Olympus) and a 15-bit EDGE/sCMOS camera (PCO) with GFP live filter wheel settings. Image sequences were recorded for 2 min intervals at 0.5 s per frame at 3.2% illumination intensity. Time series analysis was done using ImageJ (1.49s), plotting y-axis profiles for regions of interest (ROIs). Neuronal calcium events were defined as a sharp transient increase in fluorescence intensity (Fluo-4 AM, dF/F >5%, fast rise, slower decay). Images are presented using Green Fire Blue lookup tables.

**Immunocytochemistry**

Borosilicate glass coverslips containing neuronal cells were fixed in paraformaldehyde 4% for 15 min at room temperature and stored at 4°C. Permeabilization and blocking were done in PBS containing 0.1% Triton, 1% BSA and 5% normal goat serum for ~60 min at RT. Primary antibody incubation was done overnight at 6°C, followed by three PBS
washes (5 min each), after with a fluorescent conjugated secondary antibody and Hoechst 33258 (Sigma, 14530) were added. In the case of using MitoTracker® Orange, live cells were incubated prior to fixation, since the dye fluorescence is retained after fixation. Mowiol® 4-88 (Sigma) was used as mounting medium to attach coverglasses to a coverslip. Samples were stored at 4°C until further analysis.

Confocal images were acquired using a Leica SP8 confocal microscope, equipped with an HC PLAPO CS2 63x oil lens, with NA 1.4. Other epifluorescence images were acquired using a Leica AF-6000 fluorescent microscope, using a PL FLUOTAR 20x/NA 0.4, Dry lens.

**Mitochondrial trafficking using MitoTracker® Orange**

At 90 DIV, cells were incubated with 50nM MitoTracker® Orange CMTMRos (Invitrogen) for 30 min, after which medium was changed to fresh NBA supplemented medium. Live imaging was performed using a Deltavision Elite live cell imaging system (GE Healthcare) equipped with PLAPON 60x oil, NA 1.42, WD 0.15 mm (Olympus) and a 15-bit EDGE/sCMOS camera (PCO) with mCherry live filter wheel settings, using 1 % illumination intensity. During live imaging, cells were maintained in a humidified imaging chamber at 37°C with 5%CO₂. Data sets were recorded at 2.5 s intervals, with a total of 301 frames per recorded image sequence at 2024x2024 pixels.

**Analysis using ImageJ plugin Difference tracker**

Supplemental figureS3 A-C illustrates a simplified example of the method we have used for analysing mitochondrial trafficking images. The ImageJ plugin DifferenceTracker can compare time series images to their previous frames to determine if pixels have shifted. Using a frame offset value determines if pixels have moved over a number of frames.

Comparing figure S3C to figure S3B, the plugin is unable to detect changes in the red boxed pixel, since it still records a positive pixel value in this frame. However, when looking at figure S3A a difference is
observed, and the pixel can be classified as moving. In our approach, we have used a frame offset of four. Apart from frame offset, we used a difference in pixel intensity of at least 20 to be observed in an 8-bit image (256 grey values) to classify the pixel as moving. This is correlated to total number of pixels present, as this corrects between cell densities between groups. This leads to the reported % of pixels moving (i.e. % moving mitochondria), and % of moving intensities (i.e. %moving mitochondria corrected for fluorescent signal). Overlay images were generated using ImageJ software, with pseudocolor red for DifferenceTracker generated images.

**Statistical analysis**

All statistical tests were performed using Sigmaplot 13.0. Statistical significance was determined using one-way ANOVA followed by Tukey or Bonferroni post hoc test. A p-value < 0.05 was considered significant. Data are presented as the minimum to maximum whisker box plots or dot plots.

**Results**

**PD patient iPSCs DA differentiation**

iPSCs were generated from three fibroblast samples (one age-matched control and two PD patients) and characterized using standardized procedures. Supplemental figure S1 shows the characterization of iPSCs during different stages of generation.

