Oxidative switch drives mitophagy defects in dopaminergic parkin mutant patient neurons

Aurelie Schwartzentruber\(^1\), Camilla Boschian\(^1\), Fernanda Martins Lopes\(^1\), Monika A. Mysczynska\(^1\), Elizabeth J. New\(^2\), Julien Beyrath\(^3\), Jan Smeitink\(^1\), Laura Ferraiuolo\(^1\)\& Heather Mortiboys\(^1\)\*

Mutations in \textit{PRKN} are the most common cause of early onset Parkinson’s disease. Parkin is an E3 ubiquitin ligase, functioning in mitophagy. Mitochondrial abnormalities are present in \textit{PRKN} mutant models. Patient derived neurons are a promising model in which to study pathogenic mechanisms and therapeutic targets. Here we generate induced neuronal progenitor cells from \textit{PRKN} mutant patient fibroblasts with a high dopaminergic neuron yield. We reveal changing mitochondrial phenotypes as neurons undergo a metabolic switch during differentiation. Fibroblasts from 4 controls and 4 \textit{PRKN} mutant patients were transformed into induced neuronal progenitor cells and subsequently differentiated into dopaminergic neurons. Mitochondrial morphology, function and mitophagy were evaluated using live cell fluorescent imaging, cellular ATP and reactive oxygen species production quantification. Direct conversion of control and \textit{PRKN} mutant patient fibroblasts results in induced neuronal progenitor and their differentiation yields high percentage of dopaminergic neurons. We were able to observe changing mitochondrial phenotypes as neurons undergo a metabolic switch during differentiation. Our results show that when pre-neurons are glycolytic early in differentiation mitophagy is unimpaired by \textit{PRKN} deficiency. However as neurons become oxidative phosphorylation dependent, mitophagy is severely impaired in the \textit{PRKN} mutant patient neurons. These changes correlate with changes in mitochondrial function and morphology; resulting in lower neuron yield and altered neuronal morphology. Induced neuronal progenitor cell conversion can produce a high yield of dopaminergic neurons. The mitochondrial phenotype, including mitophagy status, is highly dependent on the metabolic status of the cell. Only when neurons are oxidative phosphorylation reliant the extent of mitochondrial abnormalities are identified. These data provide insight into cell specific effects of \textit{PRKN} mutations, in particular in relation to mitophagy dependent disease phenotypes and provide avenues for alternative therapeutic approaches.

Parkinson’s disease (PD) is the second most common neurodegenerative disease, with approximately 10 million people affected worldwide. Only symptomatic treatment options are available. Mutations in \textit{PRKN} are the most common cause of early onset PD (EOPD). Parkin is an E3 ubiquitin ligase and functions in the mitophagy pathway\(^1\). Mitochondrial dysfunction is well established in both familial and sporadic forms of PD (recently reviewed\(^2\)). Mitochondrial abnormalities are present in both \textit{PRKN} null Drosophila\(^3\) and mice\(^4\). We and others have shown mitochondrial abnormalities in peripheral cells from patients with \textit{PRKN} mutations\(^5-8\); these include cellular ATP defects, mitochondrial membrane potential deficiencies, complex I defect and altered mitochondrial morphology. Recent work suggests mitophagy is defective across many PD types\(^9\). Several reports have found alterations in the same mitochondrial parameters in iPSC derived \textit{PRKN} deficient neurons\(^10-17\). These studies provide insight into a mitochondrial phenotype in PRKN deficient neurons; several have identified mitochondrial functional and morphological abnormalities as well as increased cell death occurring in \textit{PRKN} mutant neurons. Dopaminergic (DA) neurons are particularly vulnerable to mitochondrial abnormalities due to their high basal oxidative load, tonic activity and highly complex arborisation of the dendritic network\(^18\). The studies so far have utilised the iPSC reprogramming route and subsequent differentiation into DA neurons, which can generate a

\(^1\)Sheffield Institute for Translational Neuroscience, University of Sheffield, Sheffield, UK.  \(^2\)School of Chemistry, University of Sydney, Sydney, Australia.  \(^3\)Khondrion BV, Philips van Leydenlaan 15, 6525 EX Nijmegen, The Netherlands. \(^*\)email: H.Mortiboys@sheffield.ac.uk
relatively poor yield of DA neurons as reflected by % TH positive cells reported in these studies.\textsuperscript{13,14,19} Therefore, there remain some unanswered questions about the specific role and importance of mitochondrial abnormalities in a \textit{PRKN} deficient background in DA neurons. In particular, a study has suggested the differentiation method from iPSCs is important to be able to identify mitochondrial dysfunction in DA neurons.\textsuperscript{10}

The role of parkin in the mitophagy pathway is extremely well documented mainly in tumour cell lines over expressing parkin with mitophagy induction due to treatment with uncoupling agents such as CCCP. Limited studies have investigated the role of parkin dependent mitophagy in cells expressing endogenous parkin and fewer still without the induction of mitophagy via uncoupling. Recent findings from several in vivo models have called into question the relative importance of parkin dependent mitophagy in adult DA neurons with studies in \textit{PINK1} deficient mice and \textit{PRKN} and \textit{PINK1} deficient Drosophila showing no difference in mitophagy rates in DA neurons.\textsuperscript{20,21} However an age dependent increase in mitophagy in DA neurons which is absent in \textit{PRKN} and \textit{PINK1} deficient Drosophila has also been identified.\textsuperscript{22} Therefore more studies are needed to elucidate the importance of mitophagy in \textit{PRKN} deficient EOPD.

