Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterised by the preferential loss of dopaminergic neurons in the substantia nigra. Mitochondrial dysfunction is increasingly appreciated as a key determinant of dopaminergic neuronal susceptibility in PD and is a feature of both familial and sporadic disease, as well as in toxin-induced Parkinsonism. Recently, the mechanisms by which PD-associated mitochondrial proteins PINK1 and parkin function and induce neurodegeneration have been identified. In addition, increasing evidence implicates other PD-associated proteins such as α -syn and LRRK2 in mitochondrial dysfunction in genetic cases of PD with the potential for a large functional overlap with sporadic disease. This review highlights how recent advances in understanding familial PD-associated proteins have identified novel mechanisms and therapeutic strategies for addressing mitochondrial dysfunction in PD.

Mitochondrial dysfunction in Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting 1% of the population over the age of 60. The cardinal motor symptoms of PD are a result of the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc; see glossary) causing a deficiency of dopamine in the striatum. The exact aetiology and natural course of the disease has yet to be fully characterised but involves dysfunction of numerous systems-level processes including mitochondrial function, calcium homeostasis, dopamine homeostasis and autophagy (Box 1). Although age remains the greatest risk factor for developing sporadic PD, both common, low-penetrance genetic variants, and rare genetic variants with large effect sizes, have helped inform us of the proteins and systems that modulate sporadic disease [1]. Familial forms of PD account for up to 10 % of disease cases and usually result in early-onset disease.

Many of the PD associated genes identified by both genome-wide association studies (GWAS) and Mendelian inheritance patterns encode proteins that have been shown to either directly or indirectly play a role in mitochondrial homeostasis or mitophagy (see glossary). Amongst these mutations, the proteins phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-induced putative kinase 1 (PINK1), parkin (PARK2) and DJ-1 all cause autosomal recessive PD and have well defined roles in mitochondrial homeostasis and mitophagy, with PINK1 and parkin being key activators of mitophagy and DJ-1 being a redox sensor and chaperone [2]. In contrast, mutations in a number of other PD-associated proteins primarily located in the cytosol, such as leucine-rich repeat kinase 2 (LRRK2; see glossary) and α -synuclein (α -syn; see glossary), cause autosomal dominant PD. Although these autosomal dominant genes have been shown to cause a wide range of cellular pathologies, they have also been shown to modulate mitochondrial function. Indeed, whilst neurons are lost from a number of regions in PD patients, it is the unique susceptibility profile of SNpc neurons which result in their loss and the associated PD symptoms (Box 2). Prominent among these susceptibility factors are bioenergetic demands, increased oxidative stress and calcium

buffering of SNpc neurons, all of which may converge on mitochondrial function. PD-causing mutations have been demonstrated to either increase mitochondrial damage or impair clearance of damaged mitochondria resulting in cellular stress (Box 1). This review will explore recent advances in our understanding of the roles of LRRK2 and α -syn, PINK-1, parkin and DJ-1 and how these mechanisms may be operative in sporadic PD. Finally, we will discuss how these recent findings influence the development of therapeutics targeting mitochondrial pathways.

PINK1, parkin and DJ-1 as key mediators of mitochondrial homeostasis and mitophagy

PINK1-dependent activation of parkin is recognised as a major route of mitophagy and is essential for mitochondrial quality control in a number of models. Recently, significant advances have been made in defining the precise mechanism of PINK1-dependent parkin activation (Box 3). PINK1 activity is primarily regulated by mitochondrial import across polarised mitochondrial membranes and subsequent cleavage. This import of PINK1 into mitochondria occurs via the translocase of the outer-membrane (TOM) complex and into the mitochondrial matrix via the TIM complex whereupon it is cleaved by mitochondrial processing peptidase and the presenilin-associated rhomboid-like protease (PARL) [3, 4]. These cleaved forms of PINK1 are degraded by the cytosolic proteasome, although recently, cleaved PINK1 has been shown to act as a repressor of parkin translocation [3, 5]. Mutations located around the A103 cleavage site in PINK1 such as C82F, Q115L and R147H, result in impaired cleavage by mitochondrial proteases and aberrant parkin recruitment [4]. Blockade of mitochondrial protein import results in PINK1-dependent parkin recruitment to TOM-complex components TOM70A and TOM40 resulting in degradation of outer mitochondrial membrane (OMM) proteins [6]. Indeed, the activation of PINK1, resulting from loss of mitochondrial membrane potential (MMP) or accumulation of misfolded proteins, is dependent on autophosphorylation and phosphorylation of ubiquitin for parkin recruitment and activation (see Box 3 for details)[7, 8].

Targets of activated parkin

Once activated, parkin acts as an E3 ligase, catalysing ubiquitination of numerous substrates with diverse roles in mitochondrial homeostasis including the mitochondrial fusion regulators Mfn1/2, the fusion regulator Drp1 as well as DJ-1 [9-11]. Recently, an effort to fully map the parkin-dependent ubiquitylome (see glossary) has identified a number of novel parkin substrates [12]. A key parkin substrate appears to be the mitochondrial motility regulating protein Miro, which promotes anterograde mitochondrial movement via kinesin motor (KIF5) association with microtubules [13, 14]. Indeed, PINK1 knockdown results in increased anterograde mitochondrial movement, which has been suggested may be linked to peripheral neuropathy seen in PD patients with either PINK1 or parkin mutations through accumulation of damaged mitochondria in axons [14]. In support of these observations, LC3-dependent mitophagy of mitochondria damaged by ROS in distal axons of hippocampal neurons is PINK1/parkin dependent [15].