We successfully differentiated all iPS lines into neurons with tyrosine hydroxylase (TH) expression in 10-20% of cells at 60 days in vitro (DIV) (figure1A). TH-positive differentiated cells show robust expression of α-synuclein compared to non-neuronal cells, which can be recognized by a larger nucleus (figure 1B), but no differences were observed between lines. To remove non-neuronal cells from culture, we applied a metabolic selection procedure developed by our lab (figure 1C). We found similar efficiencies in dopaminergic differentiation in all the different cell lines after
metabolic selection (figure 1D-F). Calcium imaging revealed the generation of spontaneous action potentials in PD1 derived neuronal cells at 90 DIV indicating that these neurons were viable and active at the time of mitochondrial trafficking analysis (see supplemental figure S2 and movie S2).

Figure 1. Patient hiPSCs can be differentiated into DA neurons (A)
Differentiation of hiPSCs to neuronal cells at 60 DIV. Immunofluorescence imaging for TUJ1 (green), TH (red) and Hoechst (blue). (B) Neuronal cells display expression of α-synuclein (green), TH (red) and Hoechst (blue). (C) DA cultures can be purified to enrich for neuronal cells, staining for TUJ1 (green), TH (red) and Hoechst (blue) at 60 DIV. (D) Phase contrast image overlay with MitoTracker® Orange labeled neurons used in mitochondrial trafficking experiments at 90 DIV. (E-G) Comparison of purified cultures of Ctrl (E), PD4 (F) and PD1 (G) at 60DIV. Cells are labeled for TH (red) and Hoechst (blue). All bars represent 50 µm.
MitoTracker® Orange can be used as a specific marker for mitochondria in iPSC derived DA neurons

To validate the specificity of MitoTracker® Orange as a selective live dye for mitochondria in iPSC-derived dopaminergic neurons, a triple staining was performed using TOM20, an outer membrane protein of mitochondria, TH and Hoechst33342. Fluorescence co-localization analysis was done using the ImageJ plugin Coloc2. The resulting Pearson’s R² value of 0.89 points to a strong co-localization of TOM20 with MitoTracker® Orange in whole image analysis, indicating that most mitochondria are stained with the live dye in dopaminergic neurons (figure 2A). Zooming in on axonal structures (figure 2B) reveals a higher correlation with a Pearson’s R² value of 0.91, further validating proper use of this live dye. On average, bleaching during 5 minutes of live imaging when using this dye was to less than 10% and was not significantly different between groups (see supplemental figure S3H).
Figure 2. MitoTracker® Orange co-localizes with TOM20 (A) Staining of MitoTracker® Orange labeled cells at 60DIV labeled with TH (magenta), MitoTracker® Orange (red), TOM20 (green) and Hoechst (blue) (bars 50 µm). Pearson’s correlation $R^2$ for TOM20 and MitoTracker® Orange in the whole image has a value of 0.89. (B) An axonal excerpt of figure A, for MitoTracker® Orange (red) and TOMM20 (green) has a Pearson’s correlation $R^2$ value of 0.91.

Analysis of mitochondrial trafficking in hiPSC-derived neurons

To evaluate the process of mitochondrial trafficking, fission and fusion we used an automated approach to identify trafficking of these organelles by employing the ImageJ plugin DifferenceTracker (see Materials & Methods) [14]. A simplified explanation, based on the authors’ instructions is illustrated in supplemental figure S3. All trafficking events were recorded at 90 DIV, without use of neurotoxins, and under identical experimental conditions.

An analysis of an image of the control cell line at a window of 222.5 µm by 222.5 µm is depicted in figure 3B. In this image, moving pixels are pseudocolored in red, while stationary pixels are pseudocolored green. An excerpt of this image in figure 3A shows a more detailed image measuring 70 µm by 70 µm. A time series of 40 seconds of an even more detailed excerpt with dimensions 32.5 µm by 32.5 µm is presented in figure 3C. In this time series, we show a moving mitochondrion in red followed by the arrow over different frames, moving approximately 25 µm before moving out of frame. Calculation of the whole image with difference tracker on this excerpt revealed that 11.6 % of the pixels are moving over a total of 297 imaging frames. A movie is available as Movie S1.

