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Mitochondrial DNA and Primary Mitochondrial Dysfunction in Parkinson's Disease

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ABSTRACT: In 1979, it was observed that parkinsonism could be induced by a toxin inhibiting mitochondrial respiratory complex I. This initiated the long-standing hypothesis that mitochondrial dysfunction may play a key role in the pathogenesis of Parkinson's disease (PD). This hypothesis evolved, with accumulating evidence pointing to complex I dysfunction, which could be caused by environmental or genetic factors. Attention was focused on the mitochondrial DNA, considering the occurrence of mutations, polymorphic haplogroup-specific variants, and defective mitochondrial DNA maintenance with the accumulation of multiple deletions and a reduction of copy number. Genetically determined diseases of mitochondrial DNA maintenance frequently manifest with parkinsonism, but the age-related accumulation of somatic mitochondrial DNA errors also represents a major driving mechanism for PD. Recently, the discovery of the genetic cause of rare inherited forms of PD highlighted an extremely complex homeostatic control over mitochondria, involving their dynamic fission/fusion cycle, the balancing of mitobiogenesis and mitophagy, and consequently the quality control surveillance that corrects faulty mitochondrial DNA maintenance. Many genes came into play, including the PINK1/parkin axis, but also OPA1, as pieces of the same puzzle, together with mitochondrial DNA damage, complex I deficiency and increased oxidative stress. The search for answers will drive future research to reach the understanding necessary to provide therapeutic options directed not only at limiting the clinical evolution of symptoms but also finally addressing the pathogenic mechanisms of neurodegeneration in PD. © 2017 International Parkinson and Movement Disorder Society

Key Words: Parkinson disease; parkinsonism; mitochondrial DNA; mtDNA; quality control; mitophagy

Parkinson's disease (PD) is one of the most frequent neurodegenerative age-related disorders, it affects 0.3% of the entire population and about 1% of people older than 60 years of age,[1] and it is clinically characterized by the association of bradykinesia with tremor or rigidity.[2,3] Pathological hallmarks are the loss of dopaminergic neurons in substantia nigra pars compacta and the presence of Lewy bodies (LB) in spared neurons, typically containing aggregates of α-synuclein, neurofilaments, ubiquitin, and other compounds.[4,5] A great debate is ongoing about the presence or absence of LB in relation to the identification of inherited forms of PD that may lack LB.[6-8] The pathogenesis of PD remains poorly understood. However, the seminal descriptions of 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced parkinsonism in humans in 1979-1983 led to the discovery that inhibition of the respiratory complex I (nicotinamide adenine dinucleotide [NADH]: unibiquinone oxidoreductase) in mitochondria was the biochemical defect related to MPTP intoxication.[9-12] This

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observation generated the hypothesis that mitochondrial dysfunction may be relevant for PD pathogenesis and consequently led to consider also the role of mitochondrial DNA (mtDNA) defects.

Mitochondrial Involvement in Parkinson's Disease: Complex I Impairment

Historically, the first observation involving mitochondria in PD relates to the evidence that an impairment of complex I was present in different forms of PD and parkinsonism.[13] Research has focused on 2 main areas. The first investigated the occurrence of parkinsonism in relation to intoxication with a variety of compounds, mostly acting as complex I inhibitors. The second area of investigation concerned the occurrence of a complex I defect in patients with idiopathic PD as a result of genetic predisposition, possibly related to mtDNA.

MPTP: A Complex I Inhibitor

The first observation that intoxication with MPTP, a byproduct of the chemical synthesis of meperidine, could induce a parkinsonian syndrome in a group of young drug abusers dates back to 1979.[9,10] A few studies reproduced the parkinsonian features induced by MPTP in both primate and murine models,[14-16] and the biochemical details of MPTP toxicity were hence elucidated, in particular the inhibitory effect on mitochondrial complex I.[11,12] These studies clarified that MPTP is metabolized to MPP + by MAO-B in glial cells[17] and that MPP + through the dopamine transporter (DAT) concentrates in dopaminergic neurons of the substantia nigra, where it exerts an inhibitory action on the mitochondrial complex I.[18,19] The observation that MPP + inhibits complex I led to a wide interest for all molecules with known complex I inhibitory activity, in particular compounds commonly used as pesticides such as rotenone and paraquat.

Rotenone, Paraquat, and More: All Converging on Complex I Inhibition?

Epidemiological studies on PD prevalence in relation to environmental exposure to toxins suggested the role of some pesticides,[20-22] namely, rotenone and paraquat attracted attention. In 2000, Betarbet and colleagues[23] produced a rat model of parkinsonism by chronic, systemic administration of rotenone, a classic complex I inhibitor. This report showed some striking pathological similarities with PD, such as cytoplasmic inclusions in nigral neurons reminiscent of LB. A subsequent *in vitro* study showed that chronic complex I inhibition could increase oxidative stress and apoptotic cell death with ubiquitin/α-synuclein accumulation and aggregation.[24] However, an independent rotenone-based rat model failed to reproduce the specificity of lesion for substantia nigra, probably owing to ubiquitous complex I inhibition induced by the systemic administration of rotenone, which would not be selectively concentrated in dopaminergic neurons as MPP+.[25] Other discordant results have been published challenging complex I inhibition as the central mechanism of action for MPP+, rotenone, and paraquat.[26]

Notwithstanding these controversies, epidemiological investigations on pesticides continue,[27,28] supporting the view that a subgroup of idiopathic PD patients may have had a chronic exposure to toxins, most of them being complex I inhibitors (Fig. 1).[29] The list of these potentially harmful compounds is remarkably long[30] and also includes commonly used drugs such as neuroleptics.[31] Their role in triggering parkinsonism is well known and ascribed to a blockade of the dopamine D2 receptors in the nigrostriatal pathway,[32] even if the possible biochemical inhibition of complex I has not yet been fully explored.[31]

Complex I Defect in Idiopathic PD

About a year after the seminal discovery of causative mtDNA mutations in human diseases,[33,34] many reports described complex I deficiency or a wider impairment of respiratory complexes in different tissues from PD patients.[35-44] Depending on the tissue investigated and the biochemical assay used, contrasting results were reported. One controversy was centered on the tissue specificity of complex I deficiency, which according to Mann and colleagues[39] was limited to the substantia nigra, whereas other studies supported a systemic defect of complex I, recognizable in muscle biopsies[36,38,41,44] or circulating platelets.[37,42] However, other authors failed to recognize defective complex I in muscle[39,40] or circulating blood cells.[39,43] Another question was whether the enzymatic defect was specific to complex I[35,39] or more widespread, extending to complex IV or other complexes.[36,41,42,44] Further issues regarded the biochemical assays methodology, the sensitivity of enzymatic activities to postmortem time and/or tissue conservation, the confounding effect of age-related decline of mitochondrial efficiency, and the possible influence of therapy or cigarette smoking.[39,42,44] Immunoblotting and immuohistochemical studies of postmortem substantia nigra pointed to the specific deficiency of complex I subunits in PD patients.[45,46]