Under severe stress, elicited by valinomycin, PINK1/parkin acts as molecular switch regulating mitochondrial homeostasis and apoptosis through the ubiquitin-mediated degradation of Mcl-1 (see glossary) [16]. This observation suggests that the nature/severity of the stress is an important factor in the extent of PINK1/parkin activation and ultimately the initiation of mitophagy or apoptosis.

Parkin-independent functions of PINK1

A recent, comprehensive study of mitochondrial respiratory activities and protein abundance revealed that PINK1 KO fibroblasts show decreased complex I, III and IV activities, at least partially due to decreased complex abundances [17]. These data are consistent with previous reports showing oxidative phosphorylation (see glossary) deficits and synaptic dysfunction in both *Drosophila* and mice with PINK1 mutations [18]. A number of independent papers have also demonstrated mitochondrial complex I deficiencies in PINK1 knockouts and mutants. For example, mitochondrial complex I subunit Ndufa10 is directly phosphorylated by PINK1 at Ser250, which is important for the ubiquinone reductase activity of complex I [19]. In fibroblasts and iPSC-derived neurons from PD patients with mutations in PINK1, phosphomimetic complex I subunit Ndufa10

rescued complex I activity and MMP [19]. Interestingly, the drosophila homologue of NdufA10 restores complex I activity and partially rescues locomotor deficits which cannot be rescued by parkin overexpression [20].

A further example of parkin-independent PINK1 activity is the observation that PINK1 but not parkin mutants can be rescued by both the GDNF receptor Ret and TNF receptor-associated protein-1 (TRAP1)[21-23]. Both Ret- and TRAP1-dependent rescue of bioenergetics and mitochondrial morphology was independent of mitophagy or parkin recruitment in *Drosophila*. In addition, Ret activation and TRAP1 expression rescue complex I deficiency in PINK1 but to a lesser extent in parkin mutants [21, 23]. Together, these data demonstrate the existence of a possibly convergent PINK1-dependent pathway for mitochondrial homeostasis this is distinct from the PINK1-parkin axis.

DJ-1- a link between mitochondrial and cytosolic dysfunction?

DJ-1 (*PARK7*) has a diverse range of cellular functions, including as a redox sensor/reductase [24] and mutations in DJ-1 are known to cause familial PD. Redox regulation of DJ-1 mediates its role as a chaperone, protease, glyoxalase and regulator of transcription, influencing autophagy and mitochondrial dysfunction. Mitochondrial localisation of DJ-1 is neuroprotective and is dependent on oxidation of the free cysteine (C106) residue by ROS and the formation of a cysteine sulfinic (SO₂) acid through ROS scavenging modulates protein function [24] (Figure 1). Mitochondrial localisation of DJ-1 during oxidative stress is dependent on S to G2 phase nuclear autoantigen (SG2NA) and DJ-1 associated PD-associated mutations M26I, C106A and L166P inhibit this process [25]. However, *DJ-1* knockout mice show increased antioxidant enzyme activity suggesting either increased H₂O₂ production or a compensatory response to deal with lack of DJ-1 [26].

DJ-1 directly interacts with both monomeric and oligomeric α -syn, inhibiting oligomer formation (as discussed in detail below). Additionally, this interaction protects cells from α -syn toxicity in a yeast model [27]. Importantly, DJ-1- α -syn interactions are disrupted by several (L10P, M26I and P158del),

but not all, PD-linked DJ-1 mutations [27]. It is not clear yet if this interaction occurs in a specific subcellular compartment, such as in mitochondria or in response to stimuli, such as DJ-1 oxidation.

Mutated and aggregated α -synuclein as mediators of mitochondrial dysfunction

Roles of α -synuclein aggregation in mitochondrial dysfunction

The aggregation of α -syn and its incorporation as a major component of Lewy bodies (see glossary) is the hallmark of Parkinson's disease. Oligomerisation/aggregation of α -syn in response to oxidation or phosphorylation likely mediates its deleterious effects. Highly penetrant familial mutations multiplications in the α -syn gene (*SNCA*) have been shown to cause aggressive early-onset PD. Additionally, a common SNP in the *SNCA* gene which is enriched in PD patients has been demonstrated to increase expression *in vitro* [28]. Furthermore, recent evidence suggests that α -syn may play a role at the mitochondrial-associated membrane (MAM) [29] and extracellular α -syn may act as a prion to propagate α -syn aggregation and cell stress in PD [30], suggesting novel roles of wild-type and pathogenic α -syn.