Our study uses a direct conversion route from patient fibroblasts to induced neuronal progenitor cells (iNPC’s) and subsequently to DA neurons with a high yield. Direct reprogramming methods result in cells that both retain the genetic background and the age phenotype of the donor fibroblasts.\textsuperscript{23} With a high yield of DA neurons we are able to study the mitochondrial and mitophagy phenotypes throughout differentiation in this specific cell population. This study aims to provide evidence for mitochondrial abnormalities in \textit{PRKN} mutant patient derived DA neurons, using an alternative method to previous studies, in addition to investigate if the reprogramming and differentiation methodology generates different mitochondrial phenotypes. We show that mitophagy defects are dependent on the metabolic status of the cell, with high mitophagy rates in \textit{PRKN} deficient neurons early in differentiation when the cells are mainly glycolytic and mitophagy rates which are extremely low in \textit{PRKN} deficient neurons reliant upon oxidative phosphorylation. We also show treatment with known potent intracellular redox-modulating agents improves the neuronal phenotype of the neurons without restoring mitochondrial function or morphology.

**Results**

**iNPC derived DA neurons display high yield and purity.** Unlike iPSC reprogramming of fibroblasts, the direct conversion method first developed by Meyer et al. produces iNPC’s which are tripotent and able to differentiate into neurons, astrocytes and oligodendrocytes.\textsuperscript{24} The iNPC’s produced using this methodology can be differentiated into specific neuronal populations with a high purity yield, as demonstrated for motor neurons.\textsuperscript{25} We successfully converted fibroblasts from 4 control and 4 \textit{PRKN} mutant patients producing iNPC’s which displayed a clear change in cell morphology, proliferation rate and stained positive for the NPC markers, Pax6 and nestin as found previously for all iNPC’s reprogrammed using this methodology\textsuperscript{24–27} (data not shown).

In order to be able to investigate biochemical parameters in DA neurons without other neuronal types or non-neuronal cells contaminating the culture; a high yield of DA neurons is required. We optimised the DA differentiation protocol based upon Swistowski and co-workers\textsuperscript{28}. Differentiation is in three stages, first iNPC’s are treated with DAPT, a Notch inhibitor that enhances neuronal differentiation; in stage 2 the cells are driven towards a rostral midbrain neuronal lineage and finally in stage 3 DA neuron differentiation is complete. As differentiation proceeds the cellular morphology alters; at day 15 of differentiation cells have become elongated and beginning to form connections as compared to iNPC morphology and by day 27 the cells have formed longer, larger connections (Fig. 1A shows brightfield images of cells at two stages of differentiation). The morphology is distinct from the parental iNPC morphology. However, we note, the processes are shortened and thicker than those usually seen in neurons differentiated from iPSCs or primary embryonic cultures. Therefore, we sought to characterise the expression of several pan-neuronal markers and DA specific markers throughout differentiation. We assessed expression of pan neuronal markers βIII tubulin, MAP2 and NeuN at various stages of differentiation (day 12, day 17 and day 27). The amount of cells staining positive for each of these markers increases throughout differentiation, resulting at day 27 in 94.5% of cells staining for βIII tubulin, 87.9% for MAP2 and 76.5% for NeuN (quantification throughout differentiation is shown in Fig. 1B–D with representative images at day 27 shown for each marker in a control and a \textit{PRKN} mutant line). Next, we investigated the expression of two DA markers, tyrosine hydroxylase (TH) and the dopamine transporter (DAT). Again, expression increases throughout differentiation; with undetectable levels of TH and DAT expression at day 17 of differentiation. At day 27 of differentiation however 89.9% of cells stain positive for TH and 82.4% for DAT (Fig. 1E,F quantification throughout differentiation and representative images at day 27 of differentiation). In addition, we quantified mRNA transcript level for TH at day 27 as compared to iNPC’s and found a 4.2 fold increase in DA neurons compared to iNPC’s (Fig. 1G). Neurosensor 521 dye labels both noradrenaline and dopamine in live cells, and we found that at end stage of differentiation 87.8% of cells stained positive with Neurosensor 521 (Fig. 1H). We next sought to measure the dopamine content and stimulate release of dopamine from our neuronal cultures. We found that the neurons had measurable intracellular dopamine and the neurons could be stimulated to release dopamine using potassium chloride in a magnesium containing buffer. \textit{PRKN} mutant neurons contain less intracellular dopamine than controls (mean ± SD, controls 111.8 ± 2.6, parkin mutants 88.4 ± 3.4 nmol/l dopamine; p < 0.001; Fig. 1D). Upon stimulation with potassium chloride the amount of dopamine in the media increases in controls by 51% and only by 15% in \textit{PRKN} mutant patients (Fig. 1I). The neurons do not release dopamine when stimulated with potassium chloride without magnesium present. Finally, we also assessed the cellular membrane potential to assess neuronal properties of our cells in culture. We find positive staining for membrane potential and fluctuations as expected in active neuronal cultures (Fig. 1J); however, the \textit{PRKN} mutant neurons are less responsive to stimuli.
**Figure 1.** Control and PRKN mutant iNPC derived dopaminergic iNeuron characterization throughout differentiation. (A) Bright field images (scale bar = 100 μm) at day 15 and day 27. The insets show magnified regions highlighting the processes of the neurons. (B–F) (i) Quantification of each neuronal marker throughout differentiation in controls (black bars) and PRKN mutants (grey bars) and (ii) shows representative images of a control and PRKN mutant at day 27. (Bi) Green is βIII tubulin and blue nucleus. (Ci) Red is MAP2 and blue the nucleus. (Di) Red is NeuN and blue nucleus. (Eii) red is tyrosine hydroxylase and blue the nucleus. (Fii) green is DAT and blue the nucleus. (G) The quantification of mRNA expression levels for tyrosine hydroxylase in iNPC’s and day 27 neurons, showing a four fold increase in expression (black bars controls and grey bars PRKN mutants). (Hi) The quantification at day 27 of Neurosensor staining which stains dopamine and noradrenaline showing 90% of cells staining positive in both controls (black bars) and PRKN mutants (grey bars). (ii) shows representative images of control and PRKN mutant at day 27. (I) The dopamine content and release assay in control and PRKN patient neurons. Intracellular and extracellular dopamine content assessed in neurons from three rounds of differentiation for each condition. Neurons are either untreated, stimulated with potassium chloride or potassium chloride without magnesium in the buffer. Extracellular dopamine levels increase when neurons are stimulated with potassium chloride in both control (**p < 0.001) and PRKN mutant patient neurons (**p = 0.0032). J shows neuronal membrane potential in control (black line) and PRKN mutants (grey line) recorded at baseline and after stimulation at time 200 s. All quantification was done on at least three different differentiations of four control and four PRKN mutant neurons; two way ANOVA with Tukey multiple comparisons correction was used.