The need to link the biochemical findings in different tissues with the upcoming molecular investigation of mtDNA at single cell level was envisaged by DiMauro in 1993.[47] Overall, it is currently accepted that a partial complex I deficiency affects a subset of patients with idiopathic PD (Fig. 1). The hypothesis

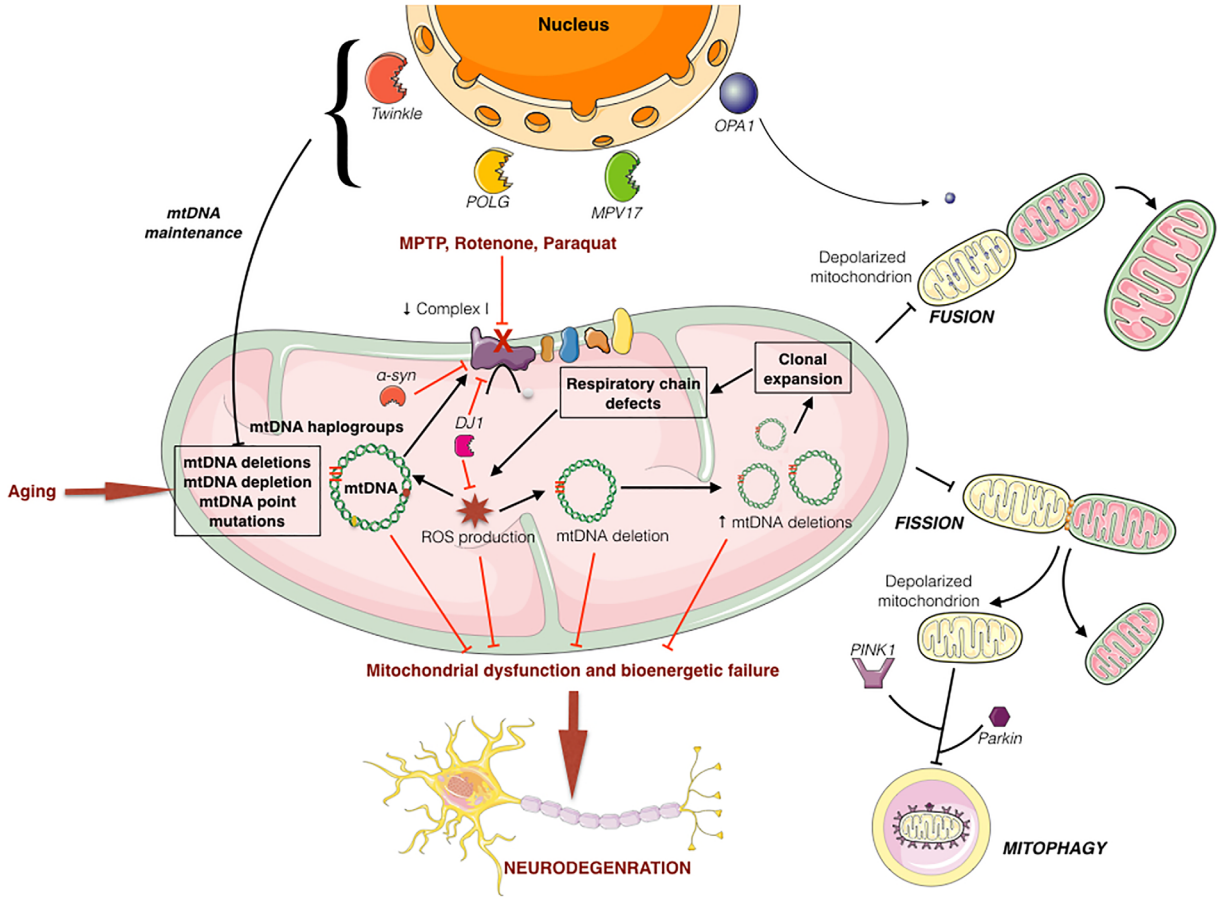

FIG. 1. Mitochondrial DNA and Parkinson's disease. The mitochondrial genome may be affected by somatic age-related accumulation of genetic errors, or secondarily to nuclear DNA (nDNA) mutations in genes involved in mitochondrial DNA (mtDNA) maintenance (POLG, Twinkle, MPV17, OPA1). The haplotype-dependent mtDNA genetic variation can influence longevity and predispose to or protect from neurodegeneration. These mtDNA alterations, along with exotoxins, impact on respiratory function determining ATP production failure and increased ROS production, which may contribute to further mtDNA damage. Also, mutations in PD-related genes, that is, DJ1 and α-synuclein, could contribute to reactive oxygen species accumulation and complex I inhibition. The burden of mtDNA errors, clonal expansion of mutant mtDNA, and mtDNA depletion may lead single neurons to energetic failure and degeneration. However, the damaged mitochondria can be rescued by fusion with normal mitochondria or targeted to mitophagy by fission. Impaired fusion (as a result of OPA1 mutations) or impaired mitophagy (as a result of PINK1/Parkin mutations) promote neurodegeneration. ROS, reactive oxygen species.

was hence raised that not only environmental exposure to complex I inhibitors but also genetic predisposition could have a role in the pathogenic mechanism of PD.[48]

Complex I Defect Is Transferred Into Cybrids: mtDNA Becomes the Candidate

Complex I transfers electrons from NADH to CoQ, thereby generating ubiquinol ($CoQH_2$), which then shuttles 2 electrons to complex III (ubiquinol:ferricytochrome *c* oxidoreductase, cytochrome bc_1 complex).[49] Complex I contributes to energy conservation by coupling the electron transfer to CoQ with proton translocation across the mitochondrial inner membrane, thus charging the membrane potential.[50] Complex I also contributes to reactive oxygen species (ROS) production, generating the superoxide anion.[51] The mammalian complex I architecture has been recently elucidated, and it results from the assembly of 45 subunits, 7 of which are mtDNA encoded, the remaining being encoded by nuclear DNA (nDNA).[52] It is now established that complex I assembles into supercomplexes with complex III and IV, constituting the "respirasome."[53] Thus, a new global hypothesis of how respiratory chain functions is proposed, with particular reference to electrons channeling through the alternative pathways of complex I and II.[54]