Multiple studies have demonstrated the effects of α -syn overexpression/oligomerisation on mitochondria both *in vitro* and *in vivo*. These dysfunctions include inhibition of mitochondrial complexes [31], increased mitochondrial fragmentation in the presence of α -syn oligomers in cells [32], and the permeabilization of mitochondrial-like lipid vesicles by α -syn oligomers [33]. Interestingly, the effects of overexpression appear to be brain region- and oligomeric state-dependent. For example, α -syn overexpression induces complex I, II, IV and V inhibition in the midbrain but complex IV and V dysfunction the striatum [31]. In addition, intermediate α -syn aggregates (pre-fibrillar forms), but not monomeric or fibrillar forms, reduce mitochondrial Ca^{2+} retention resulting in Ca^{2+} dependent mitochondrial dysfunction including loss of MMP and NADH oxidation in isolated mitochondria via complex I dysfunction [34]. The neurotoxin MPTP/MPP⁺ causes dopaminergic cell death through complex I inhibition and ROS production. α -syn protein is required for MPTP induced neurodegeneration, a function retained in the mutated forms of the

protein, potentially indicating an essential role for α -syn in the propagation of complex I-induced dysfunction [35, 36]. Knockdown of α -syn also protects cells from MPP⁺ toxicity *in vitro*, potentially through mechanisms involving increased levels of the antioxidant tetrahydrobiopterin, which may serve to neutralise mitochondria-derived ROS [37].

PD-causing point mutation in α -syn induce mitochondrial dysfunction

A number of PD-associated mutations in α -syn occur in the amphipathic N-terminal of the protein (A30P, E46K, H50Q and A53T). This region of the protein responsible for both self-interactions and interactions with lipids with varying effects of mutations observed. The recently discovered H50Q mutation [38] has been demonstrated to induce only a small number of pathological effects, including oligomerisation and aggregation in SH-SY5Y cells and increased mitochondrial fragmentation in hippocampal neurons [39]. This is consistent with the observations showing increased mitochondrial fragmentation induced by fibrillar α -syn [34]. BAC-transgenic mice expressing 2-3 fold higher E46K α -syn demonstrate a mild neurodegenerative phenotype and increased sensitivity to rotenone-induced cell death [40]. However, overexpression of wild-type α -syn at similar levels using BACs, has demonstrated a similar pre-clinical phenotype, but with the development of age-dependent loss of dopaminergic neurons and the onset of locomotor deficiencies, suggesting that these effects may be attributed to α -syn overexpression [41].

The A53T mutation is the most well-studied α -syn mutation and results in the presence of more fragmented mitochondria and increased ROS production both of which also correlate with aggregation-inducing S129 phosphorylation of α -syn [42]. This correlation between mitochondrial phenotypes and S129 phosphorylation suggests that phosphorylated α -syn may be an important mito-toxic α -syn species, possibly through increasing α -syn aggregation.

Indirect modulation of mitochondrial function by α -syn

Induced pluripotent stem cells (iPSC) neurons derived from A53T patients produce more ROS as well as reactive nitrogen species (RNS; see glossary) in response to stimulus, compared to isogenic control lines [43]. One of the consequences of this oxidative stress is increased oxidation and S-nitrosation of the transcription factor, myocyte enhancer factor 2C (MEF2C). These post-translational modifications result in decreased MEF2C binding to the several targets including the PGC-1 α promoter resulting in decreased mitochondrial biogenesis [43].

Mitochondrial associated ER membranes (MAM) are increasingly being appreciated as key regulators of many mitochondrial processes including dynamics, Ca²⁺ homeostasis and lipid synthesis, as previously reviewed [44]. In addition, it has been demonstrated that the MAM is a major site of syntaxin-17-dependent autophagosome formation in starved cells [45]. MAM also act as key regulatory sites of mitochondrial fission. MAM are contact sites between ER tubules and mitochondria, formed prior to Drp1 translocation representing sites of fission [46]. PD-associated mutations to α -syn result in reduced association of α -syn with the MAM and reduced ER-mitochondrial association, concomitant with increased Drp1-independent fission, possibly through altered OPA1 processing [29]. These data suggest that increases in α -syn levels or post-translational modifications may affect MAM.

The role of increased LRRK2 kinase activity in mitochondrial dysfunction

LRRK 2 is a multifunction protein with important kinase activities, the PD-associated mutation G2019S (kinase domain) thought to increase kinase activity of the protein [47]. Wild-type LRRK2 interacts with a number of key regulators of mitochondrial fission/fusion, co-localising with them either in the cytosol or on mitochondrial membranes, indicating it has multiple regulatory roles (Figure 2) [48, 49]. It has been established that in murine primary neurons and human neuroblastoma, endogenous LRRK2 directly interacts with Drp1 at the mitochondrial membrane, increasing Drp1 phosphorylation and mitochondrial fission [49, 50]. This LRRK2-Drp1-dependent mitochondrial fragmentation is enhanced by overexpressing wild-type LRRK2 and by expressing the

PD-associated G2019S form of the protein but may be rescued by inhibiting Drp1 or increasing fusion [49, 51]. Furthermore, kinase-dead or GTP-binding-deficient LRRK2 displays greatly decreased Drp1 interaction [49]. Phosphorylation of S616 of Drp1 has been shown to promote fission and increased S616 phosphorylation has been observed in sporadic PD patients [52] [53]. However, other data suggest that the G2019S mutant primarily phosphorylates Drp1 at T595 resulting in aberrant mitochondrial fragmentation [51]. LRRK2 also interacts with the mitochondrial fusion regulators Mfn1/2 and OPA1 modulating their activities; PD patients carrying the G2019S mutation demonstrate decreased levels of mature OPA1 [48]. Together, these data demonstrate that the increased kinase activity of LRRK2 results in decreased mitochondrial fusion in addition to increased fission and suggest that regulation of LRRK2 kinase activity may be an important factor in mitochondrial fission/fusion in sporadic PD.

