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Review

Mitochondrial homeostasis in cellular models of Parkinson's Disease

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Abstract

Mitochondrial function is known to be an important factor in maintaining cellular homeostasis and its dysregulation has become a hallmark for multiple disease conditions. This review aims to sythesise the extent of this knowledge by analysing changes of mitochondrial physiology parameters in Parkinson's disease (PD) and to evaluate the contribution of cellular models of PD in the field. The analysis provided here constitutes a platform for further elucidation of mitochondrial function parameters relative to factors that may potentiate disease progression.

Keywords–Parkinson's Disease, cellular models, mitochondrial homeostasis

1. Mitochondria and Parkinson's Disease (PD)

Mitochondria comprise a dynamic organellar network which take a central position in maintaining eukaryotic homeostasis. Besides their role in the cellular bioenergetics, namely ATP synthesis, these organelles are supporting essential metabolic processes, calcium and reactive oxygen species (ROS) homeostasis regulation as well as a multitude of signalling cascades. The mitochondrial compartments host functional molecular groups which coordinate protein import and sorting, transport of metabolites, mitochondrial DNA (mtDNA) replication and expression, oxidative phosphorylation (OXPHOS) respiratory system, metabolic enzymes, and organelle quality control mechanisms as well as fusion and fission regulators. Dysfunction in these mitochondrial

components leads to impaired mitochondrial homeostasis and has been linked to diseases, of which we shall focus here on Parkinson's Disease (PD).

Parkinson's disease (PD) is a progressive neurodegenerative disease characterised primarily by loss of dopaminergic neurons in the nigrostriatal pathway, presenting with motor and non-motor signs and symptoms. It is the most prevalent cause of parkinsonism, a broader clinical syndrome with motor features that include: bradykinesia, hypokinesia, muscle rigidity, joint stiffness, resting tremor, shuffling gait, expressionless face and micrography. Non-motor features entail anxiety and depression, REM-sleep behaviour, olfactory disorders constipation and hallucinations [1]. The neuropathological hallmark of PD is the presence of intracellular protein inclusions called Lewy bodies, consisting predominantly of alpha-synuclein. Ageing, environment and genetic susceptibility are the most significant risk factors for PD and cellular defects leading to dopaminergic dysfunction are connected with defects in proteostasis, mitochondrial function, vesicle trafficking and lysosomal activity.

1.1. Respiratory physiology dysfunction as a key feature of PD

The investigation of mitochondria in PD began in early eighties when independent reports revealed that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was the likely cause of permanent parkinsonism in several patients. The parkinsonism phenotype was associated with degeneration of nigrostriatal dopaminergic neurons [2,3] and appearance of Lewy bodies in the Substantia Nigra (SN) [4]. It was found that the MPTP metabolite, 1-methyl-4-phenylpyridinium (MPP+), can be taken up by dopaminergic transporters (DAT), and inhibit complex I (CI) of the mitochondrial electron transfer system (ETS), resulting in nigrostriatal degeneration [5–8]. Subsequently, it was shown that complex I activity was reduced by 20–30% in the substantia nigra of patients with sporadic PD [9,10], with reports of enzyme complex dysfunction that may affect other tissues as well [11–14]. In addition, mitochondria from post-mortem tissue, show greater age-dependent accumulation of mtDNA deletions and somatic mosaicism, compared to control subjects [15–18].

1.2. Dissecting mitochondrial dysfunction in PD

Parkinsonism is a consistent and predominant feature in 23 monogenic disorders [19,20]. Many of the identified genes encode proteins that have strong links to mitochondrial function, and these include PTEN-induced kinase 1 (PINK1), Parkin, DJ-1, LRRK2, alpha-synuclein and vacuolar protein sorting 35 (VPS35).

PINK1, encoding the PTEN-induced serine/threonine kinase 1, and PRKN, encoding an E3 ubiquitin ligase, Parkin, have been shown to have pivotal roles in regulating mitochondrial function, particularly through mitophagy, the process of organellar quality control [21–25]. DJ-1 encodes a protein related to a family of molecular chaperones, expressed in most cell types and localized to the cytosol, mitochondrial matrix, and intermembrane space[26]. DJ-1 appears to play a role in maintaining respiratory complex stability and mitochondrial quality control, alongside other cellular functions, including maintenance of redox balance in the cell[27,28].

LRKK2 mutations are a common cause of familial PD[29], the protein functions as a kinase, a GTPase, and a scaffolding protein and its roles in PD etiopathology are linked to cellular trafficking[30]. PD caused by LRRK2 mutations, has been associated with changes in mitochondrial dynamics, membrane potential and cellular ATP levels[31,32].

