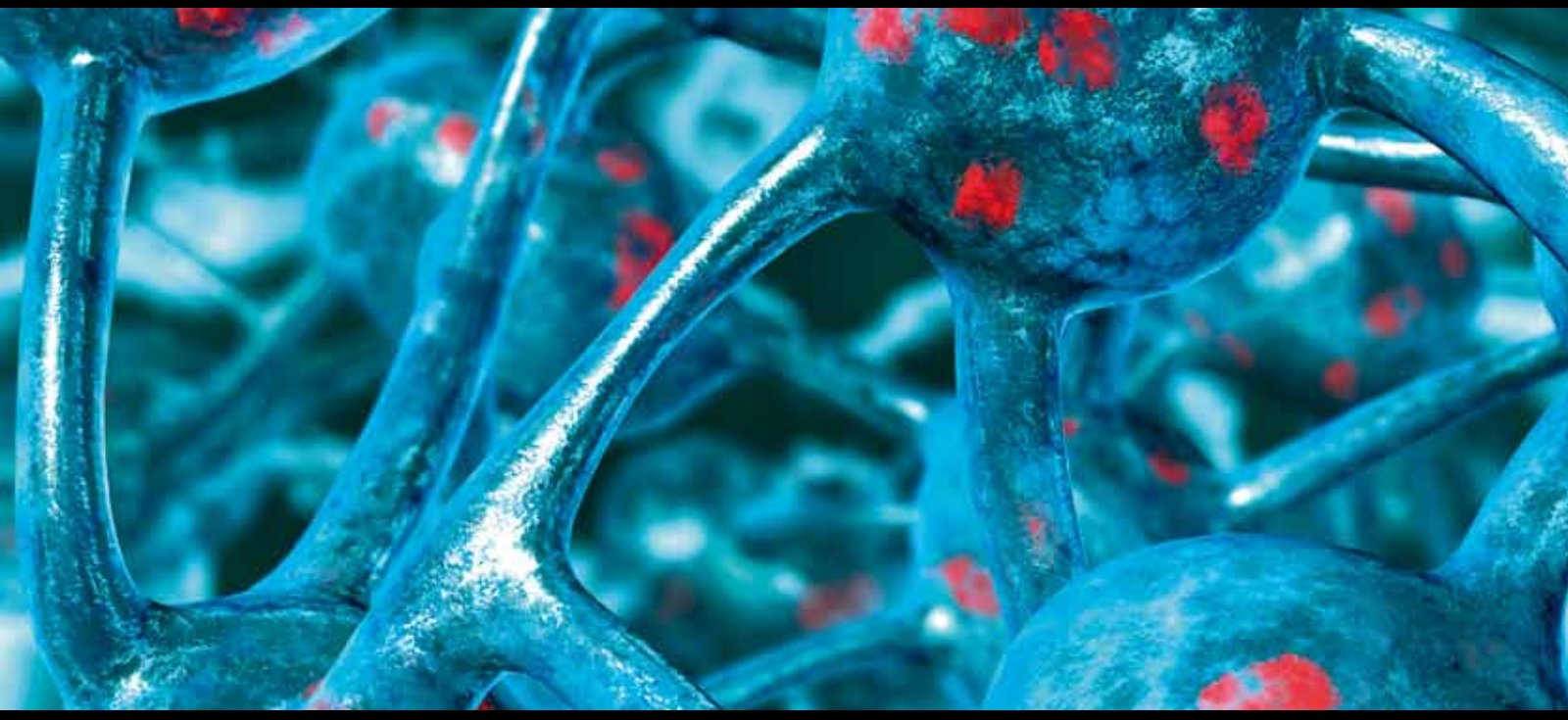
Mitochondria and Parkinson's Disease

Guest Editors: David K. Simon, Charleen T. Chu, and Russell H. Swerdlow



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Editorial

Mitochondria and Parkinson's Disease

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A large body of evidence implicates a central role for mitochondrial dysfunction in the pathogenesis of Parkinson's disease (PD), although the precise causes of mitochondrial dysfunction in PD remain to be determined. Mitochondrial complex I activity is impaired in the substantia nigra at early stages in PD. This complex I dysfunction appears not to be simply a consequence of neurodegeneration as toxins that inhibit mitochondrial complex I can reproduce many of the key pathological features of PD in animal models. Several genetic causes of PD have been linked directly or indirectly to mitochondrial function, and thus a convergence of data highlights the role of mitochondrial dysfunction in PD. This special issue now explores several key issues relating to the role of mitochondrial dysfunction in PD, potential causes and consequences of the dysfunction, and implications regarding neuroprotective strategies.

The first paper by A. H. V. Schapira and M. Gegg provides a brief but broad overview of mitochondrial dysfunction in PD. In addition to a discussion of parkinsonism associated with mutations in mitochondrial DNA (mtDNA) polymerase gamma (POLG), links to abnormal mitochondrial dysfunction are reported for several genetic causes of PD, including PINK1, Parkin, DJ-1, alpha-synuclein, and HtrA2. The role of PINK1, Parkin, and DJ-1 in the degradation of dysfunctional mitochondria (mitophagy) is discussed also.

The second paper by J. Clark, Y. Dai, and D. K. Simon reviews data regarding the potential contribution of mtDNA mutations to PD. This review briefly discusses evidence for mitochondrial dysfunction in PD, as well as data from cytoplasmic hybrid ("cybrid") cell lines that implicate a role for mtDNA mutations as a cause of the mitochondrial dysfunction. The paper then focuses primarily on data suggesting a role for somatic mtDNA mutations. The significance to PD of data from the POLG "mutator" mouse model of premature aging also is discussed, including the issue of point mutations versus large deletions.

The third article by C. T. Chu reviews the evidence for altered autophagy or mitophagy in the major genetic models of parkinsonian neurodegeneration, α-synuclein, leucine-rich repeat kinase 2 (LRRK2), Parkin, PTEN-induced kinase 1 (PINK1), and DJ-1. In recent years, autophagy dysregulation has been implicated widely in toxic/environmental and genetic approaches to studying PD pathogenesis. Diminished autolysosomal surveillance leads to alterations in mitochondrial oxygen utilization or calcium buffering. Interactions of two recessive PD-linked proteins, PINK1 and Parkin, contribute to a mechanism by which dissipation of the inner mitochondrial membrane potential triggers selective mitochondrial targeting for autophagy. These data are discussed in the context of emerging knowledge in the basic regulation of autophagy induction and cargo targeting, with discussion of neuron-specific mechanisms that may differ from those observed in other cell types.

The fourth article by A. Rakovic et al. is a research article in which tandem affinity purification and mass spectrometry were utilized to confirm interactions of PINK1 with HSP90 and CDC37 molecular chaperones, while highlighting 4 mitochondrially localized proteins that may interact with PINK1. Although no changes in protein expression were observed in control versus PINK1 mutant fibroblast lines, mutations in the *LRPPRC* gene encoding one of these proteins cause a form of Leigh syndrome, a cytochrome c oxidase

deficiency that results in childhood brain infarcts. Further studies of PINK1 regulation of mitochondrial function may well reveal functions beyond those linked to Parkin-regulated mitophagy.