The effects of LRRK2 mutations in patient-derived iPSCs

Expression of mutant LRRK2 and/or overexpression of wild type LRRK2 induce a variety of negative effects on mitochondrial and cellular health. These effects include increased fragmented mitochondria producing more ROS, less ATP and an increased vulnerability of cells to stressors. iPSC-derived dopamine neurons generated from R1441C mutation carriers show increased oxidative stress, higher cell death and impaired neuronal differentiation [54]. Furthermore, G2019S expressing fibroblasts and SH-SY5Y cells show up to 80% increase in basal oxygen consumption and a marked decrease in MMP, due to mitochondrial proton leak caused by increased mitochondrial uncoupling protein 2 (UCP2) and UCP4 expression, with inhibition of LRRK2 normalising UCP4 expression [55]. Additionally, UCP2 upregulation has been observed in iPSC neurons from PD patients carrying the G2019S mutation [56].

Mature iPSC-derived LRRK2 G2019S dopamine neurons are more vulnerable to H_2O_2 , 6-hydroxydopamine (6-OHDA), rotenone and the proteasome inhibitor MG-132, compared to wild type LRRK2 neurons [57, 58]. Increased susceptibility to MPP⁺ was also found in human LRRK2 G2019S fibroblasts, again with an increase in caspase-mediated cell death [59]. Together, these

findings demonstrate how LRRK2 mutations increase cellular susceptibility towards environmental insults. This increased susceptibility may explain the incomplete penetrance of the LRRK2 mutations observed in PD in that low penetrance mutations such as those found in LRRK2 may require an additional environmental or genetic disposition to elicit/propagate neurodegeneration.

Protein aggregation and autophagy inhibition as catalysts for mitochondrial impairment?

Impairments of autophagy, particularly chaperone-mediated-autophagy (CMA), are a widely reported feature of both LRRK2 and α -syn models [60, 61]. In addition, accumulation of α -syn, a CMA substrate, has been detected in multiple iPSC-derived models [57, 58, 62]. Given that LRRK2 has been shown to impair CMA [60], this may be a contributing factor towards increased α -syn aggregation which may initiate α -syn-dependent mitochondrial damage. Mitochondrial damage results in increased autophagic cargo and α -syn aggregation, further inhibiting CMA, LRRK2 mutations may initiate a self-perpetuating insult on the cell (Figure 2). Feedback mechanisms such as this suggest potential ways in which stochastic protein aggregation, due to environmental factors or aging, and potentially common PD-associated genetic variants, may contribute to sporadic PD.

Novel mitochondrial therapeutic targets

Models of autosomal dominant and autosomal recessive forms of PD demonstrate markedly different mechanisms for the induction of mitochondrial dysfunction and cellular stress. However, the outcomes of this dysfunction- increased ROS production and bioenergetic stress- are common to all forms of mitochondrial dysfunction (Box 1). The wealth of mechanistic information on the mitochondrial functions of PD-related proteins has identified a wide range of points for therapeutic intervention. Translation of these promising therapeutic targets to both patients with familial mutations and further towards sporadic disease is a major challenge for PD research.

Modulating mutated proteins directly

The autoinhibition exhibited by parkin may also be a target for small molecules which increase parkin activation in a PINK1 independent manner [63]. Given that parkin activation requires both

mitochondrial translocation and ubiquitin phosphorylation, as evidenced by phosphomimetic parkin [64], it will be interesting to see whether merely decreasing autoinhibition restores regulation and impacts mitochondrial phenotypes in the absence of PINK1.

Upregulation of the glyoxalase activity of DJ-1, using small molecules to increase catalytic rate/recycling of the protein, has the potential to reduce the levels of reactive aldehydes such as methylglyoxal that can damage proteins through the formation of advanced glycation endproducts (AGEs) [65]. Furthermore, the products of DJ-1 glyoxalase activity, D-lactate and glycolate, can rescue the loss of MMP observed after PINK1 knockdown suggesting an additional mechanism by which upregulation of DJ-1 glyoxalase may be protective [66].

Much interest has been generated by the modulation of the LRRK2 kinase domain as a potential therapy for LRRK2 mutation carriers. Treatment of neuronal cultures with the LRRK2 inhibitors LRRK2-IN-1 and GW5074 lead to an increased cell survival in LRRK2 G2019S iPSC dopaminergic neurons [58] and reduced cell dysfunction and death in response to valinomycin and concanamycin A treatment [67]. However, the lack of specificity of many of the inhibitors developed, particularly for G2019S LRRK2 has impaired their ability to be robust and translatable [68, 69].

It has been shown recently that oxidative stress promotes the uptake of α -syn by various cell types resulting in mitochondrial inhibition [70, 71]. This raises the possibility that mitochondrial dysfunction and oxidative stress may play a key role in the propagation of α -syn prion-like behaviour. Interestingly, the cell to cell α -syn transmission has recently been shown to be blocked in both primary culture and mice using antibodies targeting α -syn, suggesting the future potential for development of vaccines targeting extracellular α -syn [72].