The double genetic determination of complex I subunits raised the question if the genetic contribution to complex I impairment in PD belonged to nDNA or mtDNA. To tackle this issue, a cell model was implemented based on the transfer of the cytoplasmic mitochondrial organelles from enucleated cells (from patients or controls) into a hosting immortalized cell line (osteosarcoma-derived 143B.TK-) devoid of its original mtDNA (rho^0 cells) and with constant nDNA.[55] The result of this cellular fusion is the transmitochondrial cytoplasmic hybrid or "cybrid."[55] Any biochemical defect occurring in the original cell line if

linked to mtDNA is transferred into cybrids, but if determined by nDNA is complemented by the cybrid nucleus. Cybrids have been useful for investigating the mtDNA pathogenic mutations associated with mitochondrial diseases.[56-58] Cybrid studies to dissect complex I deficiency in PD were subsequently performed by several research groups in the United States[59,60] and the United Kingdom.[61] These studies consistently showed that the complex I defect is transferred into PD-derived cybrids, increases ROS production, induces mitochondrial depolarization and reduces ATP, and enhances sensitivity to MPP + and other toxins involved in PD, enhancing cell propensity to undergo apoptosis.[62,63] Notably, a few of these studies documented the formation of α-synuclein aggregates reminiscent of LB in the PD-derived cybrids.[64,65] However, several methodological concerns have been raised by experienced researchers in the field about these cybrid studies when applied to neurodegenerative disorders such as Alzheimer's disease (AD) and PD.[66] Although all PD-derived cybrid results pointed to mtDNA involvement,[60,61] it remained problematic that none of these studies provided the sequence analysis of the cybrid mitochondrial genome. Thus, there was an overall lack of direct evidence that complex I defect assessed in patient's tissues (in most cases platelets used to generate cybrids) and transferred into cybrids was truly a result of defective mtDNA. Notwithstanding these controversies, mtDNA remained the most natural candidate to investigate.

Mitochondrial DNA in PD

In the early 1990s, mtDNA mutations were first associated with different sporadic or maternally inherited neuromuscular disorders.[33,34,67-69] A new class of mtDNA-based diseases, segregating in a Mendelian fashion, was further discovered. These disorders were characterized by either the accumulation of multiple mtDNA deletions in postmitotic tissues[70] or by tissue-specific mtDNA depletion,[71] and a genetic defect affecting nuclear genes involved in mtDNA replication and maintenance was postulated to be the cause.[72] Thus, mtDNA gained attention as the primary candidate for mutations possibly causing complex I defects in PD patients[48] and, over the years, there have been 3 different areas of investigations including mtDNA sequence analysis, the assessment of mtDNA rearrangements (single and/or multiple deletions), and mtDNA copy number.

Are There mtDNA Pathogenic Mutations Specific for PD?

mtDNA sequence analysis has discovered an increasing number of mutations associated with diverse clinical phenotypes.[73] However, specific PD-linked mutations have not been found, and parkinsonian features were only occasionally observed in mitochondrial disorders.[74,75] For example, a serendipitous identification of the common Leber's hereditary optic neuropathy (LHON) mutation m.11778G>A/MT-*ND4* was associated with a maternally inherited multisystem neurodegenerative disease including parkinsonism, but without optic atrophy.[76] Despite the fact that cybrid investigations pointed to mtDNA, only a few studies aimed at sequencing partially[77-79] or completely[80-82] the mtDNA in PD patients. Some studies focused on DNA derived from postmortem striatum, whereas others analyzed the DNA extracted from peripheral tissues, frequently platelets from blood. Among the numerous variants observed, the m.4336A>G/MT-tRNA^{Gln} was suggested to be associated with both AD and PD,[81,83] but this was not confirmed.[79] Many authors focused on mtDNA variants, subsequently defined as specific to mtDNA haplotypes, as possibly relevant in predisposing or protecting from PD.[77-80]

Are mtDNA Haplogroups Relevant for PD?

Since the early 1990s, Ozawa and colleagues[84,85] proposed that PD patients could be characterized by distinct clustering of mtDNA variants. van der Walt and colleagues[86] published the first systematic investigation in a sufficiently powered cohort in 2003, finding that haplogroups J and K, harboring the common 10398G variant in the ND3 subunit of complex I, had a significantly reduced risk of developing PD than the most common haplogroup H in European populations. Other studies on geographically distinct cohorts also associated a lower risk to haplogroups K,[87] UK,[88] or UKJT,[89] and an increased risk to haplogroup H. Recently, a 2-stage association study followed by a meta-analysis confirmed that haplogroups J, K, and T are associated with a reduced risk of PD, whereas the super-haplogroup HV has an increased risk of PD.[90] Interestingly, the super-haplogroup HV also increases survival after sepsis,[91] prompting the authors to speculate that mtDNA haplogroups may exert antagonistic pleiotropic effects impinging on predisposition to age-dependent neurodegenerative diseases.

The most relevant risk factor for PD remains age,[1-3] and increased longevity is paralleled by an increased incidence of PD.[92] Interestingly, haplogroup J presents the apparent paradox of being associated with longevity in some populations,[93-95] but it is also solidly established to increase penetrance in a mitochondrial neurodegenerative disorder such as LHON, characterized by mtDNA mutations impairing complex I function.[96,97] One interpretation for this paradox is that the genetic variants defining some specific haplogroup J subbranches may lower the energetic efficiency by a slightly uncoupled respiration.[98] This, in turn, could reduce

ROS production promoting longevity, but if a pathogenic mutation arises on the haplogroup J background, such as LHON mutations, their pathogenic effect is enhanced as confirmed in cybrids,[58] therefore increasing disease penetrance. Haplogroup J, which branches with haplogroup T from a shared phylogenetic root, is in fact characterized by nonsynonymous variants affecting amino acids in complexes I and III.[96,97,99]

Functional studies on control cybrids harboring mtDNA with different haplogroups showed that cybrids carrying haplogroups UK and J present with lower mtDNA copy number, oxygen consumption, and ATP levels when compared with haplogroup H cybrids.[100,101] This certainly fits the effect of haplogroup J on LHON penetrance and points to the co-occurrence of nonsynonymous variants in complexes I and III.[96,97,99] In fact, increased penetrance for LHON is associated with the specific sub-branches J1c and J2b, characterized by the m.14798T>C and m.15257G>A variants, respectively.[96,99] Interestingly, the m.14798T>C variant (J1c) is also shared by haplogroup K, which emerged as a protective background for PD.[87-90] However, there is currently no major insight on the possible branches in haplogroups U, and in particular K, that may be responsible for the protective effect on PD.