The fifth paper by P. C. Keane et al. reviews mechanisms through which mitochondrial dysfunction may impair function and induce neurodegeneration in PD, as well as mediate the adverse consequences of gene mutations known to associate with Mendelian PD. Relationships between mitochondria and oxidative stress, cell calcium homeostasis, dopamine metabolism, and Lewy bodies are addressed. What we have learned over the years regarding PD-associated toxins, as well as mutations in genes such as Parkin, Pink1, alpha synuclein, and DJ1, are discussed from the perspective of mitochondria and cell bioenergetics. Overall, this review makes a compelling case for why mitochondria should be considered to play a key role in both PD etiology and physiology.

The sixth paper, a review by K. U. Tufekci et al., focuses on compensatory transcriptional responses to oxidative stress mediated by the Nrf2 transcription factor. Through the electrophile response element, Nrf2 participates in upregulation of antioxidant and anti-inflammatory genes and affects mitochondrial biogenesis. Interestingly, the survival-death outcome in injuries associated with increased mitophagy may be governed by whether or not there are concurrent deficiencies in mitochondrial biogenesis, as observed in a chronic culture model of MPP+ intoxication (Zhu et al., *Cell Death and Disease,* 2012 May 24; 3:e312. doi: 10.1038/cddis.2012.46). Given recent data implicating crosstalk between autophagy and Nrf2, Nrf2 may represent a promising therapeutic target addressing several mechanisms implicated in PD pathogenesis.

In the seventh paper, D. M. Arduíno et al. address the fact that, in addition to mitophagy, other aspects of mitochondrial dynamics have been implicated in PD models. These authors review recent studies implicating changes in mitochondrial fission and fusion, mitochondrial biogenesis, degradation, and transport along microtubules in the major genetic models of PD. A model whereby deficits in mitochondrial bioenergetics may underlie each of these changes is proposed, although several PD genes may also show direct effects on mitochondrial transport or mitochondrial fission.

The eighth paper by A. R. Esteves et al. provides a broad review of evidence for mitochondrial dysfunction in PD as well as potential causes and consequences of this dysfunction. The topics include oxidative stress, calcium homeostasis, the NAD+/NADH ratio, autophagy, and mitophagy. The "mitochondrial cascade" hypothesis is discussed in which each of these mechanisms ultimately contributes to alpha-synuclein aggregation followed by neuronal death.

Finally, the ninth paper is a review by R. B. Mounsey and P. Teismann that provides a historical overview of data implicating mitochondria in PD pathogenesis. This paper spans early data from idiopathic PD through consideration of the MPTP, rotenone and 6-hydroxydopamine models, to recent work exploring how genetic mutations influence mitochondrial activity. The preclinical data underlying several potential mitochondrially targeted therapies are discussed.

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Review Article

Mitochondrial Contribution to Parkinson's Disease Pathogenesis

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The identification of the etiologies and pathogenesis of Parkinson's disease (PD) should play an important role in enabling the development of novel treatment strategies to prevent or slow the progression of the disease. The last few years have seen enormous progress in this respect. Abnormalities of mitochondrial function and increased free radical mediated damage were described in post mortem PD brain before the first gene mutations causing familial PD were published. Several genetic causes are now known to induce loss of dopaminergic cells and parkinsonism, and study of the mechanisms by which these mutations produce this effect has provided important insights into the pathogenesis of PD and confirmed mitochondrial dysfunction and oxidative stress pathways as central to PD pathogenesis. Abnormalities of protein metabolism including protein mis-folding and aggregation are also crucial to the pathology of PD. Genetic causes of PD have specifically highlighted the importance of mitochondrial dysfunction to PD: PINK1, parkin, DJ-1 and most recently alpha-synuclein proteins have been shown to localise to mitochondria and influence function. The turnover of mitochondria by autophagy (mitophagy) has also become a focus of attention. This review summarises recent discoveries in the contribution of mitochondrial abnormalities to PD etiology and pathogenesis.

1. Introduction

Mitochondria are ubiquitous organelles, critical for cell survival and for correct cellular function [1]. Furthermore, they play an important role in mediating cell death by apoptosis and in determining their own destruction by mitophagy. Mitochondria are recognised to play an important role in neurodegenerative disorders. This may be a consequence of a primary mutation of mitochondrial DNA (mtDNA), for example, the *A3243G* mutation—a cause of myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), a mutation of a nuclear gene regulating mtDNA, for example, the mtDNA depletion syndromes, a nuclear gene encoding a mitochondrial protein, for example, frataxin in Friedreich's ataxia, secondary effects of disordered cell metabolism, for example, free radical stress, or environmental toxin exposure [2, 3]. This review will focus on the contribution of mitochondrial pathology to the pathogenesis of Parkinson's disease (PD), and it is notable that the mitochondrial involvement covers the entire etiological spectrum detailed above.

The first report of a mitochondrial defect in PD identified deficiency of complex I activity in substantia nigra compared to age-matched controls [4] and was followed by reports of mitochondrial defects in skeletal muscle, platelets, and lymphoblasts in a proportion of cases (see [5] for review). The mitochondrial deficiency within the brain appeared to be confined to the nigra [6, 7] although other reports have identified defects in the frontal cortex [8]. These mitochondrial abnormalities, identified in pathologically confirmed, apparently sporadic PD, were seen against a background of increased oxidative stress and elevated brain iron levels—and emphasised the importance of interconnecting pathways even at this early stage [9–14]. It was a fortuitous accident of timing that these observations of abnormal mitochondrial metabolism in PD were being made when important insights were gained into mitochondrial diseases by identification of mutations of mtDNA.

2. Mitochondrial Diseases and Parkinsonism

Primary mutations of mtDNA, as opposed to, for instance mutations secondary to a nuclear housekeeping gene, rarely manifest with parkinsonism [15, 16]. In part this may be a result of regional distribution of the mutation

with a relatively lower level in nigral cells (although this has never been investigated), or alternatively, related to better physiological compensatory mechanisms in the younger patient, that is, those that usually manifest with the encephalomyopathies. In any event, tissue specificity of an ubiquitously expressed mutation remains common in mitochondrial disorders and is poorly explained, but may in part be related to the dependence of a tissue on high energy demands, for example, brain and muscle. Inherited mtDNA-mediated defects of complex I usually manifest with encephalomyopathic features rather than parkinsonism [17, 18], as do other inherited primary specific respiratory chain defects, for example, affecting complex IV [19, 20].

Mutations of mtDNA polymerase gamma (POLG) are a recognised cause of parkinsonism, usually, but not always, preceded by ophthalmoplegia and are often associated with a peripheral neuropathy [21–23]. These cases have multiple deletions of mtDNA, sometimes with mtDNA depletion, and usually exhibit ragged red fibres in muscle biopsies. They have reduced dopamine transporter density by single photon emission tomography scanning, respond well to levodopa, and have Lewy bodies at postmortem. Patients with POLG mutations can also present with other phenotypes including childhood onset liver failure, myopathy, and renal disease [24, 25]. Mutations of POLG in sporadic PD are rare [26, 27].