Modulating alternative/parallel pathways

Expression of UCP4a in flies carrying PINK1 and parkin mutations significantly reduces but does not abolish both dysfunctional mitochondrial phenotypes and gross defects in both PINK1 and parkin

mutants [73]. Conversely, the UCP inhibitor genepin restores MMP in G2019S LRRK2 mutants [55]. These conflicting effects of UCP modulation demonstrate that, whilst mitochondrial phenotypes elicited by PINK1 and G2019S mutations are similar, the mechanisms by which these mutations are operative are very different. Modulation of UCP responses in sporadic PD, using genepin-like compounds, would require establishment of whether activation/inhibition of UCP responses is useful in sporadic disease and furthermore, selective targeting of specific neuronal populations.

Upregulation of the mitochondrial unfolded protein response (mtUPR) via NAD⁺ activation of sirtuin 2.1 (potentially by the pentose phosphate pathway; Box 1) has been demonstrated to increase life span in *C. elegans* suggesting bioenergetics and mtUPR are crucial for successful aging with clear parallels to dysfunction observed in PD. These observations suggest that increasing the pentose phosphate pathway or activators of sirtuin 2.1 may have therapeutic utility in both familial and sporadic PD.

Cholesterol oximes such as olesoxime and TRO40303 are small molecules that are known to interact with OMM proteins including VDAC and limit opening of the mitochondrial transition pore in response to oxidative stress [74]. Olesoxime protects differentiated SH-SY5Y cells from α -syn overexpression-mediated toxicity [75], whereas TRO40303 upregulates a number of mitochondrially- and PD-related genes including Drp1, VDAC and tyrosine hydroxylase in mice overexpressing α -syn [76]. However, subtle modulation of behavioural phenotypes in TRO40303 mice suggest the effect may not be straightforward [76].

Modulating the downstream consequences of mitochondrial stress

Modulation of the downstream consequences of mitochondrial dysfunction may offer therapeutic opportunities applicable to both familial and sporadic PD. Recently, both transcriptional and metabolic changes in nucleotide metabolism were identified in *Drosophila* lacking *Pink1* [77]. Genetic supplementation of deoxyribonucleoside kinase in *Drosophila*, or supplementation with deoxyribonucleosides or folic acid in human neuroblastoma cells with *PINK1* knockdown, resulted in improvement in a number of markers of mitochondrial dysfunction [77].

ATP depletion, which is observed in models of severe mitochondrial dysfunction, may be a limiting factor for kinases, such as PINK1, in cells under bioenergetic stress. Therefore, it is interesting that the ATP analogue kinetin triphosphate (KTP) or its precursor kinetin (through KTP) are able to increase the activity of both native and mutant PINK1, resulting in increased parkin recruitment to damaged mitochondria and decreased sensitivity to oxidative stress under conditions of ATP depletion [78].

Supplementation with either the native mitochondrial complex I electron acceptor coenzyme Q₁₀ (CoQ₁₀) or a mitochondrially-targeted CoQ₁₀ (MitoQ; CoQ₁₀ coupled to the lipophilic molecule triphenylphosphonium) have previously been demonstrated to be beneficial in a number of pre-clinical models of PD. Unfortunately, phase 3 trials of high doses of both CoQ₁₀ and MitoQ demonstrated no clinical benefit in PD patients relative to placebo [79, 80]. Interestingly, vitamin K₂ like CoQ₁₀ acts as an electron acceptor and reduces PINK1/parkin associated phenotypes [81]. Therefore, activators of the vitamin K₂ producing enzyme UBIAD1 or supplementation may be hypothesised to exert a therapeutic effect in PD.

A library screening approach using fibroblasts from patients carrying parkin mutations identified 15 compounds that normalised MMP and ATP levels in patient cells, including ursocholic acid which acts through glucocorticoid receptors and Akt signalling. This compound has also been shown to rescue mitochondrial phenotypes in LRRK2 patient fibroblasts [82]. This screening approach using fibroblasts and potentially iPSC neuronal cultures represents a paradigm for screening compound libraries for novel and repositionable compounds for PD. Screening of individual patient-derived iPSCs in this manner may represent a strategy for assessing the pre-clinical efficacy of individually targeted therapies. Furthermore, normalisation of robust phenotypes, such as decreased complex I activity, in iPSCs from sporadic PD patients is a logical and potentially hugely beneficial strategy for future PD therapeutics.

PD patients with either autosomal dominant or autosomal recessive PD, or models of these mutations, demonstrate mitochondrial complex-I deficiencies, mitochondrial DNA damage and oxidative stress. However, as discussed earlier, it is likely that mitochondrial dysfunction in autosomal dominant and autosomal recessive PD does not occur by the same mechanism. Indeed, even from a clinical perspective, autosomal recessive mutations may represent a nigral-specific degeneration (nigropathy) due the selective vulnerability of the SNpc to mitochondrial stressors (as evidenced by the lack of non-motor dysfunction in PINK1/parkin patients), whereas autosomal dominant mutations in α -syn and LRRK2 may represent synucleinopathies, which are driven by protein aggregation and autophagic impairment resulting in mitochondrial dysfunction much more broadly. Together, increased understanding of the mechanisms of mitophagy and increased understanding of how pathogenic PD mutations disrupt mitophagy have increased our understanding of both mitophagy and PD aetiology, identifying exciting novel therapeutic avenues. Our understanding of the connection between PD and mitophagy would be further advanced by answering several outstanding questions (Outstanding Questions Box). Key for the development of novel therapeutics is how observations in familial forms of PD translate into sporadic PD. The complex nature of PD and our experience with the failure of monotherapies targeting single systems in PD hint at the need for modulation of multiple causes of mitochondrial dysfunction in concert with other systems-level processes in PD.