**PRKN mutant DA neurons display increased cell death and morphological abnormalities.** Others have previously reported fewer surviving neurons at the end of differentiation towards a DA enriched population from iPSCs derived from PRKN mutant parental patient fibroblasts. We investigated this during the differentiation from iNPC’s to DA neurons. There was significant cell death occurring throughout differentiation specifically in the PRKN mutant patient derived DA neurons; the percentage of cells surviving until the end of the differentiation was significantly reduced (mean ± SD, controls 83.62 ± 4.8; parkin mutants 52.72 ± 11.98); however, the same % yield of surviving neurons expressed DA markers between controls and PRKN mutants. We quantified cell death using activated caspase 3 staining. The number of activated caspase 3 positive spots was higher in PRKN mutant neurons compared to controls at day 17, with a subsequent dramatic increase during the final stage of differentiation (% cells with activated caspase 3 staining at day 17 15% and at day 27 64%); whereas in control neurons the level remains constant at approximately 7.8% (Fig. 2A). Furthermore, DA
neurons from PRKN mutants displayed altered neuronal morphology at end stage differentiation; being more round and less elongated (Fig. 2B,C; controls 1.92 ± 0.02; PRKN mutants 2.74 ± 0.2; *p < 0.05 for cell roundness and controls 0.013 ± 0.0002; PRKN mutants 0.008 ± 0.001; p < 0.05 for cell elongation).

Metabolism shifts throughout differentiation; revealing mitochondrial morphology and functional abnormalities in PRKN mutant DA neurons. Recent work has shown direct reprogramming methods retain the age characteristics of the donor fibroblasts; importantly this also includes the switch to oxidative phosphorylation during direct reprogramming and reductions in mitochondrial function and gene expression in the neurons generated from aged donors rather than those generated from younger donors23. We therefore sought to understand the metabolism in our system, which utilises direct conversion of fibroblasts to iPSC’s rather than reprogramming to iPSC’s. In order to measure the percentage of ATP produced via glycolysis or oxidative phosphorylation in the cells throughout differentiation; we measured the ATP content of the cells...
after treatment with various inhibitors. We treated with either oligomycin, to inhibit oxidative phosphorylation (specifically complex V), 2-Deoxyglucose to inhibit glycolysis (specifically hexokinase), both inhibitors together or vehicle only. This allowed us to understand the contribution of each metabolic pathway to generating ATP in the cells as iNPC’s and throughout differentiation. The iNPC’s are reliant on glycolysis, as we can see from inhibition with 2-Deoxyglucose (glycolysis) inhibiting 70.4% of ATP produced; whereas oligomycin (oxidative phosphorylation) inhibits 3.4% of ATP levels (Fig. 2D; untreated 3.1 ± 0.6; OXPHOS inhibited 2.9 ± 0.7; glycolysis inhibited 0.9 ± 0.3 and Fig. 2E). The data shown in Fig. 2E are % calculations based on the ATP values in Fig. 2D. We have used the untreated value as 100%, and calculated how much ATP is still measurable after inhibition of either OXPHOS (with oligomycin) or glycolysis (with 2-Deoxy-glucose) or both inhibitors. However during neuronal differentiation the cells undergo a metabolic switch from glycolysis to oxidative phosphorylation; such that by the end stage of differentiation neurons are reliant on oxidative phosphorylation for 77% of their energy generation (Fig. 2D, untreated 15.1 ± 1.4; OXPHOS inhibited 3.5 ± 1.2; glycolysis inhibited 8.5 ± 2.5 and Fig. 2E). This switch occurs in both control and PRKN mutant DA neurons (PRKN mutant data shown in Fig. 2F, calculated in the same way as described above for control cells). At day 27 of differentiation PRKN mutant neurons are reliant on OXPHOS for 80.8% of their ATP generation (Fig. 2G). It is interesting to observe the time point at which the dramatic increase in activated caspase 3 and cell death occurs in the PRKN mutant neurons (day 22) correlates with the metabolic switch towards OXPHOS reliance.