Overall, we emphasize 2 main considerations. First, these results have been frequently contradictory because of 2 different methodological approaches. In 1 approach, the aggregation into super-haplogroups valued most the ancient polymorphisms fixed by selection, whereas the highest definition reached by complete mtDNA sequence analysis took into consideration all the recent and even private variants.[102] Second, all results obtained from haplogroup studies should undergo rigorous functional validation, as partially provided by the few cybrid studies that have been recently undertaken.[100,101] The functional investigations by multiple metrics assessing mitochondrial function and homeostatic regulation (mtDNA copy number, cell respiration, ROS production, etc.) are relevant for the correct interpretation of these results. For example, similar mitochondrial respirations may be maintained by different homeostatic settings of different haplogroups[103,104] based on differential ROS production that leads to different efficiencies in mitochondrial biogenesis.[104] This becomes important for predisposition to neurodegenerative disease, such as PD, as well as for longevity itself (Fig. 1). However, during aging, the mtDNA haplogroup differences may be overshadowed by the accumulation of somatic mutations, in particular of deletions,[105] affecting mtDNA.

mtDNA Maintenance: Age-Dependent and nDNA-Driven Multiple mtDNA Deletions

After the seminal discovery that single heteroplasmic mtDNA deletions can cause both mitochondrial myopathy[33] and a more severe multisystem disorder known as Kearn-Sayres syndrome,[106] many research groups searched for[107,108] and some described the occurrence of mtDNA deletions[109,110] in various brain areas of PD patients. The issue of deletion amount and the techniques used to detect them became immediately evident. In fact, those studies employing southern blot analysis failed to recognize mtDNA deletions in the postmortem substantia nigra and frontal cortex.[107,108] Instead, the use of the newly introduced PCR technique, designed to amplify and detect only the "common" mtDNA deletion of 4,977-bp already observed in patients with mitochondrial myopathy and Kearn-Sayres syndrome,[33,106] highlighted its occurrence in the striatum. Thus, the failure of detection by southern blot,[107,108] as opposed to the PCR-based approach,[109,110] pointed to the very low amount of mtDNA-deleted molecules. These latter studies also noted that this mtDNA deletion was recognized in both PD patients and age-matched controls, found in higher amounts in PD patients.[109] Furthermore, when the striatum was compared with the cerebral cortex, the amount of mtDNA-deleted molecules was far more abundant in the striatum.[109] These observations were consolidated by a subsequent study of normal adult brains, showing that 3 regions with high dopamine metabolism—caudate, putamen, and substantia nigra—had the highest levels of deleted mtDNA molecules.[111] The use of more sophisticated PCR protocols (in situ and long-extension PCR) showed that in the substantia nigra, and other brain regions, a mixture of mtDNA deletions of different sizes was accumulating with age, being higher in PD patients[112] but also characterizing normal individuals (Fig. 1).[113]

Recently, the landscape of age-related accumulation of mtDNA defects has been redefined at the single cell level. This became possible thanks to the availability of the laser-capturing technique for the molecular investigation of single cells in postmortem tissues. Thus, 2 studies confirmed that a mixture of multiple mtDNA deletions was accumulating with age in the substantia nigra from normal people,[114] being significantly more abundant in PD patients, however.[115] Remarkably, as predicted by DiMauro,[47] the single-cell analysis showed that clonal expansion of a single mtDNA deletion was prevalent in isolated COX-negative dopaminergic neurons,[114,115] paralleling the same finding in isolated COX-negative muscle fibers from patients with mitochondrial myopathy.[116] This established a direct link in single neurons between the mtDNA damage and the functional mitochondrial impairment (Fig. 1).

The pathologic accumulation of multiple mtDNA deletions also characterizes the still expanding category of mitochondrial diseases determined by mutations in nuclear genes involved in mtDNA replication and