The kymograph, corresponding to the line drawn in the last timeframe in figure 3C, provides an accurate representation of mitochondrial trafficking (figure 3D). However, we have chosen to show DifferenceTracker overlay images since these depict whole image trafficking events. Additional kymographs are presented as supplementary images (see figure S4).
Figure 3. Difference tracker can be used to identify trafficking mitochondria. (A) MitoTracker® Orange labeled culture analyzed using difference tracker. Red pseudo colors moving mitochondria, determined using the ImageJ plugin Difference Tracker, while green shows stationary mitochondria (bars 50 μm). (B) Shows an excerpt from (A) with enhanced detail, while (C) illustrates an even more enhanced excerpt with 16 consecutive frames to show movement of mitochondria. (D) Kymograph representation of the line drawn in (C), depicts mitochondrial trafficking over 16 frames.

Mitochondrial trafficking is impaired in PD neurons

 Trafficking analysis was assessed using large frame images (as in figure 3) and images were excluded if the image moved out of focus or if cells moved, leading to false positive trafficking events. We determined the percentage of moving pixels in whole image analysis (figure 4A), and the percentage of moving pixel intensity, correlating to the percentage of moving mitochondria (figure 4B-D, see also corresponding kymographs in supplemental figure S4). Both graphs show a significant difference in trafficking of mitochondria in neurons between Ctrl and PD1 patient lines, but not between PD lines. The corresponding p-value for Ctrl and PD4 is 0.024, while p-value for PD1 and Ctrl is 0.002. When correcting for pixel intensity, the p-value between Ctrl and PD4 is 0.017 and the p-value between PD1 and Ctrl is 0.005. Movies are available as Movie S2-4.
Figure 4. PD lines show mitochondrial trafficking impairment. (A) Movement of mitochondria referenced to pixels shows a significant difference between control and PD lines (Ctrl vs. PD4 p=0.049 and Ctrl vs. PD p=0.006), but not between PD lines. (B) Moving intensity percentages are significantly different between control and PD lines (Ctrl vs. PD4 p=0.023 and Ctrl vs. PD p=0.007), but not between PD lines. (C) Example of mitochondrial trafficking analysis of control. (D) Excerpt of (C) shows less trafficking in PD4 and PD1 compared to control. Red indicates moving mitochondria, while green indicates stationary mitochondria. (Scalebar 25μm). Corresponding kymographs are illustrated in supplemental figure S4.
Whole image analysis was validated by small frame analysis

While whole image analysis has the benefit of unbiased analysis, a downside of using automated trafficking software is the inability to distinguish between mitochondrial movement and (particularly sideways) movement of axons (see Supplemental figure S3D-E). It should be noted that these events take place in both patient and control cells, but we have chosen to further analyse data in smaller excerpts that showed no sideways movement during frame acquisition. To improve the accuracy of analysis, we have chosen to analyse excerpts away from the cell body, thereby focussing on axonal mitochondrial trafficking dynamics. Analysis of 20 smaller frame images, where no sideways movement occurred, again resulted in a significant difference in trafficking between PD and control lines (see supplemental figure S5). Since axonal excerpts with mitochondria are analysed, trafficking speed was higher than those observed in whole image analysis. These results confirm that mitochondrial trafficking in DA neurons of PD iPSC lines is impaired in comparison to mitochondrial trafficking in DA neurons of control iPSC lines. The corresponding p-value for Ctrl and PD4 is 0.001 and the p-value for PD1 and Ctrl is 0.001 in both percentage of pixels and percentage of pixel intensities moving.

PD lines show mitochondrial fragmentation

In neurons, healthy mitochondria are typically elongated and undergo fusion and fission to maintain proper mitochondrial viability and functionality [15]. In our experiments, patient-derived PD DA neurons showed increased fragmentation of mitochondria (figure 5B-C), while control DA neurons (figure 5A) show healthy, elongated mitochondria. The increased presence of fragmented mitochondria hints to lowered mitochondrial integrity.
Figure 5. Mitochondrial fragmentation is observed in PD4 and PD1 line, but not in control. (A) In all imaging conditions, control line mitochondria showed an elongated phenotype, while in the PD4 (B) and PD1 (C) line mitochondrial fragmentation and round mitochondria were observed (bars 10μm).