α-Synuclein is a major component of Lewy bodies. Mitochondrial roles for α-synuclein in PD include impairment of complex I-dependent respiration[33], preferential binding to the mitochondria[34] resulting in inhibition of mitochondrial protein import, mitochondrial membrane depolarisation and impaired cellular respiration[35]. In addition, it was shown that α-synuclein induces mitochondrial fragmentation and bioenergetic alterations in iPSC-derived dopaminergic neurons[36], and that Lewy bodies from patients with PD contained fragmented mitochondria crowded with lipids and lysosomes as well as α-synuclein[37].

VPS35 has a role in recycling dynamin-like protein 1 (DRP1) complexes, regulators of mitochondrial fission, with mutations and oxidative stress increasing VPS35 interactions and mitochondrial fission[38]. Mutation in VPS35 is shown to decrease enzymatic activity and respiratory defects in complex I and II in patient fibroblasts, excessive mitochondrial fission that leads to the defects in the assembled complex I and supercomplex and causes bioenergetics deficits[39]. PD-associated VPS35 mutations caused mitochondrial fragmentation in cultured neurons, in mouse substantia nigra neurons in vivo and in human fibroblasts[38]. VPS35 mutants also showed enhanced turnover of the mitochondrial DRP1 complexes via the mitochondria-derived vesicle–dependent trafficking of the complexes to lysosomes for degradation[38].

Genome Wide Association Studies (GWAS) have uncovered increasing numbers of PD risk alleles which remain to be studied in detail[40–42]. As we go forward, idiopathic PD samples including primary fibroblasts, iPSc and iPSc-derived neurons are more readily available to elucidate etiopathological roles of the mitochondria in PD providing a test-bed for potential therapeutic strategies.

To provide a platform for ongoing and future work we have reviewed and compared studies of mitochondrial physiology in cellular PD models and compared these with animal models of PD to identify common features that may be investigated as PD risk factors.

2. Mitochondrial homeostasis parameters

Significant effort is being made by the scientific community to consolidate and disseminate protocols relevant to mitochondrial homeostasis dysfunction and neurodegeneration [43–46]. Here we have considered some of the key parameters and common assays used to assess PD phenotypes and summarised some of the most common assays employed for these analyses.

- Mitochondrial Respiration: Oxygen consumption by the mitochondria as a result of processing of fuel substrates is one of the mitochondrial physiology parameters most used to characterise the health status of a mitochondrial preparation whether it is sourced from a cellular model or tissue sampling from an animal model. Mitochondrial physiology parameters, including mitochondrial

respiration, are successfully addressed in recent years with two modern experimental platforms, Oroboros O2k[47] and Seahorse XF[48], whose advantages and experimental capabilities have been previously compared[49]. Using various experimental paradigms, including permeabilization of the cells, purification of the mitochondria and specific combinations of mitochondrial respiration substrates and inhibitors, allows for detailed dissection of mitochondrial physiology states[47] as well as a differentiation between oxidative phosphorylation and glycolysis measurement. Typically, parameters of mitochondrial respiration employed to address mitochondrial function comprise basal respiration, measurement with endogenous substrates (ROUTINE) in saturating substrate and ADP conditions (OXPHOS), respiration uncoupled to ATP synthesis (LEAK) and residual oxygen consumption (Rox)[50].

In Parkinson's models a decrease in oxygen consumption is typically associated with the presence of the disease condition. However, there are also situations where the disease condition may lead to enhanced respiration and consequent oxygen consumption of a mitochondrial preparation (see analysis below and attached tables). Biochemical assays are also employed to inform on individual mitochondrial complex activities[51]; however, the measurement of mitochondrial respiration parameters has become the gold standard for mitochondrial function evaluation.

- Adenosine triphosphate (ATP), is the primary parameter considered for studies of mitochondrial dysfunction both in *in vivo* and *in vitro* models. A variety of assays are now available based on colorimetric, fluorometric and luminescence measurements[52]. These provide information about the total ATP in the sample and would not distinguish between ATP produced from glycolysis versus oxidative phosphorylation. Developed more recently, the Ateam genetically-encoded fluorescent reporters are able to detect changes in ATP at the mitochondrial level[53].

- Mitochondrial membrane potential represents the difference in electric potential between the mitochondrial matrix and the cytosol. On its own, the mitochondrial potential regulates ion transport and protein import. Together with the pH gradient across the inner membrane, it is the driving force of ATP production during the oxidative phosphorylation process. Fluctuations in mitochondrial potential need to be interpreted in conjunction with other mitochondrial physiology parameters, e.g. respiration, in order to address mitochondrial health[54]. However significant membrane depolarisation is typically correlated with cellular death[55]. The mitochondrial potential is determined by using membrane permeable fluorescent probes able to accumulate in the mitochondrial matrix proportionally to the mitochondrial potential. Fluorophores employed for this purpose include TMRM, JC-1, TMRE, Rodhamine 123. Fluorescence readout methods include flow cytometry, fluorometry and fluorescence microscopy for detailed subcellular and temporal changes in mitochondrial potential.