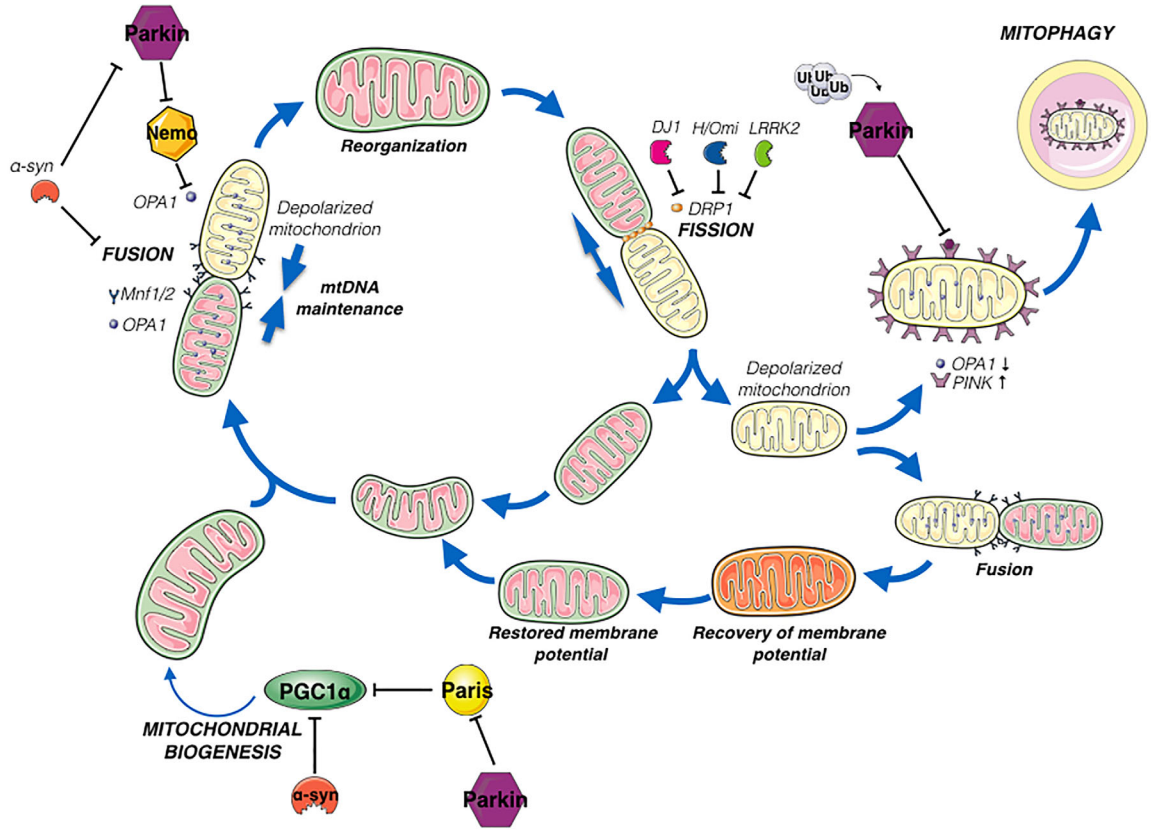

FIG. 2. The life cycle of mitochondria and Parkinson's disease. Mitochondria are a dynamic network of organelles undergoing continuous cycles of fusion and fission, with a balance between mitochondrial biogenesis and mitophagic elimination of dysfunctional organelles after their fission. Although mitochondrial biogenesis may be regulated by Parkin, through Paris and PGC-1α, the loss of membrane potential may be recovered after fusion promoted by Mitofusins and OPA1, the latter also regulated by Parkin through Nemo. Thus, mitochondria can re-enter the life cycle. Alternatively, a loss of membrane potential may be segregated by fission, mediated by Drp1, to isolate damaged components for elimination by mitophagy. Under conditions of low membrane potential, PINK1 accumulates on the outer membrane, activating a complex signaling cascade that includes the recruitment of Parkin and ubiquitination of various mitochondrial proteins, ultimately targeting the damaged mitochondria for mitophagy. Other PD-related genes may participate with the maintenance of the delicate balance between mitochondrial fusion and fission and ultimately mitochondrial homeostasis and function. Indeed, α-synuclein can increase mitochondrial fragmentation by inhibiting fusion, whereas DJ1, OMI/HtrA2, and leucine-rich repeat kinase 2 (LRRK2) regulate fission acting on Drp1. Furthermore, α-synuclein may also modulate mitochondrial biogenesis by directly interacting with PGC-1α. mtDNA, mitochondrial DNA.

maintenance,[117] as initially proposed by Zeviani.[70,72] Interestingly, there have been premolecular descriptions of patients with mitochondrial myopathy and chronic progressive external ophthalmoplegia (CPEO), manifesting parkinsonian features.[118-121] In the early 2000s, 3 genes—ANT1 (*SLC25A4*), Twinkle (PEO1/*C10orf2*), and mitochondrial DNA polymerase γ (*POLG*)—were discovered to cause these mitochondrial phenotypes.[122-124] A report subsequently described patients affected by CPEO, parkinsonism, and premature menopause in women, carrying dominant and recessive mutations in the *POLG* gene.[125] This study highlighted that parkinsonism could be a frequent manifestation of genetically disordered mtDNA maintenance. Many other reports on CPEO/parkinsonism followed,[126-130] including other genes involved in mtDNA maintenance such as *C10orf2*,[131-133] *SLC25A4*,[134] and *MPV17*[135] (Fig. 1). Interestingly, the postmortem examination of 2 individuals from the same family carrying a dominant *POLG* mutation (Family S in reference 125) revealed a severe loss of pigmented neurons in the substantia nigra and no LB in 1 case,[136] whereas in the other there was also the hallmark pathology of AD with neuritic plaques and neurofibrillary tangles.[137] This second case was coincidentally homozygous for the *APOE* epsilon 4 allele. A further compound heterozygous *POLG* case suffering with parkinsonism and cognitive decline showed LB pathology and minimal Alzheimer-type pathology.[138] The COX/SDH combined histochemistry revealed more than 20% of COX negative-spared neurons in the substantia nigra, and single-cell analysis confirmed high levels of multiple mtDNA deletions. These findings reinforced the similarities with studies on idiopatic PD patients.[114,115] Remarkably, in a large study from Norvegia, *POLG* encephalopathic patients without clinical signs of parkinsonism have been investigated by DAT imaging and fluorine-18 fluorodeoxyglucose positron emission tomography, showing severe nigral neuronal loss and nigrostriatal depletion.[139] The postmortem investigation in 6 of these patients

documented complex I deficiency in dopaminergic neurons, with a combination of mtDNA depletion and high levels of multiple mtDNA deletions.[139] Concordantly, DAT imaging in a cohort of patients with mixed mitochondrial diseases confirmed that nigrostriatal degeneration occurred exclusively in patients with defective mtDNA maintenance carrying *POLG* or *C10orf2* mutations.[140] The relatively frequent occurrence of parkinsonism associated with *POLG* mutations prompted the investigation of this gene for possible variants predisposing to idiopathic PD. In particular, a trinucleotide CAG repeat in exon 2 of *POLG*, encoding a polyglutamine tract and previously associated with male infertility,[141] was investigated yielding conflicting results.[142-151]

In 2008, patients combining dominant optic atrophy with CPEO and multiple mtDNA deletions were found to harbor heterozygous mutations in the *OPA1* gene,[152,153] a key factor involved in mitochondrial fusion and dynamics,[154] (Fig. 2), but not yet formally involved in mtDNA replication. This observation widened the spectrum of mitochondrial diseases characterized by disturbed mtDNA maintenance,[155] highlighting that mitochondrial dynamics and life cycle[156] are crucial to the preservation of mitochondrial homeostasis, and thus provided a novel mechanism for the pathologic accumulation of mtDNA deletions in postmitotic tissues.[157] This concept was recently expanded to other genes involved in mitochondrial dynamics, such as *MFN2*,[158] *DRP1*,[159] *AFG3L2*[160] and *SPG7*,[161] mutations of which may lead to deficient mtDNA maintenance. We recently reported 2 families segregating a heterozygous dominant *OPA1* mutation associated with syndromic CPEO, parkinsonism and dementia, and abnormally increased autophagy and mitophagy (Fig. 1).[162] This report established a link with the developing field of genetic forms of PD, many of which are tightly implicated with mitochondrial quality control and homeostasis (Table 1).[163]