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Figure 1. Overview of the roles of PD-associated mitochondrial proteins in mitochondrial homeostasis and mitophagy. **1)** DJ-1 acts as a redox sensor and antioxidant in mitochondria during normal homeostasis. **2)** Upon oxidative stress selective sequestration of mitochondrial components into PINK1/parkin-dependent mitochondria-derived vesicles (MDVs) occurs. These MDVs, which can fuse with lysosomes, likely act as an important mitochondrial quality-control mechanism. **3)** Mitochondrial dynamics are regulated by the proteins including mitofusins (Mfn) which promote fusion and and dynamin related protein-1 (Drp1) which promotes fission. Mitochondrial fission as a result of phosphorylation (activation) of Drp1 leads to increased fragmented mitochondria which generate more ROS and less ATP. Increased ROS production causes post-translational modification of proteins including oxidation of DJ-1 promoting mitochondrial fission and degradation. DJ-1 mutations lead to excessive fission/degradation. **4)** Increased ROS production by damaged mitochondria (particularly complex I) results in increased DJ-1 oxidation. **5)** Loss of mitochondrial membrane potential results in PINK1-dependent recruitment and activation of parkin. Parkin acts as an E3 ligase ubiquitinating mitochondrial proteins, particularly outer-membrane proteins, resulting in sequestration in autophagic vacuoles and **6)** degradation of cargo by lysosomal proteases.

Figure 2. Overview of the roles of α -synuclein and LRRK2 in mitochondrial dysfunction. 1) Reduced but not oxidised DJ-1 inhibits α -syn aggregation through chaperone-like activity. 2) Aggregated α -syn may directly permeabilize lipid membranes and inhibit mitochondrial complex activities. 3) Damaged mitochondria may be degraded by the PINK1/parkin pathway, however, cardiolipin externalisation on the mitochondrial surface may represent an alternative pathway. α -synuclein binds cardiolipin suggesting it may modulate cardiolipin-mediated mitophagy. 4) Aggregated α -synuclein inhibits lysosomal function exacerbating cellular stress. 5) LRRK2 plays a homeostatic role in activating the mitochondrial fission protein Drp1 through its phosphorylation, however increased kinase activity results in aberrant Drp1 phosphorylation and fission. 6) Increased fission in G2019S models is associated with mitochondrial dysfunction and increased ROS production from mitochondrial complexes. The increased number of fragmented mitochondria will increase autophagic flux due to the increase in mitophagic cargo. 7) Aggregated α -synuclein and LRRK2 mutations have been shown to impair CMA resulting in increased protein aggregation and further cellular stress.

Biochemical consequences of mitochondrial dysfunction (Box 1)

Impairments to mitochondrial dynamics or direct inhibition of mitochondrial proteins have a number of deleterious consequences including decreased ATP generation, increased oxidant generation and cytochrome c leakage. Numerous examples of bioenergetic abnormalities in PD have been documented including complex I dysfunction and a dysregulation in glucose metabolism, impairing the pentose phosphate pathway [83]. Dysfunctional mitochondria are a reservoir for oxidant production, with complex I previously shown to be the major site of their generation [84]. Oxidative stress defines a state of oxidant production above the antioxidant capacity of the cell/tissue and results in damage of proteins, lipids and DNA with deleterious consequences. Mitochondrial reactive oxygen species (ROS) generation will result in increased mtDNA mutations or catalytic peroxidation of lipid membranes, including mitochondrial membranes which results in decreased MMP.

Oxidative post-translational modification of proteins by ROS/RNS has been demonstrated to affect the structure, function and antigenicity of proteins, such as α -syn, in a number of diseases involving inflammation, including PD [85, 86]. It is likely that when PD-associated proteins are post-translationally modified by ROS that it can recapitulate the effects of loss-of-function mutations or toxic gain-of-function mutations (e.g. aggregation of α -syn). The pathological importance of oxidant production is typified by the effects of the complex I inhibitors MPP⁺, rotenone and paraquat, all of which have a well described ability to induce cellular dysfunction and Parkinsonism in humans and animal models.

Increased generation of ROS/RNS in PD may be due to modulation of MMP and oxidative phosphorylation (as proposed for PINK1 and α -syn), or produced during the respiratory burst by enzymes such as NADPH oxidase and iNOS. Indeed, inflammatory processes, such as the respiratory burst, in response to immune activation by cell debris released from dying cells or potentially via TLR recognition of aggregated α -syn are a potentially large source of reactive species [87]. The cellular effects of this oxidant production are several, including activation of the antioxidant response via

NRF2 and upregulation of uncoupling proteins (UCPs) that act to 'slow-down' oxidative phosphorylation and so limit oxidative phosphorylation derived ROS. The levels of ROS/RNS can be increased due to decreased antioxidant capacity (as with a number of DJ-1 mutations [88]). Therefore, by a number of different mechanisms, ROS/RNS may be produced and damage cells or inhibit cellular processes, generating more oxidative stress and cellular damage, eventually resulting in end-stage processes including apoptosis and necrosis of neurons.