- Mitochondrial Fragmentation / Elongation. Mitochondria are recognised as dynamic organelles able to regulate their structure-function through fission, fusion, exchange of components, transport, biogenesis and degradation. Recent

evidence highlights that the bioenergetic and metabolic status of the mitochondria are intertwined with its dynamics[56,57]. All these dynamic properties allow for proper function of mitochondria and their dysfunction is associated with disease etiopathogenesis. Furthermore, it has been identified as a disease hallmark in both cellular and animal models of PD.

Parameters of mitochondrial morphology are assessed in correlation with other mitochondrial function indicators and typically include mitochondrial number and size. Recent studies started to look at branching and elongation state of the mitochondria through form factor and aspect ratio measurements[45,58].

- Reactive Oxygen Species (ROS), resulting from core oxidative metabolism represent the most significant pool of ROS in the cell. Apart from electron carriers able to produce ROS mitochondria contain a battery of antioxidant defences[59,60]. Mitochondrial stress, including oxidative damage itself, can cause an imbalance between ROS production and removal, resulting in net ROS production with consequent increase in lipid, protein and DNA oxidation products impacting on neurodegenerative disease etiopathology[61]. Assessment of free radical accumulation, reduction in the antioxidant capacity, as well as accumulation of molecular oxidation products, represent common ways to address differences in oxidative stress in PD models and its modulation by pharmacological and genetic approaches. Measurements of **mitochondrial reactive oxygen species** are typically undertaken with superoxide redox-sensitive probes (mito-HEt, MitoSOX, Dihydrorodhamine 123) or redox sensitive fluorescent proteins targeted to mitochondria (e.g. reduction-oxidation-sensitive GFP probes). Using membrane permeable fluorescent probes presents the experimental challenge to ensure measurement of mitochondrially derived ROS rather that general cellular ROS while the main challenges of GFP probes are linked to their redox and pH related sensitivity.

2.1. Qualitative analysis of mitochondrial homeostasis parameters

We evaluated mitochondrial parameters in cellular PD models and compared them with those obtained in animal PD models for concordance. We have employed a scoring system to compare and classify data from experimental studies relative to controls in each study. For mitochondrial respiration parameters, ATP, ROS and mitochondrial potential we have scored '1' for an increase in the disease model versus control, '-1' for a decrease in the disease model versus control and '0' for no change. For mitochondrial fragmentation we have scored '-1' for more fragmented or damaged mitochondrial network, '1' for less fragmented and higher mitochondrial network. The controls have a score of '0'.

We have focused on studies that present mitochondrial respiration data and we have considered that mitochondrial respiration was changed (reduced or enhanced) if one mitochondrial parameter out of the several studied in the original publication presented a significant change. The scores are recorded in the Supplementary File 1. The data were processed with GraphPad Prism.

3. Studying mitochondrial homeostasis in animal models of PD

3.1. Toxicological Models

6-hydroxydopamine (6-OHDA), or oxidopamine, is a product of the dopamine metabolism which can be taken up by dopaminergic neurons via the dopamine transporter (DAT) and has also been found at elevated levels in the urine of PD patients[62]. As a synthetic neurotoxin it is widely used to generate rodent models of Parkinson's Disease. Unilateral injection of 6-OHDA in SN and median forebrain bundle (MFB) in rat[63–66] and mouse[67] causes dopaminergic neuronal death and motor behaviour defects including ipsilateral circling behaviour. Mechanistically, 6-OHDA is understood to cause the death of dopaminergic neurons in rats through inhibition of the mitochondrial ETS Complexes I and IV and through the production of free radicals[68,69]. High-resolution respirometry data from mitochondria in the SN of male Sprague-Dawley rats which had been injected with 6-OHDA in the MFB found that Complex I activity was decreased along with the respiratory control ratio (RCR) in a time-dependent manner[70]. These observations tally with the finding that 6-OHDA caused oxidative stress in the striatum[71] in agreement with evidence for oxidative stress as a common feature in PD patients.

Since its discovery as a PD inducing toxin, **MPTP** has been used of develop animal models of disease. While rats have been largely resistant to MPTP toxicity, MPTP animal models that have been successfully used in PD research include mice and non-human primates such as squirrel monkeys and macaques[72]. In mouse models, the inhibition of Complex I in brain mitochondria resulted in depleted ATP production, an increase in oxidative stress and the loss of DA neurons in the SN[73,74].