Mutations of mtDNA may be inherited or somatic. Somatic mutations of mtDNA are known to develop with aging and are thought to represent cumulative damage due to excess exposure to free radicals [28]. The mitochondrial genome resides in the matrix, probably in close proximity to the inner mitochondrial membrane, a site of high superoxide ion production. Initial studies did not demonstrate any increase in deleted mtDNA genomes in pathologically proven PD [29]. However, quantitation of deleted mtDNA molecules in individual nigral neurons showed a significant rise with age [30], and this appeared to be increased in parkinsonian brains [31]. This may be the result of the enhanced oxidative stress in the nigra in these brains. Nevertheless, the neurons with the highest load of deleted mtDNA expressed a mitochondrial defect in the form of cytochrome oxidase deficiency, indicating that the deleted mtDNA population did have a functional effect [31]. Mitochondria have an important role in calcium homeostasis. Prominent calcium influx occurs in nigral dopaminergic neurons via L-type channels and is a phenomenon not shared by neighbouring dopaminergic neuronal populations, which are much less affected in PD [32]. In a *DJ-1* knockout mouse model this created oxidative stress and resulted in increased oxidation of mitochondrial proteins specific to vulnerable nigral dopaminergic neurons [33].

Although the potential contribution of mtDNA to respiratory chain deficiency in PD has received support from cybrid studies [34–37] no abnormality of this genome has been consistently identified in PD patients.

3. PARK Genes and Mitochondria

There remains a debate as to whether the parkinsonism caused by these genes is phenotypically equivalent to "idiopathic" PD or not. In many respects this is a sterile argument given the phenotypic spectrum in idiopathic PD itself. Furthermore, mutations of several of these genes have been identified in patients who satisfy the Queen Square Brain Bank criteria for PD. The real point is that these gene mutations cause dopaminergic nigrostriatal cell death. The proteins encoded by the *PINK1*, *parkin,* and *DJ-1* genes can translocate to mitochondria and influence function within that organelle, although this does not exclude additional activities in other cell compartments.

3.1. PINK1. Recessive mutations in *PINK1* (Park6) were found to be responsible for a familial form of early-onset parkinsonism, previously mapped to chromosome 1p36 [38]. PINK1 protein has a mitochondrial targeting sequence at its N-terminus and has been shown to have an intramitochondrial location, although in which compartment(s) remains uncertain. Several reports have demonstrated abnormal mitochondrial function in models of *PINK1* knockout and in patients with *PINK1* mutations including defective oxidative phosphorylation, increased free radical damage and reduced mitochondrial levels [39–45].

Several of the reported mutations of *PINK1* are located in the kinase domain [38, 46–48] and altered phosphorylation of target proteins probably represents a key pathogenic mechanism. The phosphorylation of mitochondrial proteins is considered pivotal to the regulation of respiratory activity in the cell and to signalling pathways leading to apoptosis, as well as for other vital mitochondrial processes. The generation of monoclonal antibodies to respiratory chain subunits [49, 50] has enabled the demonstration that a number of the subunits are phosphorylated, including several subunits of complex I [51–54].

3.2. Parkin. Parkin (Park2) gene mutations were first identified in autosomal recessive juvenile onset parkinsonism (ARJPD) [55]. Pathologically there is dopaminergic cell loss in the substantia nigra pars compacta and locus ceruleus, but Lewy bodies are rarely seen [56–58]. Patients carry deletions or point mutations in various parts of the *parkin* gene [59, 60]. The relevance of *parkin* mutations to idiopathic PD has been highlighted by the identification of *parkin* mutations in apparently sporadic cases of PD and by the description of Lewy bodies in *parkin* positive patients with later onset disease than ARJPD [61, 62].

Parkin protein functions as an E3 ligase, ubiquitinating proteins for destruction by the proteasome [63, 64] or lysosome [65]. *Parkin* knockout mice have decreased striatal mitochondrial respiratory chain function and reduced respiratory chain activity [66]. *Parkin* knockout flies developed muscle pathology, mitochondrial abnormalities, and apoptotic cell death [67]. Overexpression of parkin in PC12 cells indicated that it is associated with the mitochondrial outer membrane [68]. *Parkin* mutation positive patients have decreased lymphocyte complex I activity [69]. Fibroblasts from *parkin* mutation positive patients also exhibit decreased complex I activity and complex I-linked ATP production [70, 71].

3.3. DJ-1. Mutations of *DJ-1* are a rare cause of familial PD. This protein is located in the cytosol, nucleus, and mitochondria but under conditions of oxidative stress preferentially partitions to the mitochondrial matrix and intermembranous space to mediate a protective effect [72]. This protection may also be an effect of mRNA regulation and increased translation under conditions of oxidative stress [73–75]. *DJ1* knockout mice downregulated uncoupling proteins 4 and 5, impaired calcium-induced uncoupling and increased oxidant damage [76]. DJ-1 is thought to have a protective role in reducing protein misfolding and aggregation that may be a consequence of oxidative stress and so has been reported to reduce alpha-synuclein aggregation [77].

3.4. Alpha-Synuclein. Point mutations in the *alpha-synuclein* (Park1) gene [78, 79] and more recently multiplications of the wild-type gene have been described as causes of familial PD. A triplication of the gene was identified in a large autosomal dominant kindred with PD and tremor [80] and duplication of the gene was found in one of 42 familial probands of early onset PD [81]. A further alpha-*synuclein* point mutation (E46K) has been reported in an autosomal dominant family with parkinsonism and Lewy body dementia [82]. Alpha-synuclein is a major component of Lewy bodies in idiopathic, apparently sporadic PD [83].

Alpha-synuclein protein is predominantly cytosolic, but a fraction has been identified in mitochondria [84], appears to interact directly with mitochondrial membranes, including at the neuronal synapse [85], and to inhibit complex I in a dose dependent manner that reflects the brain regional expression of alpha-synuclein [86, 87]. Alpha-synuclein has also been shown to reduce ATP synthesis and mitochondrial membrane potential, although in one study alpha-synuclein did not affect respiratory chain activity or membrane potential [88, 89]. Mitochondrial abnormalities of structure and function have been observed in transgenic mice over-expressing mutant alpha-synuclein [90]. Alpha-synuclein undergoes an important posttranslational modification with phosphorylation at serine 129 [91], and it would be interesting to determine whether this might influence the effect of the protein on mitochondrial function.

4. Mitochondrial Dynamics and Mitophagy

Abnormal mitochondrial morphology and changes in mitochondrial dynamics have been reported for PINK1, parkin, DJ-1, and alpha-synuclein in a variety of cell and animal models [70, 89, 92–98]. These events could be due to direct effects on mitochondrial fission and fusion [89, 94, 97, 98], be secondary to deficiencies in oxidative phosphorylation [86], and/or be related to impaired mitochondrial turnover [99].

Recent studies have demonstrated that PINK1 together with parkin play a vital role in the turnover of mitochondria mitophagy [96, 98, 100, 101]. Parkin translocates from the cytosol to the mitochondrion in response to a fall in mitochondrial membrane potential [102]. Recent data suggest that this is preceded by phosphorylation of parkin by PINK1 [103]. Parkin translocation to depolarised mitochondria is abolished in *PINK1* knockout mouse embryonic fibroblasts (MEFS). Transfection of these MEFS with wild-type *PINK1* restored parkin translocation [104]. However, transfection of kinase-dead PINK1 could not restore mitophagy suggesting that PINK1 recruits parkin to mitochondria by a kinase pathway. Parkin and PINK1 involvement in mitophagy includes the ubiquitination of mitofusin 1 and 2 (mfn 1 and 2) by parkin [105, 106]. Recently DJ-1 has also been implicated in mitophagy [92, 107]. The increased oxidative stress as a result of DJ-1 deficiency has been suggested as a cause. Data also suggests that DJ-1 works in a parallel pathway to PINK1 and parkin [107, 108].