Monogenic PD: The Mitochondrial Perspective

The identification of PD genes[163,164] has prompted an extraordinary wave of studies demonstrating that mitochondrial dysfunction is central to PD pathogenesis. The detailed analysis of PD genetics is beyond the scope of this review, so we limit our discussion to how this relates to mtDNA (Table 1). To this end, the 2 key PD genes are parkin *(PARK2)* and *PINK1* encoding for Parkin[165] and PINK1,[166] respectively, an E3 ubiquitin ligase[167] and a PTEN-induced serine/threonine kinase 1.[166] Although PINK1 has been recognized to target mitochondria since its discovery,[166] Parkin cellular localization did not apparently target mitochondria.[168] The understanding of Parkin function focused initially on its interaction with α-synuclein in the formation of LB.[169,170] In fact, Parkin was recognized as 1 of the protein components of LB,[171] and patients with *PARK2* mutations apparently did not have LB deposition in their brains.[8] However, studies on *PARK2* mutant animal models also provided evidence of mitochondrial impairment.[172,173] In particular, Parkin-deficient Drosophila was characterized by male sterility and both flight muscle and dopaminergic neuronal degeneration.[172] The identification of mutations in *PINK1* as causative for recessive PD led to a turning point. It became clear that PINK1-deficient Drosophila had a virtually identical phenotype as Parkin deficient and that Parkin could rescue the PINK1-deficient fly, but not the opposite, thus linking the 2 proteins in the same pathway, with PINK1 being upstream of Parkin.[174-176] This was also confirmed in HeLa cells with silenced *PINK1* and patient-derived fibroblasts carrying *PINK1* mutations, which displayed fragmented mitochondrial network and altered membrane potential that could be rescued by either wild-type PINK1 or Parkin, but not by mutant PINK1 or DJ1,[177] the latter being another PD-associated gene product implicated with mitochondrial function (Table 1).[178] Altered mitochondrial dynamics consequent to both *PINK1* and *PARK2* mutations was also reported in the Drosophila models.[179-181] Further studies in mammalian cells showed that excessive fission as a result of either Parkin or PINK1 loss could be counteracted by the mitochondrial fusion proteins Mfn2 and OPA1 or by a dominant negative mutant of the fission protein Drp1.[182] Thus, mitochondrial dynamics came prominently into play, and a few reports pointed to mitochondrial fission as a powerful promoter of mitophagy (Fig. 2).[183,184] This process was mediated by the mitochondrial recruitment of Parkin from the cytoplasm, as highlighted by challenging cells with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone,[183] and was observed in *PINK1*-silenced cells.[184] Finally in 2010, the 2 proteins were locked by Narendra and colleagues[185] into the same mechanism showing that PINK1 senses mitochondrial dysfunction/depolarization and becomes stabilized on impaired mitochondria to then recruit Parkin for mitophagic targeting of these dysfunctional organelles. Others confirmed this paradigm, highlighting the quality control of mitochondria as the key pathway through which the PINK1/Parkin axis operates, 1 protein upstream of the other.[186] The cascade of events leading to Parkin recruitment on mitochondria and mitophagy activation has been greatly refined, being very complex, and we refer to specific reviews for this topic.[187-189] A direct consequence of the mitochondrial quality control on the accumulation of mtDNA mutations was shown *in*

TABLE 1. Nuclear mitochondrial genes associated with parkinsonism and PD-associated genes with a mitochondrial-related function

Gene	Protein	Transmission	Subcellular localization	Effects of mutation on mitochondria	Clinical phenotype	Neuropathology	Reference
Nuclear mitochondrial genes associated with parkinsonism							
POLG	DNA polymerase subunit gamma-1	AD/AR	Mitochondrial matrix/nucleoids	Impaired mtDNA replication, multiple mtDNA deletions	Variable phenotype reported: parkinsonism plus CPEO, ataxia, neuropathy, hearing loss	Variably reported: LB pathology; severe loss of pigmented neurons in SN without LBs; AD pathology	125-130,136-139, 252,253
PEO1/C10orf2	Twinkle (mtDNA helicase)	AD	Mitochondrial matrix/nucleoids	Impaired mtDNA replication, multiple mtDNA deletions	Parkinsonism plus CPEO	Not available	131-133
MPV17	Protein Mpv17	AR	Mitochondria	Impaired mtDNA replication, multiple mtDNA deletions	Parkinsonism plus CPEO, mitochondrial myopathy, sensorineural deafness, peripheral neuropathy, depression	Not available	135
OPA1	Mitochondrial dynamin-like GTPase	AD	Mitochondrial inner membrane and intermembrane space	Impaired mitochondrial fusion, multiple mtDNA deletions	Parkinsonism plus CPEO, mitochondrial myopathy, sensorineural deafness, peripheral neuropathy, and/or cognitive impairment	Not available	162
PD-associated genes with a mitochondrial related function							
SNCA	Alpha-synuclein	AD	Mitochondria-associated ER membranes, inner mitochondrial membrane	Binding to mitochondrial membranes causes mitochondrial fragmentation; reduced complex I activity; abnormalities in mitochondrial morphology; interaction with the voltage-dependent anion channel; impaired mitochondrial protein import	Early-onset PD. Frequently dementia and autonomic dysfunction in the course of disease	Nigral neuronal loss, cortical and brain stem Lewy bodies	8,163,234-240, 254-257
LRRK2	Dardarin	AD	10% located in outer mitochondrial membrane	Mitochondria-dependent programmed cell death through the release of cytochrome c; altered mitochondrial dynamics and increased fragmentation	Classical PD	Variable: Lewy body disease; nigral degeneration (nonspecific or with ubiquitin positive inclusions); tau or Alzheimer pathology	8,221,222,224, 258,259

(Continued)

TABLE 1. *Continued*

Gene	Protein	Transmission	Subcellular localization	Effects of mutation on mitochondria	Clinical phenotype	Neuropathology	Reference
PINK1	PTEN induced putative kinase 1	AR	Outer and inner mitochondrial membrane and cytosol	Increased mtc fragmentation; increment of mitochondrial Ca^{2+}, increased production of mitochondrial ROS, reduced complex I activity, decreased mitochondrial respiration, and a lowered threshold for Ca^{2+}-dependent opening of the mitochondrial permeability transition pore complex, overall resulting in increased apoptosis	Early-onset or, rarely, juvenile onset PD. Dystonia (often of the lower limbs), either as a presenting sign or occurring during disease progression can be present	Nigral neuronal loss, Lewy bodies and aberrant neurites in the reticular nuclei of the brain stem, substantia nigra pars compacta and Meynert nucleus	8,166,177,179-182, 184,197-201,260-262
PARK2	Parkin	AR	Cytosol; recruited to the mitochondrial outer membrane	Increased mitochondrial fragmentation, along with a decrease in the mitochondrial membrane potential and ATP production; reduced complex I activity	Juvenile onset and early-onset onset PD. Lower limb dystonia, which may be a presenting sign or occurs during disease progression	Variable (based on different mutation): nigral neuronal loss without Lewy bodies; alpha-synuclein positive inclusions in the pedunculopontine nucleus; cortical Lewy bodies, none in the brain stem but occasional Lewy neurites in the dorsal nucleus of vagus; Lewy bodies in the locus ceruleus and substantia nigra	8,172,173,179,180, 182,202-204,262
PARK7	DJ1	AR	Cytosol and mitochondrial matrix and intermembrane space	Increased mitochondrial ROS production; increased mitochondrial fragmentation; reduced complex I activity	Early-onset or juvenile onset PD	No data available	178,214-217,238
HTRA2	Serine protease HTRA2	AR	Mitochondria, intermembrane space	Mitochondrial swelling, reduced membrane potential; altered mitochondrial morphology ?	PD/ET	No data available	226-231,263