In order to fully understand mitochondrial function and morphology as this metabolic switch occurs and the role of parkin in this; we investigated mitochondrial function, morphology and mitophagy throughout differentiation in control and PRKN mutant neurons. Previous reports have shown mitochondrial fragmentation in PRKN mutant iPSC derived neurons29,30. We observe the same mitochondrial fragmentation at the end stage of differentiation accompanied by an increase in mitochondrial number (Fig. 3A mitochondrial interconnectivity: controls 0.07 ± 0.003; PRKN mutants 0.04 ± 0.005 p < 0.05; Fig. 3B mitochondrial number (% normalised to controls): controls 100 ± 3.4; PRKN mutants 204 ± 35; p < 0.0001). In both control and PRKN mutant DA neurons throughout differentiation mitochondria become more interconnected as the metabolic switch occurs. The
**A**

**Ai** Basal mitophagy (12 days of differentiation)

**Bi** Basal mitophagy (17 days of differentiation)

**Ci** Basal mitophagy (27 days of differentiation)

**ii** Induced mitophagy (12 days of differentiation)

**Bii** Induced mitophagy (17 days of differentiation)

**Biii** Induced mitophagy (27 days of differentiation)

**D**

Fixed mitophagy

**E** TOMM20 amount (27 days of differentiation)
controls then return to a 'normal' morphology once this has happened (Fig. 3A). The increase in mitochondrial number in PRKN mutant DA neurons does not seem to be driven by increased biogenesis but rather the total mitochondrial content remains fairly constant however mitochondria are smaller and more fragmented in the PRKN mutant DA neurons. There is much debate in the literature as to whether the energy defect or increased ROS production is more detrimental in PD. We found dramatically increased mitochondrial ROS levels at end stage of differentiation (controls 0.17 ± 0.018; PRKN mutants 0.6 ± 0.15; *p < 0.05; Fig. 3C). There is no change in mitochondrial ROS levels at earlier stages of differentiation. In terms of mitochondrial function, we show that mitochondrial membrane potential is significantly reduced only at end stage differentiation (MMP controls 0.02 ± 0.004, PRKN mutant 0.003 ± 0.002, ***p < 0.001; Fig. 3D). However, there is a worsening trend in MMP decreases as differentiation continues. A similar pattern is observed for cellular ATP levels with increasing deficits as differentiation progresses. The first significant decrease in ATP levels is observed when the neurons are becoming reliant on oxidative phosphorylation at day 17 of differentiation (controls 100 ± 5.5, parkin mutants 82 ± 4.3, *p < 0.05; Fig. 3E). At the end stage of differentiation the deficit is more severe (% normalised to controls: controls 100 ± 15, PRKN mutant 61 ± 15; ****p < 0.01; Fig. 3E). Taking the above data together we see dramatic changes in mitochondrial function and morphology, which are only revealed in PRKN mutant neurons as the metabolic switch occurs from glycolysis to oxidative phosphorylation; accompanied by a dramatic increase in mitochondrial ROS levels once this switch has taken place.