HtrA2 is a mitochondrial protease thought to be involved in the turnover of mitochondrial proteins. The phosphorylation of HtrA2 is dependent on PINK1, probably via a kinase cascade, rather than as a direct substrate [109]. Mutations in the *HtrA2* gene are a possible rare cause of PD [110, 111]. The mitochondrial chaperone TRAP1 has been shown to be a direct substrate of PINK1 [112]. These data suggest that PINK1 might be involved in the regulation of mitochondrial proteins as well as mitochondria as a whole.

Thus, quality control of mitochondria may play an important role in PD pathogenesis if the essential clearance of defective mitochondria is impaired and damaged mitochondria accumulate, utilising substrate and generating excess superoxide radicals. The recent description of reduced autophagy protein expression in PD nigra and amygdala may mirror defects of mitophagy [113]. Defective trafficking of mitochondria between cell compartments may be an additional consequence of impaired fission fusion and in turn may contribute to regional cellular dysfunction such as at the synapse.

5. Conclusion

Since the discovery of mitochondrial dysfunction in PD, a very large body of evidence has accrued to confirm that this organelle plays an important part in pathogenesis. Mitochondrial toxins have been used to induce dopaminergic cell death [114–116] and environmental exposure to toxins can increase the risk for parkinsonism [117]. The familial causes of parkinsonism/PD function in pathways that influence mitochondrial function directly or indirectly. This is not to aver that mitochondrial dysfunction is *the* cause of PD, but rather to suggest that it is a critical feature and one worthy of further investigation, particularly in relation to the development of interventions to modify the course of PD. Indeed, several studies have been performed using agents that influence mitochondrial function [118–120].

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Review Article

Do Somatic Mitochondrial DNA Mutations Contribute to Parkinson's Disease?

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A great deal of evidence supports a role for mitochondrial dysfunction in the pathogenesis of Parkinson's disease (PD), although the origin of the mitochondrial dysfunction in PD remains unclear. Expression of mitochondrial DNA (mtDNA) from PD patients in "cybrid" cell lines recapitulates the mitochondrial defect, implicating a role for mtDNA mutations, but the specific mutations responsible for the mitochondrial dysfunction in PD have been difficult to identify. Somatic mtDNA point mutations and deletions accumulate with age and reach high levels in substantia nigra (SN) neurons. Mutations in mitochondrial DNA polymerase γ (POLG) that lead to the accumulation of mtDNA mutations are associated with a premature aging phenotype in "mutator" mice, although overt parkinsonism has not been reported in these mice, and with parkinsonism in humans. Together these data support, but do not yet prove, the hypothesis that the accumulation of somatic mtDNA mutations in SN neurons contribute to the pathogenesis of PD.

1. Evidence for Mitochondrial Dysfunction in PD

A large body of evidence suggests that mitochondrial dysfunction is a common pathological mechanism of neurodegenerative diseases including PD. There is a significant decrease in complex I activity of the mitochondrial electron transport chain and reduced immunohistochemical staining for complex I subunits in the substantia nigra (SN) from PD patients [1–4]. A recent meta-analysis of genome-wide expression data from SN neurons of patients with symptomatic and subclinical PD identified defects in mitochondrial electron transport, glucose utilization and sensing [5]. One key set of genes found in this study to be underexpressed in the brains of PD patients is under the control of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a master regulator of mitochondrial biogenesis. Moreover, functional imaging like PET and functional magnetic resonance imaging (fMRI) show glucose metabolic defect in the frontal cortex in PD patients [6, 7]. Together, these and other data provide clear evidence for a defect in mitochondrial metabolism in PD.

2. Mitochondrial Dysfunction: Chicken or Egg?

Although evidence for defective mitochondrial energy metabolism in PD is strong, such a defect theoretically could be a consequence or marker of neurodegeneration rather than a contributor to the pathogenesis of PD. The most compelling evidence supporting a pathogenic role for mitochondrial dysfunction in PD comes from accidental human exposures to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydrodropyridine (MPTP), which causes parkinsonism in human by inhibition of mitochondrial complex-I of the electron transport chain [8, 9]. MPTP also inhibits alpha-ketoglutarate dehydrogenase; therefore there was some debate as to whether or not the mechanism behind MPTP toxicity involved complex I inhibition [10]. This question has since been addressed by the subsequent discovery that chronic systemic administration of rotenone, a highly specific complex-I inhibitor, causes highly selective and progressive nigrostriatal dopaminergic degeneration and a parkinsonian phenotype in rats, further implicating mitochondrial complex I dysfunction in PD pathogenesis [11–13].

3. Origin of the Mitochondrial Complex-I Defect in PD

The evidence cited above implicating a pathogenic role for mitochondrial complex I dysfunction in PD has led to great interest in understanding the origin of the complex I defect. Cytoplasmic hybrid (cybrid) cell lines created by transferring cytoplasmic contents including mitochondria from platelets or enucleated donor cells into cells experimentally depleted of endogenous mtDNA can be used to determine if a mitochondrial defect results from mtDNA mutations versus other causes (e.g., mitochondrial toxins or nuclear DNA mutations) [14]. Cybrids expressing mtDNA from idiopathic PD patients show reduced complex-I activity and increased susceptibility to MPP+, suggesting that mtDNA encoded defects in PD [15, 16]. Because cybrid cell lines share the same nuclear DNA background and differ only in the source of their mtDNA, these data implicate stable change in mtDNA (e.g., mutations) as a cause of the complex I defect in PD. In contrast, cybrid cell lines expressing mtDNA from subjects in the "Contursi" kindred who develop PD due to an autosomal dominant mutation in the α-synuclein gene do not manifest complex I deficiency [17]. This suggests that a nuclear genetic mutation can cause PD in the absence of mtDNA mutations, and that mtDNA mutations are more likely to contribute to idiopathic PD than to less common forms of PD associated with known nuclear genetic mutations.

4. Mitochondrial DNA and Classification of Mutations

These data from cybrids implicating mtDNA mutations in the complex I defect in PD therefore led to the search for inherited mtDNA mutations in PD patients. There are three main classes of mtDNA mutations: ancient maternally inherited polymorphisms that have given rise to various mitochondrial haplogroups, evolutionarily recent maternally inherited potentially pathogenic mutations, and somatic mtDNA mutations that accumulate during the life of the organism [18]. A number of studies have linked certain mitochondrial haplogroups with increased or decreased risk of PD in various populations of differing ethnicity [19–27]. Our laboratory found the G11778A mtDNA point mutation in a subunit of mitochondrial complex I in a family with maternally inherited parkinsonism and multisystem degeneration including nigral cell loss, demonstrating an association between an inherited mtDNA mutation and parkinsonism [28]. However, attempts to identify clearly pathogenic inherited mtDNA mutations in PD patients by direct sequencing of mtDNA have revealed that most PD patients lack such mutations [29–31]. The common 10398G variant of the NADH dehydrogenase 3 (ND3) gene (a subunit of mitochondrial complex I), present in up to 40% of Caucasians, has been reported to be present in a lower percentage of Caucasian PD patients compared to matched controls, suggesting a protective role in PD [32]. However, although a similar association was identified in another study [33], others have not replicated this result [27, 29].