Roles of mitochondria in the preferential vulnerability of dopaminergic neurons (Box 2)

The basis for the preferential vulnerability of A9 dopaminergic neurons in PD remains unresolved. However, a number of factors including increased calcium fluxes, long unmyelinated axons with large energetic demands and increased oxidative stress, contribute to the vulnerability of these neurons [89]. Whilst none of these factors is unique to A9 dopaminergic neurons, it is likely that the interplay between these factors is responsible for neurodegeneration in both familial and sporadic PD.

Neurophysiologically, it has been demonstrated that A9 dopaminergic neurons use calcium for their pacemaking activity via $\text{Ca}_v1.3$ channels and that such pacemaking using these L-type calcium channels increases mitochondrial oxidative stress [90, 91]. This link between Ca^{2+} fluxes and mitochondria is enhanced by previous demonstration that loss of the mitochondrial regulatory protein PINK1 sensitises cells to Ca^{2+} -induced cytotoxicity [92] (as reviewed in detail [93]). The result of these fluxes, if not buffered, is disruption of MMP, increased oxidative and endoplasmic reticulum stress.

Analysis of A9 dopaminergic neuroanatomy suggests that the bioenergetic cost of both maintaining the large number of synapses and propagating action potentials is vast in long, unmyelinated dopaminergic neurons is extreme [94, 95]. Conservative theoretical calculations, based on existing experimental data, suggest that a single midbrain dopaminergic neuron may form over one million synapses and that the energy cost of maintaining these synapses and the cellular membrane potential increases with a non-linear relationship [94, 95]. Indeed, mitochondrial complex I deficiency is a key feature of both sporadic and familial disease [96] and the non-neuronal specific complex-I inhibitor rotenone causes parkinsonism-like phenotypes in both humans and animal models. As mitochondria are a chief source of ROS more flux through mitochondria, this bioenergetic demand places strains on both the antioxidant and

A major source of mitochondrial deficits during aging is mutations in mitochondrial DNA (mtDNA), which are accelerated by oxidative stress. Individuals carrying polymerase gamma (POLG) mutations, the polymerase responsible for mtDNA maintenance and replication, or rats treated with rotenone, acquire mtDNA deletions at an accelerated rate in the SNpc. mtDNA mutations are concomitant with decreased complex I activity and α -syn pathology [97, 98], further demonstrating the susceptibility of the SNpc to mitochondrial dysfunction.

The presence of dopamine in A9 neurons is also a key susceptibility factor. Dopamine may generate ROS and also form adducts with proteins involved in mitochondrial homeostasis, such as DJ-1, impairing their function [99]. Thus dopamine provides an additional source of stress-inducing ROS if dopamine-uptake and release are not regulated effectively, as observed in both familial and sporadic PD.

Endogenous activation mechanisms of PINK1/parkin (Box 3)

An outstanding question in PINK1/parkin biology has been the physiological relevance of the mitochondrial uncouplers, such as CCCP, which collapse MMP to induce PINK1/parkin mediated mitophagy. Recently, the endogenous molecules short mitochondrial isoform of p19ARF has been demonstrated to elicit PINK1/parkin-dependent mitophagy in neurons, highlighting that this process can be activated endogenously without exogenous manipulation of MMP [100]. *In vivo*, endogenous activators of PINK1/parkin are likely to include unfolded protein responses and physiological factors which reduce MMP such as Ca^{2+} fluxes and acidification of the cytosol.

Prior to 2014, it was known that upon loss of MMP, PINK1-dependent phosphorylation of parkin is essential for its activation. However, introducing phosphomimetic substitutions in parkin is insufficient to elicit either its activation or mitophagy in the absence of PINK1 [101]. Recently, it has been determined that PINK1 acts as an ubiquitin kinase to phosphorylate ubiquitin, which subsequently reverses autoinhibition of parkin's catalytic C431 residue [16, 64]. These papers demonstrate that both ubiquitin and the ubiquitin like domain (UBL) of parkin is only phosphorylated at S65 in the presence of PINK1 and that this phosphorylation occurs after loss of MMP [16, 64, 102, 103]. Furthermore it has been demonstrated that phosphomimetic ubiquitin activates phosphomimetic parkin, in the absence of PINK1 and that phospho-ubiquitin and parkin physically interact [64]. Furthermore quantitative proteomics data has suggested that PINK1 phosphorylates ubiquitin chains bound to mitochondrial substrates promoting further parkin activation and ubiquitylation [104].

Mitochondrial outer-membrane- a key determinant of mitochondrial fate? (Box 4)

In response to oxidative stress, mitochondria-derived vesicles (MDVs) are formed and selectively sequester mitochondrial proteins from the matrix and inner mitochondrial membrane [105, 106]. These vesicles are enriched for oxidised proteins however, the source of the ROS is a key determinant in the nature of the cargo with intra-mitochondrial ROS (generated by antimycin A treatment) forming vesicles positive for mitochondrial matrix components but negative for the TOM20. In contrast, extra-mitochondrial ROS (generated by xanthine/xanthine oxidase system), generates matrix⁻/TOM20⁺ MDVs in a parkin-independent manner [105]. Therefore, MDVs appear to act as a mitochondrial quality control mechanism, possibly as an alternative to degradation of the whole mitochondrion by mitophagy.