Rotenone is a widely used pesticide in aquatic environments. Like MPTP, it is highly lipophilic, enabling its transport across the blood brain barrier (BBB). Similar to MPTP, rotenone also inhibits the catalytic activity of the mitochondrial Complex I enzyme, a characteristic pathology in PD patients[9,12,75], although there is now evidence for a Complex I independent activity of rotenone[76]. Alongside Complex I inhibition, rotenone administration to rats also resulted in decreased levels of DA and its metabolites in the striata[77]. However, damage to brain regions, not including the SN, had also been recorded, suggesting that rotenone may be unsuitable for producing animal models of PD[78,79]. Another significant issue with rotenone rat models was the inconsistent response to the toxin, which often resulted in high rates of mortality[80,81]. However, as a systemic inhibitor of mitochondrial function, it is capable of inducing PD-like pathology across several animal models[82,83].

Paraquat is a widely used herbicide in agriculture that was first suggested as a toxin that could induce PD due to the structural similarities that it shares with MPP^+. Investigations into paraquat as an environmental risk factor for PD have largely used mice as the animal model. It was shown in mice that paraquat treatment can cause a dose- and age-dependent decrease of DA neuron numbers in the SN, and a decline in the density of striatal DA nerve terminals[84,85]. Paraquat mice models have replicated increased presence of α-synuclein fibrils, the up-regulation of α-synuclein protein levels and the formation of aggregates that contain α-synuclein[86]. In striatal mitochondria isolated from paraquat-treated Sprague-Dawley rats LEAK (state 4) respiration was significantly

increased while the respiratory control ratio was significantly decreased in comparison with control rats[87].

The most significant drawback of toxicologic models of PD are that they are acute models of disease, contrary to the typical evolution of PD which takes place over many years, the high variability of the results between different murine strains that are administered the toxins[88] and the variability in reproducing PD features including loss of DA neurons[89], appearance of Lewy bodies and motor and non-motor behavioural changes[90,91].

3.2. Transgenic Models

The discoveries in the genetics of PD have led to development of genetic murine models harbouring genetic modifications related to PD. However, these models do not fully recapitulate the PD characteristics and present rather mild phenotypes[92,93]. To complement these, other animal models of PD, particularly using *Drosophila*, have been successfully employed to address mitochondrial homeostasis alongside behavioural and other mechanistic PD characteristics[94].

Much of the work done to elucidate the mitophagy pathway, within which **PINK1** is a crucial enzyme, was completed using *D. melanogaster* [21,22,95]. These first studies reported that in *D. melanogaster* PINK1 loss of function mutants present the PD phenotypes of dysfunctional mitochondria and locomotive defects associated with DA neurons degeneration. Further studies have reported impaired synaptic transmission, defects in mitochondrial fission and decreased ATP levels arising from reduced Complex I and Complex IV activity[96,97]. When PINK1 models of PD in mice have been investigated, the fidelity of the phenotype to PD in humans has had mixed results. RNAi silencing of the PINK1 gene in mice aged 6 months failed to cause a significant decrease in the number of TH-positive neurons in the SN[98]. Building upon this, the investigation in reported that the PINK1 null mice had no changes to the number of DA neurons or striatal DA content, but there was a significant decrease in the evoked release of DA[99]. Impaired mitochondrial respiration was observed in the striatum of PINK1 null mice[100] and similar to *D. melanogaster* PINK1 mutants were reported to present reduced respiratory activity of both Complex I and Complex II[101]. Interestingly, the PINK1 loss of function accelerates *in vivo* neurodegenerative phenotypes induced by mitochondrial stress triggered by the expression of an unfolded protein in the mitochondrial matrix[102].

Investigations into the role of the ***Parkin*** gene, like PINK1, in PD, were also first conducted in *D. melanogaster*, concurrently with the PINK1 studies[21,22,95,103]. Many of the studies that have investigated Parkin loss of function mutants as a model of PD in *D. melanogaster* have observed mitochondrial dysfunction. Parkin loss-of-function *D. melanogaster* had significantly decreased Complex I and Complex II activity when measured as a function of oxygen flux by high resolution respirometry[104]. In mitochondria isolated from the striata of 9 months old Parkin null mice, OXPHOS (state 3) respiration was significantly decreased, while in 24 months old mice respiratory reserve was instead significantly decreased on Complex I substrates, both as detected by high resolution respirometry[105].

Multiple studies have reported motor defects in **DJ-1** loss of function murine models of PD, as well as altered DA metabolism, however a loss of DA neurons and formation of Lewy bodies has been more difficult to replicate[106–109]. A recent study reported that DJ-1 deficiency in mice accelerated that accumulation and aggregation of the key Lewy body component, α-synuclein, in mice[110]. In mitochondria isolated from the cortex of DJ-1 null mice aged either 3 months or 24 – 26 months, there was no significant differences in OXPHOS or LEAK respiration for Complex I, Complex II or Complex III/IV[111].