CPEO, chronic progressive external ophthalmoplegia; lb, Lewy bodies; mtDNA, mitochondrial DNA; ROS, reactive oxygen species.

vitro by the inhibition of fission, which brought an increased tolerance to higher mtDNA mutation load,[190] whereas Parkin overexpression selected against mtDNA mutations.[191] Overall, the mitochondrial life cycle seems to be crucially involved in tolerance and complementation of mtDNA mutations through fusion[192] as well as in their selective elimination through fission and mitophagy (Fig. 2).[193]

Following the elucidation of the PINK1/Parkin axis in mitochondrial quality control, critiques were raised about the nonphysiological experimental conditions (carbonyl cyanide m-chlorophenylhydrazone) used *in vitro*,[194] and doubts were cast on the real *in vivo* occurrence of such dysfunctional quality control.[195] For both PINK1 and Parkin, many other functions and potential pathogenic pathways have been described.[188,196] For example, *PINK1*-mutant animals and cells were characterized by complex I deficiency,[197-199] the mitochondrial paradigm for PD. It was found that PINK1 could influence complex I function, as the NdufA10 subunit is phosphorylated in a PINK1-dependent manner.[200,201] Similarly, complex I deficiency was also evidenced in cells from *PARK2*-mutant patients as well as in animal models.[202-204] Remarkably, it has been reported that the PINK1/Parkin pathway promotes mitophagy with some degree of selectivity for turnover of membrane-bound subunits of respiratory chain complexes, complex I being the most represented.[205] Furthermore, a study investigating induced pluripotent stem cells derived from a mitochondrial encephalomyopathy, lactic acidosis, stroke-like syndrome patient carrying the common m.3243G>A/tRNA^{Leu} mtDNA mutation reported that upon neuronal differentiation, complex I was specifically sequestered into perinuclear PINK1/Parkin positive autophagosomes, suggesting its active degradation through mitophagy.[206] Thus, a direct link emerges between complex I deficiency and the PINK1/Parkin driven mitophagy. Parkin has been reported to have at least 36 outer mitochondrial membrane protein substrates that ubiquitinates upon activation in response to mitochondrial depolarization.[207] Of interest, Paris *(ZNF746)* has been reported to be a Parkin target and accumulates in models of Parkin inactivation and in human PD brains.[208] Paris is a repressor of the transcriptional coactivator PGC-1α, the master regulator of mitochondrial biogenesis and possibly mtDNA copy number.[209] Thus, Parkin is at the crossroad of the mitochondrial life cycle: by Paris ubiquitination, it may promote mitochondrial biogenesis[210] while regulating mitophagy through the PINK1-induced pathway (Fig. 2).[193] Similarly, *PINK1*-mutant Drosophila is also characterized by the upregulation of genes involved in nucleotide metabolism critical for mtDNA maintenance.[211] Another target that undergoes linear ubiquitination is the NF-kB essential modulator (NEMO), which, as part of the NF-kB signaling, upregulates *OPA1*.[212] OPA1 exerts many other functions besides the canonical role in mitochondrial fusion, including mtDNA maintenance and control of apoptosis,[154] and it has been shown to mediate dopaminergic neurodegeneration linked to MPP + induced complex I deficiency.[213] Overall, at least these 2 Parkin targets, Paris and NEMO, establish a link with mtDNA maintenance, mitochondrial biogenesis, and dynamics, with a central role for the complex I/OPA1 axis (Fig. 2).

We should also mention that other forms of monogenic PD relate to mitochondrial dysfunction (Table 1). *DJ-1* mutations are associated with autosomal recessive early-onset PD,[178] and DJ1 may be located both in the cytoplasm, where it senses ROS, and in the mitochondrial matrix and the intermembrane space.[214] DJ1 was recently identified as an atypical peroxiredoxin-like peroxidase able to scavenge H_2O_2.[215] Mutant *DJ1* induces mitochondrial network fragmentation by modulating Drp1 expression,[216] and suppression of *DJ1* expression has been linked to complex I deficiency.[217]

Dominant mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene are also associated with PD and a spectrum of other neurodegenerative disorders displaying variable histopathology.[218,219] LRRK2 has been implicated in regulating the α-synuclein homeostasis.[220] LRKK2 is mostly cytoplasmic, but a fraction is also associated with the outer mitochondrial membrane.[221] LRRK2 interacts with Drp1 regulating mitochondrial fission[222] and with Parkin.[223] *LRKK2* mutations have been reported to induce neurodegeneration in SH-SY5Y cells and primary neurons,[223] accompanied by mtDNA damage in the iPSC-derived neural cells from PD patients.[224]

OMI/HtrA2 is a serine protease targeted to the mitochondrial intermembrane space, where it exerts a proapoptotic function.[225] *OMI/HtrA2* variants have been controversially implicated as a susceptibility factor in PD,[226,227] but *OMI/HtrA2* suppression leads to parkinsonian features in animal models.[228] Silencing of *OMI/HtrA2* in cells results in mitochondrial dysfunction and hyperelongated network, which was attributed to a functional and physical interaction with OPA1.[229] Loss of OMI/HtrA2 also leads to mtDNA damage,[230] and its protease activity is regulated by PINK1.[231]

Finally, α-synuclein, the key protein deposited in LB,[4] was the first gene associated with dominant PD.[164] Either mutant α-synuclein or an increased expression of wild-type α-synuclein[232] may promote the pathological aggregation forming LB.[233] However, it has also been reported that both mutant and wild-type α-synuclein may bind to and enter within the mitochondria,[234,235] possibly through the voltage-

dependent anion channel,[236] ultimately affecting complex I function at the inner mitochondrial membrane.[237] The binding of α-synuclein to the outer mitochondrial membrane inhibits mitochondrial fusion in a fashion that is rescued by PINK1, Parkin, and DJ1,[238] and drives excessive fission and mitochondrial network fragmentation.[239] Finally, α-synuclein may be enriched at the mitochondrial-endoplasmic reticulum contact sites,[240] highlighting the overwhelming complexity of crossing and converging pathogenic pathways in PD.[163]