Discussion and Conclusion

In our experiments, we have demonstrated that mitochondrial trafficking is impaired in familial PD neurons generated from iPSCs compared to healthy age-matched control cells. Our data extends to previous data published by our group [16] and supports the hypothesis that intracellular trafficking dynamics impairment precedes the onset of PD [12]. Being able to culture pure neuronal cultures for extended periods of time has given us the opportunity to study mitochondrial trafficking without the use of environmental toxins, when compared to other PD studies [9, 17-19]. Use of environmental toxins has led to a link between α-synuclein and mitochondrial pathology in previous studies, but these approaches mainly highlight the vulnerability of patient DA neurons for these toxins. In contrast, our data gives insight to early pathology and mitochondrial dysfunction as opposed to a rapid onset of the disease phenotype. While mutations in parkin (PARK2) and PTEN-induced kinase 1 (PINK1) have provided a strong link between mitochondrial dysfunction and PD in iPSC studies [20], a link to early mitochondrial dysfunction in SNCA-mutated iPSCs has remained elusive.
Acknowledgements

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References


Supplemental figure S1. Characterization of iPSC lines. (A) iPSC cultures stained with Live Alkaline Phosphatase staining (Thermo Scientific). (B-D) iPSC immunocytochemistry for pluripotency markers. (B) Staining for OCT4 (green),
SOX2 (red) and Hoechst (blue). (C) Staining for NANOG (green), SSEA4 (red) and Hoechst (blue) (D) Staining for TRA1-81 (red) and Hoechst (blue) (E) Embryoid body formation in all lines. (F-H) Embryoid body immunocytochemistry for DESMIN (red) and Hoechst (blue) (F), TUJ1 (green) and Hoechst (blue) (G) and GATA4 (red) and Hoechst (blue) (H).

**Supplemental figure S2. Calcium imaging of PD1 line at 90 DIV shows spontaneous neuronal activity patterns.** (A) Shows PD1 derived neuronal cells labeled with Fluo-4 AM over a period of 120 seconds. Spontaneous depolarization events are characterized by increasing presence of the green color. (B) Depicts the change in pixel values corresponding to ROI1, ROI2 and ROI3, showing similar activation patterns of these different locations. Data is represented as $\Delta F/F_0$. 
Supplemental figure S3. Simplified example of difference tracker software and experimental validations. From (C) to (B), the plugin is unable to observe changes in the red boxed pixel, since it still records a positive pixel value in this frame. However, when looking at (A) a difference is observed, and the pixel can be classified as moving. It should be noted that in the actual 8-bit image, as opposed to this binary example, the pixel can have 256 intensity values, allowing for offset values to classify pixels as moving. (D) An axonal shift results in a positive trafficking event (E), while in reality mitochondria are stationary. The axonal positions in (F) shift down (G) again resulting in false positive events. Since the software is not able to recognize these events, screening of smaller excerpt has to be performed to prevent a false positive bias of results. (E) Fluorescence intensity loss shows bleaching effects observed during imaging. While minor differences between groups were observed, the difference in bleaching was not significant, and in amounted on average 9.8%.

Supplemental figure S4. Kymographs of trafficking experiments. (A) Grey scale images containing MitoTracker® Orange labelled neuronal cells from control and PD patient lines. (B) Kymographs corresponding to figure 4, consisting of 117 frames, containing an average of a 5 pixel line indicated in the images depicted in (A).
Supplemental figure S5. Trafficking impairment is observed when analyzing more detailed images. (A) Movement of mitochondria referenced to pixel intensities shows a significant difference between control and PD lines, but not
between PD lines. (B) Absolute moving percentages are significantly different between control and PD lines, but not between PD lines. (C) Example of mitochondrial trafficking analysis of control. (D) Excerpt of (C) shows less trafficking in PD4 and PD1 compared to control. Red indicates moving mitochondria, while green indicates stationary mitochondria. (Scalebar 10μm).