Despite the recognised importance of other mutations, like α-synuclein and LRKK2, mitochondrial homeostasis has not been thoroughly addressed in murine models comprising their mutations.

Additional PD murine models have mutations affecting mitochondrial function and quality control. Loss of function of the mitochondrial protease HtrA2, situated in the intermembrane space, induces a reduction in mitochondrial respiration, accumulation of oxidative stress markers and accumulation of unfolded proteins in the mitochondria. These correlate with sustained upregulation of the integrated stress signalling specifically in the brain, which contributes to neurodegeneration[112]. An interesting transgenic model to address mitochondrial homeostasis in PD is represented by the 'MitoPark' mouse model with deletion of the mitochondrial transcription factor A (TFAM) in dopaminergic neurons. Although the model does not present a mutation of a PD gene, it is inducing mitochondrial dysfunction and successfully reconstitutes PD phenotypes rendering the model as a tool to further etiopathology mechanistic studies in the field[113–115].

Our qualitative summary of mitochondrial homeostasis parameters shows that the parameters analysed most often in PD murine brains are mitochondrial respiratory activity, ATP level and accumulation of oxidative species (Supplementary File 1). These show a consistent decrease of respiratory activity, reduced ATP and increased oxidative species (Figure 1A).

4. Studying mitochondrial homeostasis in cell models of PD

Given the experimental challenges and the extensive time required for use of animal models to study mechanistic details of mitochondrial dysfunction in PD, an extensive range of cellular PD models has been developed. These comprise cell lines as well as primary neuronal and iPSc neuronal models undergoing combinations of PD related toxin treatments as well as relevant genetic manipulations.

PD drug treatments are frequently employed to induce or enhance disease phenotypes [116–120]**.** While in animal models MPTP itself is used as an inducer of PD etiopathology in cellular models of dopaminergic neurons presenting the DAT transporter, like SHSY5Y, its metabolite MPP+ is employed. In addition, inhibitors of mitochondrial function, particularly those demonstrated to induce PD phenotypes in animal models, e.g. rotenone, are also employed as PD relevant toxins in cellular models. MPP+ and rotenone are primarily linked to inducing a dysfunction in the complex I linked respiration. However, some studies have shown that these drugs are toxic in the absence of Complex I functionality[76]. Whether Complex I inhibition leads to compensatory mechanisms that are influenced by MPP+ and Rotenone is not fully addressed.

6-OHDA is successfully employed as a PD-relevant toxin in cellular models, both for its dopaminergic link as well as its oxidative stress-inducing properties. Generic oxidative stressors like H_2O_2 have also been employed in cellular PD models.

PD phenotypes are evaluated by a wide range of assays determining cell viability (e.g. MTS MTT, Alamar Blue), accumulation of ROS and Redox profile (ROS-DCF-DA, Amplex red, reduced glutathione content, MDA, carbonylated proteins, CAT and SOD activities), autophagy/mitophagy markers, formation of protein aggregates, electrophysiological properties of neuronal cells, Ca^{2+}/Mg^{2+} imaging, alongside the mitochondrial homeostasis parameters specified above.