Parkin mutations disrupting the ligase active site (C431F) or translocation (K211N) of parkin abolish MDV formation, whereas mutation in the UBL domain (R42P) reduces vesicle number, potentially though impaired trafficking [106] (Figure 1). These recent studies suggest that both the TOM complex and another OMM protein- voltage-dependent anion-selective channel (VDAC)- act as key mediators of PINK1/parkin-dependent mitophagy and MDV formation. These observations are of interest, given TOM40/TOM22 levels inversely correlate with OMM degradation [6, 107], that α -syn decreases TOM40 levels in mice and that VDAC1 is decreased in neurons displaying α -syn aggregation and in PD patients [108, 109]. Furthermore, rats expressing the PD-associated α -syn A30P mutant demonstrated less VDAC throughout the nigra, independent of α -syn oligomeric state [109]. However, at present, literature would favour OMM protein loss as being a consequence of α -syn -induced dysfunction rather than direct blockage of protein import by α -syn.

The phospholipid cardiolipin is an integral component of the mitochondrial inner membrane which is externalised on mitochondria after stress where it acts as an “eat me” signal that is recognised by LC3, facilitating mitophagy [110]. Interestingly, α -syn has been demonstrated to interact with cardiolipin on vesicles mimicking mitochondrial inner membranes and the A30P substitution inhibits

this interaction [111, 112]. Furthermore α -syn has been shown to translocate to mitochondria after acidification of the cytosol (reducing MMP) [113]. Taken together, these data suggest that α -syn may be recruited to cardiolipin decorating the OMM, thus facilitating removal of damaged mitochondria. These recent observations suggest a number of mechanisms by which the mitochondrial outer-membrane acts an important modulator of mitochondrial homeostasis and degradation.

Outstanding questions

1. What are the endogenous triggers for the three main pathways of mitophagy: MDV formation, PINK1/Parkin and cardiolipin externalisation? Do these triggers determine the mechanism of degradation and how do these pathways interconnect?
2. What is the role of the MAM in regulation of mitochondrial function and how do PD-associated proteins influence this association?
3. Do mitochondrial dysfunction and oxidative stress play a significant role in promoting prion-like behaviour of α -syn and do these two phenomena form part of a positive feedback loop in PD?
4. Can modulation of mitochondrial dysfunction in isolation be sufficient to provide therapeutic benefit in symptomatic PD patients?
5. How do mitochondrial phenotypes observed in familial PD translate to sporadic PD? Is sporadic PD driven by SNPs, environmental factors or post-translational modifications which mimic monogenic mutations?

Glossary

α -synuclein (α -syn): The major component of Lewy bodies- the pathological hallmark of PD, and a protein with diverse roles in cellular biology and synaptic transmission. Mutations or increases in the levels of α -syn are found in PD and increase the propensity for the protein to aggregate, a major factor in the deleterious effects ascribed to the protein.

Autophagy: A process of degradation of cellular components via breakdown in the lysosome, either by direct import: chaperone mediated autophagy (CMA) or by incorporation into autophagosomes and subsequent lysosomal fusion: micro/macroautophagy.

GWAS: Genome-wide association studies are used to identify single nucleotide polymorphisms (SNPs), which are commonly present in the population, that are associated with a phenotype, for example an increased risk of developing Parkinson's disease. GWAS can be used as a way of identifying novel mediators of disease.

Lewy bodies: intracellular protein aggregates comprised of damaged and misfolded proteins of which α -synuclein is a prominent component. Lewy bodies are the defining pathological feature of post-mortem PD brain.

LRRK2: Mutations in Leucine-rich repeat kinase 2 (LRRK2) is the most common form of autosomal dominant Parkinson's disease.

Mitophagy: A specific form of macroautophagy, relating to degradation of mitochondria via autophagosome formation.

MPTP/MPP+: A complex I inhibitor similar in structure to dopamine. MPP+ is preferentially taken up by the dopamine transporter resulting in selective inhibition of complex I in dopaminergic neurons.

NRF2: Nuclear factor (erythroid-derived 2)-like 2 is a key transcription factor activated in response to stress, resulting in upregulation of phase II (detoxifying) enzymes including many antioxidants.

PGC1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha is a transcriptional coactivator with important roles in mitochondrial biogenesis.

ROS/RNS: Reactive oxygen/nitrogen species, an umbrella term for a range of oxygen or nitrogen containing species which may damage macromolecules such as proteins, DNA or lipids.

Substantia nigra pars compacta (SNpc): The pigmented region of the midbrain in humans. The A9 region is the major site of dopaminergic cell loss in Parkinson's disease.

TH: Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of the neurotransmitter dopamine.

TOM: The translocase of the outer-mitochondrial membrane is a complex of proteins (including TOM20 and TOM40) which act to move proteins across the mitochondrial outer-membrane into the intermembrane space.

Ubiquitin: An 8.5 kDa protein that can post-translationally modify proteins influencing their function, such as targeting proteins for degradation.

UCPs: Proteins responsible for uncoupling oxidative phosphorylation from ATP generation in mitochondria through reducing MMP.