Mitophagy rates are higher in PRKN mutant neurons with glycolytic capacity before becoming defective when neurons undergo a metabolic switch. As parkin is known to function in a well characterised parkin dependent mitophagy pathway targeting dysfunctional or damaged mitochondria for degradation; we developed a live imaging assay to assess both basal and induced mitophagy rates in the neurons throughout differentiation. This assay relies upon live staining of the total mitochondrial population and the lysosomal population combined with advanced high content imaging acquisition, data processing and analysis. At 12 days differentiation, when cells are positive for the pan neuronal marker βIII tubulin but are not yet DA and are glycolytic, PRKN mutant neurons have very similar rates of basal mitophagy as controls (PRKN mutants 0.07 ± 0.03, PRKN mutants 0.09 ± 0.05, Fig. 4Bi) and again mount a higher response to mitophagy induction with mitochondrial inhibitor treatment but cannot sustain that level of mitophagy over time (Fig. 4Bii). Finally, at the end stage of differentiation when neurons are reliant on oxidative phosphorylation PRKN mutant neurons have a severe deficit in basal and induced mitophagy with mitochondrial inhibitors (Fig. 4C basal mitophagy controls 0.09 ± 0.025, PRKN mutants 0.025 ± 0.008; induced mitophagy controls 0.3 ± 0.09, PRKN mutants 0.1 ± 0.01, p <0.01). Representative images of the live mitophagy assay are shown in Supplementary Fig. 1. We used two alternative methods of evaluating mitophagy rates previously validated31,32; using these methods we found the quantification of mitochondrial mitophagy over time; basal mitophagy is unaltered in a PRKN mutants (grey lines) compared to controls (black lines) at day 12 of differentiation (Ai); however by day 17 basal mitophagy is increased in PRKN mutants (*p = 0.019, tested using ANOVA repeated measures across all time points; Bi) and at endpoint of differentiation day 27, PRKN mutant neurons have significantly reduced basal mitophagy levels (p = 0.011, tested using ANOVA repeated measures across all time points; Ci). Induced mitophagy (induced with mitochondrial inhibitor treatment) is increased in PRKN mutants initially after induction at day 12 (*p < 0.05, Sidaks post-test; Ai); the same pattern is seen at day 17, although without statistical significance (Bii) however by day 27 induced mitophagy is significantly lower in PRKN mutant neurons (*p = 0.0113, tested using ANOVA repeated measures across all time points; Ci). (D) Quantification of mitophagy using an alternative measure; showing the same pattern as the live assay. No difference at day 12, an increase at day 17 in the PRKN mutants (grey bars) compared to controls (black bars, **p = 0.0016) and a reduction at day 27 (**p =0.0035). All experiments were repeated on three separate rounds of differentiation in each control and PRKN mutant patient line (four different controls and PRKN mutant patient lines are included). Bar graphs represent mean with SD. (E) Quantification of the amount of Tomm20. PRKN mutant neurons have increased Tomm20 amounts at day 27 of differentiation as compared to control A (PRKN A *p = 0.04, PRKN B *p = 0.02). n = 3 for each line.
Redox modulating compounds KH176 and KH176m partially reverses neuronal deficits in PRKN mutant DA neurons. In order to evaluate if the driving mechanism in PRKN mutant DA neurons is the loss of energy production by the mitochondria or the dramatically increased mitochondrial ROS production, we treated the end stage day 27 neurons with the known potent intracellular redox-modulating agents KH176 and KH176m currently in clinical trials in mitochondrial patients with m.3243A>G spectrum disorders. KH176 and its quinone metabolite KH176m were identified from a screen in fibroblasts from patients with primary mitochondrial diseases. The mechanism of action is known to be interaction with the thioredoxin system and specifically the peroxiredoxin machinery, and are able to rescue both in vitro and in vivo models of primary complex I deficiency. In addition, we have previously showed that KH176 is actively metabolised to KH176m. In agreement with the mechanism of action treatment with KH176 and KH176m decreased mitochondrial ROS production to control levels after 24 h treatment (Fig. 5A, control A vehicle treated 0.16 ± 0.006; control A KH176 treated 1 µM 0.14 ± 0.02; PRKN mutant A vehicle treated 0.7 ± 0.15; PRKN mutant A KH176 treated 1 µM 0.12 ± 0.05; p < 0.001). KH176 and KH176m treatment had no significant effects on mitochondrial function (MMP or cellular ATP levels) or mitochondrial morphology parameters (Fig. 5B–D). Treatment with KH176 and KH176m did show a mild effect on neuronal morphology after only 24 h of treatment; with the resulting neurons being less round after treatment; a morphology closer to control neurons (Fig. 5E, cell roundness: control A vehicle treated 1.5 ± 0.09; control A KH176 treated 1 µM 1.6 ± 0.06; PRKN mutant A vehicle treated 3.1 ± 0.6; PRKN mutant A KH176 treated 1 µM 2.2 ± 0.6). KH176 or KH176m have not been assessed...
before for an effect on mitophagy rates and it is unclear the effect, if any, of a redox restoring metabolite would have on the mitophagy pathway. We investigated if KH176m treatment could affect basal mitophagy rates in neurons from PRKN mutant A. We chose KH176m for these experiments as it has shown the most effect in the other assays outside of mitochondrial ROS; therefore we wanted to investigate if there was any additional mitochondrial and cellular benefit with KH176m treatment which was modulated via mitophagy; in addition as mentioned above KH176 is metabolised to KH176m in cells. Our data show a significant increase in basal mitophagy rates after treatment with KH176m in both control and PRKN neurons (Fig. 5E; control A vehicle treated 1.14 ± 0.11, control A KH176m 100 nM 1.27 ± 0.11, control A KH176m 1000 nM 1.56 ± 0.15 PRKN mutant A vehicle treated 0.6 ± 0.05, PRKN mutant A KH176m 100 nM 0.9 ± 0.05, PRKN mutant A KH176m 1000 nM 0.8 ± 0.08).