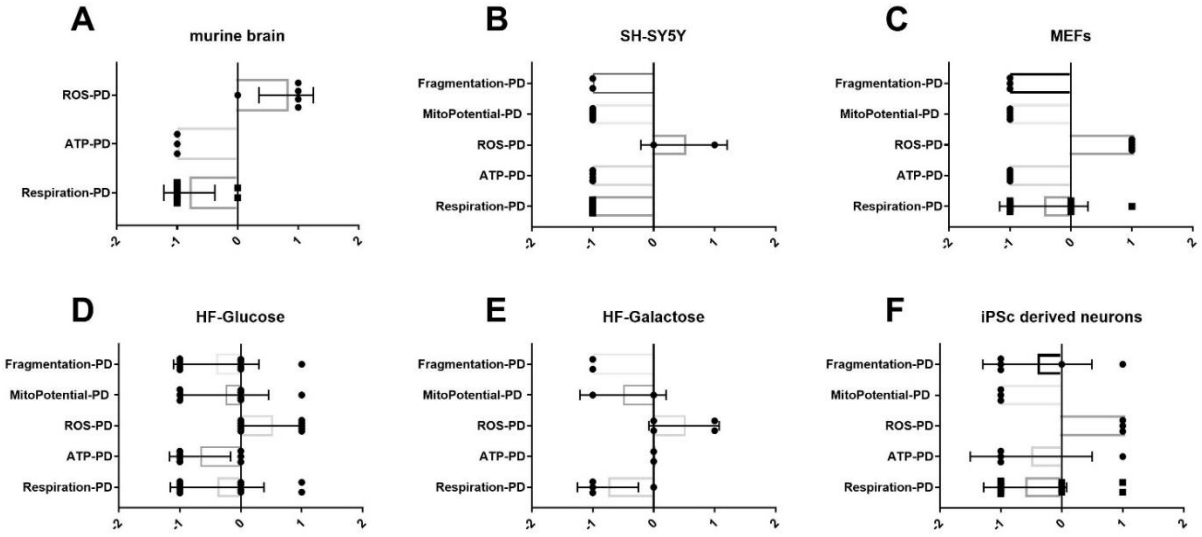

Figure 1. Variation of mitochondrial homeostasis parameters in models of PD represented as mean+/-SD versus the 'zero' line as control. The analysis includes data from (A) murine brain (12 studies), (B) SH-SY5Y (12 studies), (C) mouse embryonic fibroblast (MEFs) (6 studies) (D) human fibroblasts in glucose media (16 studies) (E) human fibroblast in galactose media (4 studies) and (F) iPSc derived neurons (9 studies).

4.1. SH-SY5Y

SH-SY5Y have gained ground as a popular cell model for PD[121]. Developed from a metastatic neuroblastoma cell line[122], has been shown to present tyrosine hydroxylase activity[123] and consequent dopaminergic phenotypes. The cell line is broadly used as a PD model in differentiated or non-differentiated conditions. A variety of protocols have been reported for culturing of the cells, as well as for differentiation[121], which makes it difficult to cross-compare data between studies.

In SH-SY5Y, PINK1/Parkin downregulation and overexpression of loss of function disease mutations are the most relevant genetic transformations employed for this model (Supplementary File 1). Also in SH-SY5Y, the R492X mutation overexpression appears to

have a dominant effect in inducing mitochondrial dysfunction and oxidative stress, particularly in the presence of MPP+[124]. Although DJ-1 has multiple roles in maintaining cellular function there is now evidence for a role in S-nitrosylation of Parkin. Thus, denitrosylation of Parkin due to DJ-1 loss of function has negative consequences on mitochondrial function reducing ATP synthesis and respiration[125]. PD-associated mutations in F-box only protein (FBXO7) have been linked to disruption in mitochondrial homeostasis. SHSY-5Y genetically modified to achieve FBXO7 loss of function have been used to demonstrate that FBXO7 deficiency is linked to mitochondrial dysfunction increased ROS and consequent poly-ADP-polymerase (PARP) overactivation which contributes to cell death[126].

MPP+ treatment in differentiated SH-SY5Y[127] has demonstrated a profound effect on decreasing coupled respiration and increasing the LEAK respiration indicating significant damage at the level of the inner membrane. However, the study did not account for loss of mitochondrial mass. Treatment of SH-SY5Y with 6-OHDA is shown to reduce NADH-linked mitochondrial respiration but detailed characterisation of mitochondrial physiological changes under 6-OHDA has not been performed[117]. A protective effect for antioxidant enzymes was correlated with increasing ROS, a result of 6-OHDA treatment, thereby uncoupling respiration and phosphorylation.

SH-SY5Ys are also used to produce cybrids by fusing dopaminergic cells depleted of mtDNA with human platelets from PD patients. The cybrids recapitulate mitochondrial dysfunction observed in PD human samples providing an additional model to study PD [128,129].

In summary, SH-SY5Y models of PD show consistent decrease of respiratory activity, reduced ATP and increased oxidative species. These are accompanied by mitochondrial potential reduction and mitochondrial fragmentation (Supplementary File 1, Figure 1B).

4.2. Mouse Embryonic Fibroblasts (MEFs)

Immortalised mouse embryonic fibroblasts offer the opportunity of high numbers of cells to employ in parallel for different experimental approaches.

PINK1 KO MEFs have been used to demonstrate that mitochondrial dysfunction in PD is not due to proton leak, but respiratory chain defects with consequences on decreased mitochondrial potential, ATP levels and increased ROS production. Mitochondrial impairments are more pronounced when the cells were grown in galactose rather than glucose medium[130]. We have also shown independently in MEFs that PINK1 KO results in impaired respiration, reduced ATP levels, increased ROS and decreased mitochondrial potential[131]. However, the individual activity of the respiratory complexes did not appear to be affected by PINK1 loss of function[132]. The data in MEFs are consistent with results on mitochondrial impairment in the brains of PINK1 KO mice[133].

DJ-1 loss of function in KO primary MEFs, does not appear to affect mitochondrial respiration, but increases ROS production, contributes to reduced mitochondrial potential and leads to higher mitochondrial transition-pore opening, rendering the cells more susceptible to death following oxidative stress[111]. The DJ-1 effect on mitochondrial physiology has shown some differences in immortalised MEFs, with reduced respiration

in the DJ1-KO, while the other mitochondrial features were consistent with data in the primary cells[134].