Thus, although mtDNA point mutations have been found in rare families exhibiting parkinsonism [28, 34–39], clearly pathogenic mutations have not been identified in the vast majority of PD patients. Although a role of common mtDNA variants and rare pathogenic point mutations remains a possibility for some PD patients, these data led to the hypothesis that somatic mtDNA mutations, which may not be detectible by standard sequencing methods, might contribute to the complex I defect in PD.

5. A Role for Somatic mtDNA Mutations

The term "somatic mutations" (also known as acquired mutations) refers to DNA mutations that were not present in the germ cells that gave rise to the organism, but occurred later in development of the organism. Somatic mutations in mtDNA may take the form of single point mutations or small or large mtDNA deletions. mtDNA point mutations and mtDNA deletions can arise through separate mechanisms, although both types of mutation are now thought to be more commonly due to endogenous processes as opposed to exposure to exogenous agents [40, 41]. Somatic mtDNA point mutations may arise in three ways: firstly, base-substitution mutations caused by mitochondrial polymerase γ infidelity [42, 43], and mitochondrial polymerase γ replicating across damaged bases [40] may arise. Secondly, the proximity of mtDNA to ROS generated from the electron transport chain (ETC), as well as the lack of protective histones [44], may lead to oxidative damage to mtDNA, which can be highly mutagenic [45, 46]. Indeed, levels of oxidative damage to mtDNA in the brain are much higher than levels of damage to nuclear DNA, particularly in elderly subjects [47]. It is possible that this is especially pronounced in the SN where dopamine metabolism, high levels of iron, and low levels of glutathione (an important antioxidant), may create an environment especially high in oxidative stress [48, 49]. Finally, although mitochondria lack nucleotide excision repair mechanisms, the mitochondrial genome is protected by base excision repair mechanisms [50]. However, a decline in the base excision repair mechanism has been observed with age [51].

A number of hypotheses have been put forward for how mtDNA deletions may occur: initially it was thought that strand slippage during replication of mtDNA molecules [52, 53] gave rise to deletions in mtDNA, but more recently a theory that mtDNA deletions are created during the repair of mtDNA double strand breaks has been proposed [41]. Conclusive experimental evidence for either theory is currently insufficient.

6. Clonal Expansion of mtDNA Somatic Mutations

The term heteroplasmy refers to the instance where mtDNA mutations affect only a proportion of the mitochondrial genome within a single cell, tissue, or organism. In contrast, the term homoplasmy is used when an mtDNA mutation affects all copies of the mitochondrial genome within

a defined unit. Heteroplasmic somatic mutations may become functionally significant for two reasons: firstly, mtDNA mutations may undergo clonal expansion within individual cells. Clonal expansion of mtDNA mutations has been observed for both mtDNA deletions [54–56] and mtDNA point mutations [57]; however, the mechanisms behind clonal expansion are still under investigation. The high mtDNA content per cell and dynamism of the mitochondrial genome may be a contributing factor: each mitochondrion may contain several mitochondrial genomes, and each cell contains multiple mitochondria (the number of which varies according to the energy requirements of the cell), resulting in an estimated 10^3 to 10^4 copies of the mitochondrial genome per cell [58]. In addition, mitochondria are dynamic organelles and frequently undergo fusion and fission as well as frequent rounds of mtDNA replication [59, 60]. This raises the possibility of clonal expansion of a particular mtDNA mutation through random genetic drift, which has been demonstrated experimentally after induction of "relaxed" mtDNA replication [61], which refers to replication of mitochondria in nondividing cells. In addition, theories of directed segregation have been proposed. Directed segregation has been demonstrated to select *against* mtDNA mutations [62] and seemingly paradoxically, to also select *for* mutant mtDNA via selection for advantageous replicative mechanisms [63]. A definitive answer to the mechanisms behind clonal expansion is not yet available. It is possible that there will be more than one mechanism and that this may vary depending on the mitotic or postmitotic nature of the cells [64].

7. Aggregate Burden of Somatic Mutations and Failure of Functional Complementation

Once a certain "phenotypic threshold" of clonal expansion has been reached (around 50%–95% of mtDNA copies per cell depending on the mutation, cell type, and other factors; [65–69] a deleterious mutation may lead to impaired mitochondrial function [70, 71] as beyond that level, functional mitochondrial complementation may no longer provide restoration of functional ETC complexes [72]).

To elaborate further, the term "functional mitochondrial complementation" refers to the exchange of genetic information between mitochondria to alleviate a respiratory deficit caused by the mutation of an ETC gene in one of the mitochondria [73]. mtDNA is packaged in genetically autonomous mtDNA-protein complexes called nucleoids [74–76], these are mobile elements that are located throughout the mitochondrial genome [77] and include the transcription factor TFAM, mitochondrial polymerase γ, and the mitochondrial helicase Twinkle [78]. Nucleoids work to maintain heterologous mtDNA as different populations, preventing intermixing between mtDNA populations [79]. However, complementation can occur in stably separate nucleoid populations through transcomplementation by transcripts and polypeptides from nonmutant mitochondria freely diffusing through the mitochondrial matrix and becoming translated or assembled into ETC complexes, leading to a restoration of mitochondrial function [80].

Because functional complementation occurs in mitochondrial genomes, it is possible that the presence of functional ETC complexes may phenotypically mask the mtDNA mutational burden of the cell. Therefore, even when the majority of the copies of a particular mitochondrial gene within a single cell harbor a particular mutation, the cell may remain healthy, although eventually clonal expansion or the accumulation of new mutations may lead to a mutational burden that exceeds what the cell can tolerate. This phenotypic threshold is reached when complementation is no longer sufficient to allow sufficient numbers of normal mitochondrial complexes to be formed.

In addition to the clonal expansion of a single mitochondrial mutation, cellular respiration and organismal health may also be affected by the aggregate mutational burden resulting from the net impact of a number of different individually rare mtDNA mutations. For example, 100 different mtDNA mutations, each of which is present in a mitochondrial genome at a frequency of 1%, leads to an aggregate mutational burden of 100% (meaning an average of 1 mutation per mitochondrial genome). The functional consequences of this situation may be different from the impact of a single specific mutation clonally expanding to reach 100% (homoplasmy), particularly with respect to the potential role of complementation. Complementation still may allow functional ETC complexes in the case of multiple distinct mutations, whereas this may not be the case for the clonally expanded mutation. This is the case, for example, if the clonally expanded mutation affects a critical amino acid, making it impossible for that cell to make any functional copies of that subunit. Thus, the phenotypic threshold may be much higher for multiple individually rare mutations than for a single clonally expanded mutation.