Discussion

Our study is the first to report successful reprogramming via the iNPC route of PD PRKN mutant patient fibroblasts; varying reprogramming methods depend on competent energy generation for successful reprogramming[23]. We have previously reported severe mitochondrial abnormalities in PRKN mutant fibroblasts[5]; reprogramming of fibroblasts with a reduction in metabolic function can be challenging using iPSC routes[24] however here we show metabolically challenged fibroblasts can be reprogrammed using this direct reprogramming route. Recently other researchers have used alternative direct reprogramming methods to generate dopamine like neuronal cells from sporadic and LRRK2 Parkinson’s patient cells[25–27]. These studies showed several alternative reprogramming routes can lead to viable dopaminergic neuronal like cells; with each group assessing the dopaminergic qualities of the cells produced. Furthermore we have recently reported use of this reprogramming route to generate dopaminergic neurons from sporadic PD fibroblasts; in that study we found the mitochondrial abnormalities exasperated in the neurons compared to the fibroblasts from the same patient[28]. The specific method we have used here, the iNPC derived route has proved a useful model to study familial and sporadic forms of neurodegenerative diseases thus far; astrocytes derived from Motor Neuron Disease (MND) patients display neuronal toxicity when in co-culture with WT neurons similar to that seen with primary astrocytes from post-mortem biopsies from MND patients. Furthermore both iAstrocytes and iNeurons were recently used to investigate mechanisms of cell toxicity in C9orf72 dependent Motor Neuron Disease[24–27,41,42]. We report the generation of high yield of DA neurons from iNPC’s. The DA yield we achieved via this method is higher than reported via the iPSC differentiation route and similar or higher than that found with alternative direct reprogramming routes. We also note, the processes of the neurons generated via iNPC reprogramming are shorter than those from iPSC derived neurons. We hypothesise this is due to the retention of age characteristics during direct reprogramming methods; whereas iPSC derived neurons are more embryonic in nature and similar to primary cultures generated from mice. However, this requires further investigation to fully understand the mechanisms involved. We find more cell death in the PRKN mutant DA neurons throughout differentiation; less efficient differentiation has been reported by others for PRKN mutant patient neurons via iPSC reprogramming route[23]. It is interesting that less efficient differentiation from PRKN mutant neurons has now been reported using both direct and indirect reprogramming methods; this would suggest this phenotype could be determined by genotype as opposed to reprogramming route. Our control and patient groups were aged matched, however ageing changes in the primary fibroblasts could also alter reprogramming and differentiation properties of cells. This would be interesting to study in a larger cohort over a wide age range. We also report iNPC derived PRKN mutant DA neurons are smaller and less elongated than controls. The increased cell death has been suggested to be dependent on the mitochondrial status of the cell rather than genotype[43]. Our data would support this however further work to systematically test this would be useful to assess correlation between genotype and metabolic status.

Our study investigating mitochondrial function and morphology throughout differentiation suggests the increased cell death seen in PRKN mutant DA neurons coincides with the neurons undergoing a metabolic switch from glycolysis to oxidative phosphorylation. We show the iNPC derived neurons have a clear switch in metabolism at day 22 with dependence on oxidative phosphorylation rather than glycolysis with a concurrent increase in the total amount of ATP in the neurons. This allows us to study mitochondrial function in these neurons which are metabolically more aligned to adult neurons in vivo (which are oxidative phosphorylation dependent) than embryonic neurons (glycolysis dependent)[44]. Our data show that, while this metabolic switch is occurring, mitochondrial morphology changes; as the neurons become more oxidative phosphorylation dependent the mitochondria become more interconnected in both controls and PRKN mutants; we suggest this a change in mitochondrial morphology to allow the neurons to become reliant on oxidative phosphorylation. Once the metabolic switch has occurred mitochondrial morphology can return to the normal shape; however PRKN mutant neurons once OXPHOS dependent have increased mitochondrial fragmentation. This is opposite to the mitochondrial morphology phenotype we have previously reported in PRKN mutant fibroblasts[5]; however others in the literature have previously reported a more fragmented mitochondrial network associated with PRKN deficiency[45]; this is likely to be a cell type specific effect; our data suggesting this is dependent on the metabolic status of the cells. As outlined in the introduction, several studies have found mitochondrial abnormalities in PRKN mutant patient derived neurons derived via iPSC reprogramming route[10,12]. Here we show some of the same abnormalities are present in PRKN mutant generated via a direct reprogramming route; in addition we have shown how this mitochondrial phenotype develops throughout differentiation and built upon previous studies in patient derived neurons to investigate multiple parameters related to neuronal health and mitochondria in the same population of neurons.

Previous studies utilising iPSC derived PRKN mutant neurons have found mitochondrial abnormalities including defective mitophagy when induced using CCCP[12]; however recent in vivo data from mouse and Drosophila models have shown little reduction in mitophagy on a PRKN or PINK1 deficient background[29]. Here we
show basal and induced mitophagy levels in PRKN mutant patient derived DA neurons; furthermore we find in a PRKN mutant background mitophagy levels are dependent on cellular energetic status. In cells which are dependent on glycolysis for energy production, basal and induced mitophagy are increased (or at least the same as controls) in PRKN mutant patient cells however upon the switch to OXPHOS dependency the PRKN mutant DA neurons have impaired basal mitophagy and are unable to mount a response to global mitochondrial dysfunction. Our data support the finding in PRKN deficient Drosophila that adult neurons increase levels of mitophagy during ageing however PRKN deficient neurons cannot\(^2\). The specific mitophagy pathway being utilised in these PRKN mutant neurons is not clear and requires further investigation.