LRKK2 mutant MEFs have been obtained through genetic manipulation *in vitro* rather than from transgenic mice and shows the LRKK2 kinase activity sustains mitochondrial function via tethering of mitochondria to the ER[135].

In summary, mitochondrial homeostasis parameters show that, in MEF PD models, there is consistent decrease of respiratory activity, reduced ATP and increased oxidative species. These are accompanied by mitochondrial potential reduction and mitochondrial fragmentation (Supplementary File 1, Figure 1C).

4.3. Human Fibroblasts (HF)

A number of studies have reported decreased respiratory activity in fibroblasts from PD patients with Parkin mutations[14,136]. In contrast, some studies reported higher mitochondrial respiratory rates in Parkin-mutant fibroblasts, while exhibiting more fragmented mitochondrial networks and ultrastructural abnormalities[137]. Zanelatti *et al.* reported higher respiratory activity, reduced ATP levels and mitochondrial potential. While the mitochondrial size did not appear affected, a peculiar mitochondrial network with "chain-like" structures was observed in mutant fibroblasts[138]. In human fibroblasts with Parkin mutations, Grunewald *et al*. observed an overall decrease in the ATP level, increased oxidative stress associated with enhanced mitochondrial mass and higher sensitivity to oxidative stress treatments[139]. The high variability between the different fibroblast lines made it difficult to find significant differences between controls and PD[140]. Respiratory chain dysfunction is also identified in fibroblasts from patients with PINK1 mutations[141].

The impaired respiratory chain complex assembly in genetic PD together with reduced mitochondrial potential has been reported in samples with DJ-1 mutation [134,142]. VPS35 mutations result in defective mitochondrial function[38,39], whereas inefficient response to mitochondrial challenges was seen in fibroblasts with LRRK2 mutation G2019S, suggesting compromised bioenergetic function[143]. Fibroblasts from non-manifesting carriers of LRRK2 mutation showed an increase in mitochondrial network in standard growing conditions (glucose) and an improvement of mitochondrial dynamics under mitochondrial challenging conditions (galactose), while in PD patients carrying the same mutation, mitochondrial dynamic pattern is similar to controls (glucose condition) and there were less branched networks and shorter mitochondria with galactose[143].

CHCHD2 encodes a protein that modulates mitochondrial function in conjunction with the ALS/FTD-associated gene CHCHD10. CHCHD2 accumulates in damaged mitochondria and regulates CHCHD10 oligomerisation and has been linked to PD[101,144]. The CHCHD2 mutation in PD patient fibroblasts causes fragmentation of the mitochondrial reticular morphology and results in reduced oxidative phosphorylation at Complex I and Complex IV[145], as well as its precipitation in the intermembrane space and apoptosis induction *via* cytochrome c destabilization, impaired respiration and increased mitochondrial ROS production[146,147].

Studies have focussed on idiopathic PD (IPD) patient stratification based on identification of pathological mechanisms linked to mitochondrial homeostasis in peripheral tissues using dermal fibroblasts. These studies demonstrate high variability in mitochondrial parameters between patients. Thus only a small number of IPD samples present significant mitochondrial dysfunction in skin fibroblasts[148] as reflected in ATP production, IPD mitochondria present morphometric changes in IPD leading to enhanced resistance to FCCP depolarisation[58] and mitochondrial bioenergetics are changed more significantly by metabolic stress in IPD cases versus control[149]. Deus *et al.* have shown that idiopathic PD fibroblasts present hyperpolarised mitochondria associated with reduced ATP and enhanced ROS[150], while Ambrosi *et al*. demonstrated proteolytic and bioenergetic deficits in IPD fibroblasts[151].

Human fibroblasts show a circadian mitochondrial and glycolytic activity[152]. This has impacted on how mitochondrial function appears in PD versus control samples. Thus, human fibroblasts with mutated Parkin present mitochondrial dysfunction and reduced respiration that is evident when the cell culture is synchronised, while these differences are not evident under basal asynchronous conditions. This may explain some of the difficulties in observing significant differences in patient primary fibroblasts.

The qualitative analysis of mitochondrial parameters in HF follows the same pattern as in other cellular models of PD (Supplementary File 1, Figure 1D and E). However, there is much higher variability in the data and increasingly the mitochondrial properties of these samples are observed in parallel in glucose versus galactose conditions to highlight the impact of the disease mutation or treatment in predominantly glycolytic versus oxidative phosphorylation metabolic conditions. When galactose is used instead of glucose, to create mitochondria-challenging conditions, HF showed stronger decrease in mitochondrial parameters: respiration[140,149], and mitochondrial membrane potential[145], as well as stronger increase in fragmentation[143,145] and ROS production[143]. This data might suggest that PD derived HF have lower oxidative capacity to cope with an extra metabolic requirement such as galactose condition as compared to HF from healthy individuals.