Breaking News on mtDNA Depletion and Conclusions

In 2016, a new investigation of mtDNA in the dopaminergic neurons[241] expanded on the previous results showing a prevalent deletion in single neurons on a background of multiple mtDNA deletions.[114,115] This new study focused on complex I deficiency and by combining a multiple-label immunofluorescence protocol and laser capture microdissection, it showed that complex I and complex II are most consistently affected in single neurons, which also displayed a reduced mtDNA copy number.[241] Occasionally, complex IV deficiency was also observed, but only in neurons that had already complex I deficiency, confirming a primary role for this biochemical defect. mtDNA copy number reduction has been confirmed by another study showing that an overall mtDNA depletion affects the substantia nigra, but not the frontal cortex.[242] Moreover, a significant reduction of mtDNA copy number was also found in the blood cells of PD patients when compared with controls.[242] In a parallel study, the same laboratory reported that PD patients have an increase in mtDNA mutational burden in, but not limited to, the substantia nigra when compared with controls.[243] Recently, a further study tackled the same issue by investigating mtDNA copy number, deletions, and point mutations in laser-captured single neurons from 3 brain areas, the dopaminergic neurons from the substantia nigra, pyramidal neurons from the frontal cortex, and Purkinje cells from the cerebellum.[244] Remarkably, the mtDNA copy number increased with age in the dopaminergic neurons of healthy controls, maintaining the pool of wild-type mtDNA despite accumulating deletions. Strikingly, this compensatory up-regulation was blunted in PD patients, resulting in relative mtDNA depletion unable to cope with the increasing occurrence of clonally expanded mtDNA deletions leading to the well-documented respiratory deficiency of these neurons. This did not apply to the frontal cortex or cerebellar neurons, and mtDNA point mutational loads did not differ either, and, in particular, did not significantly increase with age in dopaminergic neurons of PD patients, in contrast with other reports.[243,245]

These novel studies defined conclusively the pathological features of mtDNA in substantia nigra dopaminergic neurons in PD, revealing a loss of compensatory biogenesis in mitochondrial homeostasis. This is consistent with the evidence that PGC-1α plays a key protective role in PD by orchestrating the compensatory control of mtDNA copy number and mitochondrial biogenesis, which includes the coexpression of key ROS-detoxifying enzymes. In fact, in animal models the suppression of PGC-1α sensitizes the neurodegenerative effects of MPTP and other stressors, whereas the overexpression of PGC-1α exerts a protective role, qualifying as a potential therapeutic target in PD.[246,247] Remarkably, α-synuclein can also suppress the expression of PGC-1α under conditions of oxidative stress, further reinforcing the importance of protective mitochondrial biogenesis.[248]

This history, 3 decades long, of mtDNA and primary mitochondrial dysfunction in PD provides paramount evidence that mitochondrial function is vital to dopaminergic neurons and mitochondrial failure is the ultimate event leading to their degeneration. There is a "fil rouge" that connects complex I with mitochondrial dynamics and life cycle (mitobiogenesis and mitophagy) and with mtDNA maintenance. The common ground to all of these connections remains age, the most important risk factor for PD, and the related features of mtDNA. The slight differences in mtDNA haplogroup sequence and mitochondrial functionality, fixed by natural selection, impinge on both longevity and predisposition/protection for PD. Mitochondrial genome damage that somatically accumulates with age—including low heteroplasmy point mutations, multiple mtDNA deletions and their clonal expansion in single cells, and the mtDNA copy number set up—is a major contributor to the final functional failure that leads to the neurodegeneration of dopaminergic neurons. Although this seems now fairly accepted, we are still left with important areas of incomplete understanding. For example, do we really appreciate the intimate nature of complex I dysfunction in PD, in particular its molecular basis in relation to mtDNA? The last studies highlighted a multilayered contribution of mtDNA, with a new focus on mtDNA copy number control, which shifts our attention from mitophagy to mitobiogenesis, or their upstream balanced control.[241,242] Do we correctly weigh the elegant model of PINK1/Parkin-dependent mitophagy into the context of *in vivo* PD pathogenesis? The paradigm that the key factor is how efficiently dysfunctional organelles and mutant mtDNA are cleared by mitophagy is probably too simplistic. If mitochondrial quality control exerts such an efficient surveillance, this pathway should prevent the occurrence of primary mitochondrial diseases as a result of

heteroplasmic mtDNA mutations, whereas there is no evidence of *in vivo* negative selection against such mtDNA mutations in postmitotic tissues. We predict that studying mitophagy in neurons from patients with common mitochondrial diseases will be informative. Primary mitochondrial diseases can be considered the extreme far end of the spectrum that starts from age-related accumulation of mtDNA errors. Furthermore, how much does the current mitophagy paradigm fit the super-specialized functional architecture of dopaminergic neurons? Noticeably, the MitoPark mouse model with dopaminergic neuron-specific knockout of mitochondrial transcription factor A, needed for mtDNA replication, failed to confirm Parkin recruitment on the mtDNA-depleted mitochondria of degenerating dopaminergic neurons.[195] This model, characterized by mtDNA depletion, now becomes relevant in the light of recent findings in humans.[241,242,244] These authors also showed that the possible mechanism for neuronal degeneration is a "dying back" axonopathy as a result of an impaired supply of mitochondria to axons and synapses, with large aggregates of enlarged mitochondria engulfing the axon hillock.[195] Interestingly, another study using the Mutator mouse, characterized by the accumulation of multiple mtDNA deletions as a result of a proofreading mutation in *POLG*, reported the activation of compensatory mitochondrial biogenesis in dopaminergic neurons that exerts a neuroprotective effect, thus avoiding their neurodegeneration.[249] Remarkably, by crossing the Mutator mouse with the *Parkin* knock-out mouse, which does not display a loss of dopaminergic neurons, the resulting double mutant animal convincingly reproduces the PD pathology, highlighting both the protective role exerted by Parkin and the key role of accumulated mtDNA mutations.[250] These last 3 animal model studies demonstrate how the balance between mitochondrial biogenesis and mitophagy remains central to PD pathogenesis (Fig. 2). The final question is whether we should proceed with a more thorough "deep phenotyping" of patients with *PINK1* and *Parkin* mutations as well as with other genetic forms of PD to really understand the multisystem nature of mitochondrial dysfunction? To emphasize this point, it is standard for CPEO patients with ptosis to undergo muscle biopsy as a diagnostic procedure. This usually reveals the accumulation of multiple mtDNA deletions and/or a partial depletion, which in turn leads to the molecular identification of mutations in *POLG, C10orf2, MPV17*, or *OPA1*. Interestingly, there is 1 report of a patient with an early-onset parkinsonism developing over time ptosis who had muscle biopsy alterations along with 2 heterozygous *Parkin* mutations.[251] This might suggest that we are still missing components of the phenotypic expression of PD patients.

In conclusion, although the story is quickly evolving, it remains incomplete, and the puzzle needs more critical units to be filled in. Mitochondrial dysfunction is central to a continuum of clinical phenotypes, and parkinsonism may be just the expression of mitochondrial dysfunction in dopaminergic neurons. The increased understanding of PD pathogenesis casts hope for finally making available a true neuroprotective therapeutic strategy for PD that can be applied at early stages of the disease. ■

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