Although mitochondrial abnormalities have been clearly identified by many in PD models; there is debate as to whether the detrimental component of this is actually loss of energy or increased ROS production. Here we show that mitochondrial ROS levels are significantly increased only at end stage of differentiation when the neurons are OXPHOS dependent and have severe mitochondrial abnormalities. The increase in mitochondrial ROS is striking in all four PRKN mutant patient neuron lines. Previous studies have shown an increase in ROS in some PRKN mutant patient neurons but not in others and have measured total cellular ROS rather than mitochondrial specific ROS which could explain why the data we present here is more consistent across the group of patients. Targeting mitochondrial dysfunction for a potential therapeutic to slow or stop disease progression is an attractive option with many mitochondrial targeted therapeutics shown to be effective in various models of PD (recently reviewed\(^2,46\)). Different therapeutic strategies are being developed; some primarily acting to boost energy deficits whilst others are targeting ROS production. Here we show that treatment with the known redox-modulating compounds KH176 and KH176m dramatically reduces the mitochondrial ROS production with no significant effect on MMP or cellular ATP levels; however, KH176 and KH176m do have a mild beneficial effect on the neuronal morphology of the PRKN mutant neurons. These effects could be modulated by an increase in basal mitophagy after treatment with KH176m. The increase in basal mitophagy is perhaps counter intuitive with a compound which reduces mitochondrial ROS production; however we hypothesise the mitophagy effects are due to a restoring of the redox balance in the cell, enabling some of the damaged mitochondria which have built up to be recycled. This mechanism requires further investigation in this and other models. Our data suggest a reversal of the energy deficit may not be required to have beneficial neuronal effects; however further work needs to be done to fully investigate this, particularly over a longer term treatment.

In conclusion, our study utilises the iNPC technology to generate a high DA population of neurons which both express markers of DA neurons and release dopamine upon induction. Our data shows a predominant mitochondrial dysfunction present in these neurons which is far more pronounced than that found in the primary patient fibroblasts\(^3\). Our study builds on previous work as for the first time neuronal properties, mitochondrial functional, morphological and mitophagy parameters are assessed in the same neurons; neurons which express TH and contain dopamine. Finally, our study highlights mitophagy as an energetic dependent process, which, in a PRKN mutant background varies considerably if the cells are glycolytic or OXPHOS dependent. This underlines the need to study mitophagy processes with endogenous levels of proteins in cell types which are relevant for disease and understand the energetic profile of the cells in order to be able to relate the findings to disease mechanism. Further studies to undertake detailed biochemical assessments of neuronal metabolism in this model in addition to utilising this model to assess putative neuroprotective compounds are warranted.

### Methods

#### Culture of primary fibroblasts, generation and culture of iNPC’s.

Primary fibroblasts were obtained from Coriell Cell Repository (coriell.org) controls: ND29510, GM09400, GM23967 and AG06882; PRKN mutant patient: ND30171, ND31618, ND40067 and ND40078 (details of mutation, age and sex are given in Table 1). Control and PRKN mutant groups were age and sex matched (controls 57 ± 6.8; parkin mutants 53 ± 8 years). Fibroblasts were cultured in EMEM as previously described\(^4\). iNPCs were generated as previously described\(^5\). iNPC’s were maintained in DMEM/Ham F12 (Invitrogen); N2, B27 supplements (Invitrogen) and FGFb (Peprotech) in fibronectin (Millipore) coated tissue culture dishes and routinely sub-cultured every 2–3 days using accutase (Sigma) to detach them.

| Reference | Age M/F | Mutation | Line ID |
|-----------|---------|----------|---------|
| ND30171   | 54 M    | ARG42PRO EX3DEL | Parkin A |
| ND40067   | 44 F    | EX4-7DEL c203_204 DEL AG | Parkin B |
| ND31618   | 63 F    | ARG42PRO | Parkin C |
| ND40078   | 51 F    | ARG275TRP/ARG275GLN | Parkin D |
| AG06882   | 64 M    | Control A |
| ND29510   | 55 F    | Control B |
| GM23967   | 52 M    | Control C |
| GM09400   | F       | Control D |

Table 1. Table giving details of the control and PRKN mutant patient lines used in this study. Further information can be found at [https://coriell.org](https://coriell.org) and [https://stemcells.ninds.genetics.org](https://stemcells.ninds.genetics.org).
Neuron differentiation of iNPC's. Neurons were differentiated from iNPC's as previously described [40]. Briefly, iNPCs are plated in a 6-well plate and cultured for 2 days in DMEM/F-12 medium with Glutamax supplemented with 1% NEAA, 2% B27 (Gibco) and 2.5 µM of DAPT (Tocris). On day 3, DAPT is removed and the medium is supplemented with 1 µM smoothened agonist (SAG; Millipore) and FGFR8 (75 ng/ml; Peprotech) for additional 10 days. Neurons are replated at this stage. Subsequently SAG and FGFR8 are withdrawn and replaced with BDNF (30 ng/ml; Peprotech), GDNF (30 ng/ml; Peprotech), TGF-β3 (2 mM; Peprotech) and dCAMP (2 mM, Sigma) for 15 days.

Immunofluorescence staining, live fluorescent imaging and ELISA. Neurons were plated and underwent immunocytochemistry staining as described previously [46]. Cells are plated into 96 well plates and fixed using 4% paraformaldehyde for 30 min. After PBS washes cells are permeabilised using 0.1% Triton X-100 for 10 min and blocked using 5% goat serum for 1 h. Cells are incubated with primary antibodies (Pax6 (Abcam); nestin (Abcam); GFAP (Abcam); tyrosine hydroxylase (Abcam); DAT (ThermoFisher); β III tubulin (Millipore); Tomm20 (BD Biosciences); LC3 (MBL); activated caspase 3 (Cell Signalling); Map2 (Abcam); NeuN (Abcam); nestin (Abcam); GFAP (Abcam); tyrosine hydroxylase (Abcam); DAT (ThermoFisher); β III tubulin (Millipore); Tomm20 (BD Biosciences); LC3 (MBL); activated caspase 3 (Cell Signalling); Map2 (Abcam); NeuN (Abcam)); at 4 degrees for 16 h. Cells are washed using PBS-Tween and incubated with Alexa Fluor conjugated secondary antibodies 488 and 568 (Invitrogen) and Hoescht (Sigma) 1 µM prior to imaging. Imaging was performed using the Opera Phenix high content imaging system (Perkin Elmer). Twenty fields of view were imaged per well; in seven z planes. Images were analysed using Harmony software; maximum projections were used for analysis.