4.4. iPSc derived neurons

In recent years iPSc technologies have allowed the development of human neuronal models to avoid the impact of the genetic differences between primary neuronal models from mouse and rat[153] and human neurons. These have been developed either from fibroblasts of patients with idiopathic PD or with characterised mutations through genetic modifications approaches including CRISPR-Cas9 editing to produce KO lines.

iPSc derived dopaminergic neurons with α-Syn A53T mutation as well as α-Syn triplication cause impairment in several mitochondrial function parameters, including respiration (basal, maximal, spare capacity), reduction in mitochondrial potential and change in mitochondrial morphology associated with decreased DRP1 phosphorylation[36]. Interestingly non-differentiated iPSc did not present the mitochondrial respiration dysfunction. Additional mechanistic findings from this study were perturbation of lipid

biology, enhanced ER stress and autophagic dysfunction in the PD models. iPSc derived neuroepithelial cells genetically engineered to harbour α-Syn A53T and α-Syn A30P mutations also present reduced energy performance, reflected in lower basal respiration and ATP level[154].

iPSc derived dopaminergic neurons with Parkin loss of function show no change in respiration with glucose as a substrate, but reduced respiration with lactate[155]. Interestingly iPSc derived neurons show strong mitochondrial dysfunction phenotypes at the end stage of differentiation, when the metabolic shift from glycolysis to oxidative phosphorylation has completed, consisting of decreased ATP levels, decreased mitochondrial potential, increased mitochondrial fragmentation, and increased mitochondrial ROS production[156].

LRRK2 G2019S iPSc-derived neurons analysis has demonstrated that dopaminergic neurons present an enhanced number of mitochondrial abnormalities when compared with glutamatergic and sensory neurons including decreased respiration, and trafficking abnormalities[157]. Decreased respiration in LRKK2 iPSc neurons has been observed in another independent study which reported an increase in respiratory activity in iPSc derived neurons harbouring PINK1 Q456X mutation[158]. Mutation of another component of the vesicle trafficking machinery, VPS35, has been found to lead to decreased respiration and mitochondrial potential, increased ROS and defective trafficking and consequent impaired mitophagy[159].

iPSc derived PD models appear to have generally higher sensitivity to cellular stressor affecting mitochondrial activity or PD toxins[158], while the details of mitochondrial function present much higher variability and appear to be strongly dependent on the PD mutation, as well as the neuronal type the iPSc have been processed into (Supplementary File 1, Figure 1F).

5. Prospective

Information provided by different disease models has largely been complementary and has offered a panoramic prospective on the mitochondrial contribution to mechanisms of dysfunction in PD. Relying on cellular models (cell lines), where glycolysis has an important role in the budget of total ATP production may be justified when reflecting on whole brain homeostasis. In modelling PD, immortalised cell lines offer the advantage of being able to use large amounts of cells that can be manipulated genetically and pharmacologically in order to address mechanistic details of the disease and to investigate novel pharmacological approaches to tackle cellular dysfunction. Despite differences between individual studies, the data indicate that PD models present reduced mitochondrial respiration activity, reduced ATP levels, reduced mitochondrial potential and enhanced ROS, typically together with mitochondria fragmentation. The qualitative analysis presented here indicates that lines SH-SY5Y and MEFs provide relatively consistent results, which correlate well with the data obtained in brain in murine models of PD. Human fibroblasts from PD patients, whether idiopathic or harbouring genetic mutation, reflect best the high individual variability of mitochondrial function

parameters. Similarly, the results of iPSc derived neurons reflect the variability of the models and present additional experimental challenges to maintain rendering the experiments fairly expensive. Choosing a 'best model' to recapitulate disease, particularly for a disease with multifactorial etiopathology like PD, is still challenging as each of them offers advantages and disadvantages. Thus, establishing mechanistic details in cell lines and validating such data in patient HF or iPSc derived neurons can perhaps give an integrated view on the disease aspect that is investigated. Addressing the disease condition and pharmacological approaches at whole body level may still require the use of animal models. However, the current developments in cellular PD models, including the enhanced availability of patient derived cells, supports the increased use of these models to uncover key information in PD studies.

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Author contributions

Data collection and evaluation was performed by NKJ, BE, NM. All authors, NKJ, BD, LC, IM and NM wrote the manuscript. IM and NM designed the framework of the review.

Conflicts of interest

The authors declare they have no conflict of interest.

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