8. Somatic mtDNA Mutations in the SN

The first published study to quantify the number of multiple somatic mtDNA point mutations in the SN used a highly sensitive cloning and sequencing strategy to characterize the aggregate burden of mtDNA mutations in the frontal cortex and SN of postmortem tissue from PD patients as well as young and old control subjects [71]. This study found that aggregate levels of point mutations increased with age in the frontal cortex of elderly subjects (65–91 years of age) compared to young controls (age 1–24 years). However, this study did not detect a statistically significant increase in the frequency of point mutations in the frontal cortex or SN of PD patients compared to age-matched controls. The authors hypothesized a number of reasons for this. Firstly, the study used postmortem tissue, which likely represented late stage PD. Therefore, the only dopaminergic neurons included in the analysis were those few that survived to late-stage PD when as many as 98% of the dopaminergic neurons may have already been lost [81]. It is possible that these neurons survived because they were somehow protected from the accumulation of mtDNA mutations. Secondly, these studies used brain tissue (as opposed to individual neurons); therefore the glial contribution may have been increased relative to the neuronal contribution in the PD

cases because of the scarcity of dopaminergic neurons in late-stages of PD. This could have reduced the relative level of mutations detected in the PD cases as the mean level of mutations is greater in SN neurons compared to glia [82].

To determine whether the frequency of somatic mtDNA point mutations differed between SN neurons and glia, the same authors performed a similarly sensitive cloning and sequencing strategy on laser-capture microdissected single neurons and glial cells from six control subjects [82]. This study did indeed show that the mean level of somatic mtDNA point mutations in single neurons was significantly higher than the mean level in glia with extrapolation of the data suggesting 3.3 somatic point mutations per mitochondrial genome in neurons and 2.2 mutations per mitochondrial genome in glia. In addition, this work demonstrated that heteroplasmy is common in both the D-loop and coding regions even at the level of single neurons and glia. Whether or not somatic mtDNA point mutations reach higher levels in SN neurons in PD remains an important question.

9. ND5 Somatic Mutations in PD

Other studies have used a similar cloning and sequencing approach to investigate the frequency of mtDNA complex I somatic point mutations in PD patients and aging. One study investigated mutational burden in all seven of the mitochondrial genes encoding the complex I subunits in frontal cortex tissue homogenates [83]. In agreement with previous work [71], the authors found that there was no significant difference in the overall number of point mutations between PD patients and controls. However, this study found that the percentage of transitions was lower than in the previously published work at 39% in PD and 29% in controls compared to 88% of the mutations found in the previous work. The reasons for this apparent discrepancy are not immediately clear. In addition, this work identified low levels of somatic mutations in the ND5 gene that were observed only in PD patients and not seen in controls [83]. Although the same authors subsequently confirmed this finding in another set of PD samples [84], levels of the mutations were quite low, generally less than 1%, well below the levels that would be expected to lead to functional consequences.

10. mtDNA Deletions in SN Neurons in PD and Aging

In early 2006, back-to-back papers in Nature Genetics reported the first findings on the frequency of mtDNA deletions in human SN neurons [85, 86]. Both groups used cytochrome C oxidase (COX) deficiency in SN neurons as a marker of a mitochondrial respiratory chain defect. The Bender et al. study [85] used long-range PCR to amplify mtDNA from COX-deficient and control neurons, and the Kraytsberg et al. study [86] developed a novel single-molecule PCR technique to quantify the total cellular burden of mtDNA deletions in COX-deficient neurons harboring a mitochondrial respiratory chain defect and control neurons. Both studies found that mtDNA deletions were present at higher levels in aged controls compared to younger individuals and that these deletions were clonally expanded. In addition, the work from Bender and colleagues identified that mtDNA deletions were higher still in individuals with Parkinson's disease compared to aged controls (52.3% and 43.3% resp.), although the difference was of borderline significance ($P = .06$).

These findings of surprisingly high levels of clonally expanded mtDNA deletions in SN neurons from elderly subjects, and a correlation of mutations with COX-deficiency, support the hypothesis that the age-related accumulation of somatic mtDNA mutations may contribute to the aging of the human brain and to the pathogenesis of Parkinson's disease. However, because these studies of human samples can be only observational in nature, it is difficult to accrue definitive evidence for functional consequences of mtDNA mutations. For such studies, the POLG "Mutator" mice are informative.

11. Evidence from the POLG "Mutator" Mice

The mitochondrial theory of aging proposes that the accumulation of mtDNA mutations lead to the aging process and to age-related neurodegenerative diseases [87]. However, experimental evidence in support of this fundamental theory of aging has been limited. Several years ago, two groups reported that transgenic mice expressing a proofreading deficient form of POLG are phenotypically normal at birth but accumulate very high levels of somatic mtDNA point mutations as they age, resulting in a premature aging phenotype that includes weight loss, reduced subcutaneous fat, hair loss, thinning of bones, anemia, reduced fertility, and early death [88, 89]. These mice were heralded as evidence in support of the mitochondrial theory of aging. However, it has been argued that POLG mutator mice accumulate mtDNA point mutation levels that are more than an order of magnitude higher than typical levels in aged humans, and so their aging-like phenotypes do not imply that levels of mtDNA mutations achieved during normal aging are functionally relevant [90]. Furthermore, heterozygous POLG mutator mice also accumulate higher mtDNA mutation levels than seen in normal aged mice and yet lack an overt phenotype, leading to further questions as to the relevance of these mice to the normal aging process [91].

In addition, the lack of overt parkinsonism in the POLG mutator mice could be interpreted as arguing against a role for somatic mtDNA mutations in PD. However, there are several important caveats to this argument. It remains possible that a more subtle phenotype may be present in heterozygous POLG mutator mice. For example, testing on behavioral measures sensitive to dopamine deficiency has not been reported. Also, levels of mutation accumulation within individual neurons vary substantially, and a subset of neurons may accumulate levels of mutations during normal aging that cross the phenotypic threshold. Furthermore, the POLG mutator mice are not a good model for assessing the role of mtDNA deletions in humans (see discussion on this issue below). Thus, the role for somatic mtDNA

mutations during normal aging and in neurodegenerative disease remains an open question.

12. Point Mutations versus Deletions

It has been argued that large mtDNA deletions rather than point mutations are the driving force behind the premature aging phenotype in homozygous POLG mutator mice [91]. This argument was based on a "random mutation capture" assay that demonstrated a large-fold relative increase in deletions in the POLG mutator mice, but could not determine the absolute levels of mutations. Other methods revealing the absolute level of deletions revealed that levels of deletions are quite low even in the homozygous mutator mice, especially when compared with the much higher level of point mutations [86, 92]. Moreover, another mouse model, "mito-mice", genetically engineered to accumulate mtDNA deletions, appear healthy without features of premature aging despite reaching deletions in up to 30% of all mtDNA molecules [93]. Therefore it appears likely that mtDNA point mutations rather than deletions drive the aging phenotype in the POLG mutator mice. In SN neurons from both aged controls and individuals with PD, both point mutations [71, 82] and large deletions [85, 86] accumulate to high levels. In contrast, as discussed above, in the POLG mutator mice, although point mutations reach high levels, absolute levels of mtDNA deletions remain low. Thus the POLG mutator mice may not be a valid model for understanding the functional impact of mtDNA deletions.