Dopamine ELISA was performed as per the manufacturer’s instructions (Labor Diagnostika Nord GmbH&Co. KG). Dopamine release experiments, neurons were incubated in HBSS with Ca2+ and Mg2+ (Gibco by Life Technologies) for 30 min, or HBSS with Ca2+ and Mg2+ for 15 min and 56 mM KCl (Fisher chemical) for another 15 min or HBSS without Ca2+ and Mg2+ (Gibco by Life Technologies) with 2 mM EDTA for 15 min and then 56 mM KCl is added for another 15 min. Media is collected immediately; cells are harvested using accutase, 20 mM HEPES, 20 mM Glucose, pH 7.4).

Mitochondrial function, morphology and mitophagy measurements. Neurons were plated in 96 well plates; for MMP and morphology live imaging cells are incubated for 1 h at 37° with 50 nM tetramethylrhodamine (TMRM), 1 µM rhodamine 123 and 1 µM Hoescht (Sigma), after removal of dyes and replacement with phenol red free media plates are imaged using the Opera Phenix. Fifteen fields of view are imaged per well, in seven z planes. Images are analysed using Harmony software (Perkin Elmer). Segmentation protocols were established to segment the nuclei, mitochondria, and image region containing cytoplasm including projections. Analysis of number, size, intensity and morphology of mitochondria was calculated per image region using Harmony software (Perkin Elmer) using similar methodology as previously established [31]. Cellular ATP measurements are undertaken using ATPLite kit (Perkin Elmer) as per manufacturer's instructions. To assess dependency on OXPHOS or glycolysis, cells were pre-treated with oligomycin (Sigma) 10 µM to inhibit OXPHOS, or 2-Deoxy Glucose (Sigma) 50 mM to inhibit glycolysis, or both inhibitors simultaneously, for 30 min at 37° and then ATP measurements were performed [49]. From this we can calculate the % ATP levels setting untreated at each time point to 100%. Mitochondrial reactive oxygen species generation was assessed using mitochondrial NpER2 probe [50] incubated with cells at 20 µM and 1 µM Hoescht for 30 min at 37 °C, probes were removed and phenol red free media replaced. Cells were imaged using the Opera Phenix (Perkin Elmer). In order to assess mitophagy in live cells, cells were incubated for 1 h at 37 °C with 1 µM tetramethylrhodamine (TMRM), 1 µM Lysotracker Green (Invitrogen) and 1 µM Hoescht, before washing to remove fluorescent probes. For the measurement of induced mitophagy 2 µM Antimycin A (Sigma) and 5 µM oligomycin (Sigma) were added prior to imaging. Images were captured in time lapse every 18 min in the same fields of view, minimum 6 fields of view per well. Images generated from the live imaging experiments were analysed using Harmony (Perkin Elmer software). We developed protocols in order to segment nucleus, image region containing cytoplasm, mitochondria, lysosomes, autolysosomes containing mitochondria. Maximal projection images were used for analysis. Mitochondria contained within lysosomes segmentation was set up in such a way to identify a mitophagy event when the overlap between mitochondria and lysosome was 100%.

Staining of cells using LC3 and Tomm20 (as described above) and subsequent imaging using Opera Phenix and image analysis was used to validate the live imaging mitophagy assay. The image segmentation and analysis was set up in Harmony software as previously published [31]. Furthermore as an additional mitophagy read out we utilised the loss of Tomm20 signal from cells as previously determined [31].

Statistical tests. All experiments were performed on at least triplicate differentiations for each control and PRKN mutant neuron or iNPC unless otherwise stated. Data are presented as mean ± standard deviation. Stu-
dents t test was used when comparing between control and PRKN mutant patients. When comparing different timepoints throughout differentiation a matched two way ANOVA was used with multiple comparisons using Sidak or Tukey correction. Treatment data was analysed using two way ANOVA and multiple comparisons. All statistical tests were carried out using GraphPad Prism software.

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Author contributions
A.S. undertook much of the experimental work culturing the neurons, performing assays and staining and putting figures together. C.B. undertook the remainder of neuron culture and assays. F.M.L. undertook some of the experimental work specifically the Tomm20 loss mitophagy validation and KH176m mitophagy assays. M.M. was involved in the experimental work generating the iNPCs from patient and control fibroblasts. E.N. designed and synthesised the mitochondrial ROS probes and advised on their use. J.B. and J.S. provided the Khondrion compounds and expertise of use in assays. L.F. was instrumental in the generation of iNPCs both experimentally and intellectually. H.M. conceived the study, planned the study, undertook some experimental work, analysed and interpreted the data and wrote the manuscript. All authors contributed to the editing and re-drafting of the manuscript.

Competing interests
JS is the founding chief executive officer of Khondrion. JB is the chief scientific officer of Khondrion. All other authors have no competing interests to declare.

Additional information
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Correspondence
Requests for materials should be addressed to H.M.

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