13. POLG Mutations Lead to Familial Parkinsonism

First identified as a nuclear encoded protein in 1972, POLG is the sole DNA polymerase in animal mitochondria [94, 95]. Essential for synthesis, replication, and repair of mtDNA, POLG contains three enzymatic activities: a DNA polymerase activity, a $3' \rightarrow 5'$ exonuclease activity involved in proofreading, and a $5'$-deoxyribose phosphate (dRP) lyase activity required for base excision repair [95]. Over 100 pathogenic mutations within the POLG gene have been found to cause a vast array of both neurological and nonneurological disorders including progressive external ophthalmoplegia (PEO), ataxia, and Alpers' syndrome [96]. POLG mutations have been linked to parkinsonism. Clinical assessment of several families with PEO or premature ovarian failure showed significant co of POLG mutations with levodopa responsive parkinsonism, and PET findings were consistent with dopaminergic neuron loss [37, 38, 97]. Two missense mutations (G737R and R853W) in the POLG gene were also detected in two siblings in a family with early-onset familial parkinsonism [98]. Moreover, in a large-scale systematic analysis of POLG gene in patients with sporadic idiopathic PD, there is significant clustering of rare variants of the POLG1 CAG-repeat as compared with their matched controls [99]. However, a study conducted on a large number of sporadic PD patients from UK and Italy does not conform to the possibility of linking common POLG polymorphisms in sporadic PD [100]. Nonetheless, the association of POLG mutations in humans with SN neuronal loss and parkinsonism, in combination with the premature aging phenotype in the POLG mutator mice, supports the hypothesis that the accumulation of somatic mtDNA point mutations in SN neurons may be functionally significant and may contribute to the pathogenesis of PD.

14. Conclusions

Mitochondrial dysfunction appears to play an important role in the pathogenesis of PD. Although the origin of the mitochondrial dysfunction in PD is unknown, somatic mtDNA point mutations and deletions have been found to accumulate with age and reach high levels in SN neurons. Experimental induction of POLG mutations cause the accumulation of mtDNA mutations leading to a premature aging phenotype in mutator mice. Although overt parkinsonism has not been reported in these mice, mutations in the same gene in humans can lead to dopaminergic dysfunction and parkinsonism. Thus, the accumulation of somatic mtDNA mutations in SN neurons may contribute to the pathogenesis of PD. This hypothesis, if proven to be correct, implies that strategies to block the accumulation of somatic mtDNA mutations may be protective in PD.

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Review Article

Diversity in the Regulation of Autophagy and Mitophagy: Lessons from Parkinson's Disease

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Selective mitochondrial degradation through autophagy (mitophagy) has emerged as an important homeostatic mechanism in a variety of organisms and contexts. Complete clearance of mitochondria can be observed during normal maturation of certain mammalian cell types, and during certain forms of neuronal cell death. In recent years, autophagy dysregulation has been implicated in toxin-injured dopaminergic neurons as well as in major genetic models of Parkinson's disease (PD), including α-synuclein, leucine-rich repeat kinase 2 (LRRK2), parkin, PTEN-induced kinase 1 (PINK1), and DJ-1. Indeed, PINK1-parkin interactions may form the basis of a mechanism by which dissipation of the inner mitochondrial membrane potential can trigger selective mitochondrial targeting for autophagy. Multiple signals are likely to exist, however, depending upon the trigger for mitophagy. Similarly, the regulation of basal or injury-induced autophagy does not always follow canonical pathways described for nutrient deprivation. Implications of this regulatory diversity are discussed in the context of neuronal function and survival. Further studies are needed to address whether alterations in autophagy regulation play a directly injurious role in PD pathogenesis, or if the observed changes reflect impaired, appropriate, or excessive autophagic responses to other forms of cellular injury.

1. Introduction

Macroautophagy represents an evolutionarily conserved response to nutrient stresses, which also plays an increasingly recognized role in basal cellular maintenance and in cellular responses to injury. The important role of macroautophagy in brain development and quality control was highlighted by observations that mice engineered for deficiency in key autophagy genes exhibit spontaneous neurodegeneration with ubiquitinated protein aggregates [1, 2]. Macroautophagy (hereafter, autophagy unless otherwise specified) is also implicated in mitochondrial quality control, as altered mitochondria accumulate under basal conditions of autolysosomal dysfunction [3–6], and mitophagy is further induced in cells exhibiting damaged mitochondria [7, 8]. Mitochondrial autophagy induced in response to mitochondrial damage or neuronal injury may play either prosurvival [7, 9] or prodeath roles [10, 11]. As mechanisms underlying different models of injury-induced autophagy and mitophagy are discovered, the concept of distinct regulatory inputs to a core autophagy pathway has emerged.

In sympathetic neurons, it was noted that the phosphoinositide 3-kinase inhibitor 3-methyladenine (3-MA) delayed apoptosis by reducing cytochrome c release and caspase activation [12]. Subsequent studies in the same system resulted in the first report of complete and selective clearance of mitochondria from neurons [13]. Combined with developmental observations of selective mitochondrial clearance in reticulocytes, lens, and lymphocytes [14, 15], these findings implicate the existence of specific mechanisms targeting mitochondria for autophagic clearance (although nonautophagic mechanisms may also contribute [16, 17]). Autophagy dysregulation is observed in a growing number of toxic/environmental and genetic models of Parkinson's disease (PD). Recent breakthroughs show a key role for two recessive Parkinsonian genes, PINK1 and parkin, in the specification of depolarized mitochondria for sequestration in aggresomes and/or autophagosomes [18, 19]. The potential

role of autophagy in PD models is reviewed in relation to diverse regulatory pathways feeding into the core autophagy machinery.

2. Autophagy Dysregulation in Parkinson's Disease

2.1. Sporadic PD. In PD patient tissues, evidence of apoptosis and of autophagy is observed in substantia nigra neurons [20]. Additional studies demonstrate mitophagy in the substantia nigra neurons of patients with PD spectrum diseases [21], and in Alzheimer's disease [22]. The ultrastructural observations of mitophagy in PD are correlated with a peculiar punctate/vesicular staining pattern for phosphorylated extracellular signal-regulated protein kinases (ERK1/2) [23, 24]. Although there have not been quantitative ultrastructural studies of autophagolysosomal structures in PD tissues, analysis of punctate/vesicular phospho-ERK1/2 indicates preferential involvement of the substantia nigra ventrolateral tier [21]. Incidental Lewy body disease, which is thought to represent a preclinical form of PD, exhibits an intermediate level of involvement [21]. In contrast, substantia nigra dopaminergic neurodegeneration associated with a distinct disease, progressive supranuclear palsy, do not show these changes (author's unpublished data), suggesting specificity for synucleinopathies.

2.2. Toxin Models of PD. Autophagy has been implicated in neurotoxin and environmental toxin models of dopaminergic cell death. Early and late autophagosomes can be identified by ultrastructural analysis, or by monitoring autophagosome-associated microtubule-associated protein 1 light chain 3 (LC3). Cytosolic LC3 migrates as an LC3-I band; upon stimulation of autophagy, LC3 is covalently conjugated to phospholipids, resulting in a faster migrating LC3-II band and correlating with punctate redistribution of the LC3 immunofluorescent signal. Increased autophagosomes have been described in acute injury models involving methamphetamine [25, 26], high doses of dopamine [27], 1-methyl-4-phenylpyridinium (MPP+) [10], 6-hydroxydopamine [11], the environmental toxicants rotenone [28], and paraquat [29]. In the case of toxins that are weak bases, it is not clear whether increased autophagosomes reflect increased autophagy induction or impaired completion of autophagic degradation from lysosomal pH elevation. Moreover, the role of autophagy in cell survival and cell death has been model-dependent. In some cases the "autophagy inhibitor" 3-MA exacerbated cell death [26] and autophagy stimulation conferred protection [28], but in other cases 3-MA ameliorated cell death [27, 30].

3-MA is a phosphoinositide 3-kinase (PI3K) inhibitor, which acts to inhibit autophagy by blocking the activity of the beclin 1-Vps34/class III PI3K complex. Interpretation of increased cell death in the presence of 3-MA, however, is complicated due to the ability of 3-MA to inhibit not only the class III PI3K involved in beclin 1-dependent autophagy pathways, but also the neuronal survival kinase Akt that is downstream of class I PI3K [12]. Due to opposite effects of class I and class III PI3Ks on autophagy [31], 3-MA can reduce or promote autophagy depending on the relative activation state of the two pathways [32]. 3-MA also has direct effects on glucose/glycogen metabolism independent of its autophagy-modulating effects and elevates lysosomal pH in living hepatocytes, but not isolated lysosomes [33]. Thus, it is essential to verify that 3-MA inhibits autophagy in the particular experimental condition being studied, and confirmation using more selective molecular inhibition of autophagy may be preferable.

While neuroprotective effects of autophagy in toxin models have been correlated with α-synuclein sequestration [26], noncanonical beclin 1-independent autophagy/mitophagy contributes to MPP+ toxicity, as shown by RNA interference knockdown of Atg7 and LC3/Atg8 [10]. This implicates excessive activation of autophagy, since the participation of beclin 1 in autophagy is downregulated by binding to Bcl-2 [34] or rubicon [35, 36]. Blunting the autophagy response has also been shown using dominant negative Vps34 to prevent hydrogen peroxide-mediated lysosomal leakage and caspase activation [37]. Likewise, a compound that activates mTOR to suppress autophagy confers protection from oxidative stress in neurons [30] while rapamycin exacerbates toxicity in this system and in primary neurons treated with MPP+ [38].

2.3. Genetic Models of PD. The dominant PD-linked protein α-synuclein exhibits consensus motifs for lysosomal degradation by chaperone-mediated autophagy (CMA), which is distinct from macroautophagy in its regulation. Mutant forms of α-synuclein bind the CMA receptor but are not internalized, inhibiting this degradative pathway in isolated liver lysosomes [39]. Moreover, dopamine-oxidized forms of α-synuclein show the same effect [40]. Both CMA and macroautophagy are involved in degrading wild-type α-synuclein in neurons [41], and A53T α-synuclein expression impairs CMA in living cells [42]. While upregulation of macroautophagy can mediate clearance of α-synuclein aggregates in metabolically intact cells [43], reports that α-synuclein can affect mitochondrial metabolism [44] and macroautophagy efficiency [45] raise additional questions. Indeed, CMA impairment induces upregulation of macroautophagy, which appears to contribute to neuron cell death [42]. Interestingly, low-dose application of the fusion inhibitor bafilomycin can protect against α-synuclein pathology in *C. elegans* [46]. The reciprocal cross-regulation of autophagy and of α-synuclein complicates analysis and creates the possibility of damaging feed-forward cycles.

One of the prominent phenotypes attributed to the dominant and sporadic PD-implicated protein LRRK2 is modulation of the neuritic arbor. Increased LRRK2 activity and PD-linked LRRK2 mutants cause simplification and shortening of neuritic projections while knockdown of LRRK2 expression results in enhanced neuritogenesis [47]. LRRK2-G2019S elicits neuritic autophagy, which mediates neurite shortening in retinoic acid-differentiated SH-SY5Y cells [48] and in primary cortical neurons [49]. LRRK2 associates with multivesicular bodies, and LRRK2-R1441G

elicits increased autophagosomes attributed to disrupted autophagic flux in HEK-293 cells [50]. Whether cell type differences or somatic versus neuritic differences affect flux responses to mutant LRRK2 remain to be established, as estimates of autophagy induction and flux rates are inferred unless pulse-chase techniques are used.

Parkin deficiency causes different phenotypes in different model systems. In parkin knockout mice, the primary defect relates to neurotransmission [51, 52]. In Drosophila, however, prominent mitochondrial degeneration in flight muscles and sperm is observed [53]. A pivotal discovery for parkin function was made in HeLa cells treated with the mitochondrial depolarizing agent FCCP or CCCP [18]. Parkin translocation to FCCP-depolarized mitochondria results in their eventual clearance through Atg5-dependent mechanisms, and this observation has led to an explosion of papers on the subject, each of which sheds additional insight into molecular mechanisms of mitochondrial cargo specification (discussed below). While overexpressed parkin enhances mitophagy in FCCP-treated cells [18] and in PINK1-deficient cells [7], the role of endogenous parkin in this setting is less clear. Translocation of tagged parkin to mitochondria and its ubiquitinating activity is essential for enhanced mitochondrial autophagy in FCCP/CCCP-treated cells. However, parkin monoubiquitination of Bcl2 enhances the ability of Bcl2 to bind beclin 1 and suppress autophagy, and RNAi knockdown of parkin increases the LC3-II band in 293, SH-SY5Y, and primary neuron cultures [54]. Thus, depending on subcellular localization and/or target accessibility, parkin can act to either promote mitochondrial specification for autophagy or to downregulate general autophagy.

PINK1 knockdown cells exhibit mitochondrial functional and morphological abnormalities [7, 55–57], with enhanced autophagic clearance of mitochondria [7]. On the other hand, overexpressed, full-length PINK1 reduces unconjugated LC3 [58] and increases parkin localization to mitochondria ([59, 60] and discussed below). Endogenous PINK1 in SH-SY5Y cells is predominantly processed [7], and PINK1 is processed in Drosophila by the membrane protease Rhomboid-7 [61]. As mitochondrial protein import and processing depends upon an intact inner mitochondrial membrane potential, stabilization of full-length PINK1 at the surface of depolarized mitochondria initiates PINK1-dependent mitophagy enhancement [62, 63].

Mitochondrial dysfunction observed in DJ-1 null cells is accompanied by a baseline decrease in the activated LC3-II band [6, 64]. However, whether this reflects increased or decreased autophagic flux remains controversial, and an increase in markers of compensatory mitophagy was recently reported in DJ-1 shRNA-expressing neuroblastoma cells [65]. DJ-1 null fibroblasts show reductions in expression of rapamycin-induced autophagosome markers in one study, interpreted as indicative of decreased autophagic induction [6]. Based on decreased basal levels of the autophagy substrate and cargo adaptor p62, however, another study concluded increased autophagic flux [64]. Flux analysis of autophagy or mitophagy can be technically challenging, but it is also possible that DJ-1 has different effects on basal versus induced autophagy. Interestingly, DJ-1 null cells exhibited decreased phosphorylation of ERK1/2 [6], which mediates autophagy/mitophagy in several systems [10, 11, 48, 66, 67]. DJ-1 siRNA has also been reported to inhibit paraquat-induced autophagy [68].

3. Diversity in the Regulation of Autophagy

3.1. Canonical Pathway of Starvation-Induced Autophagy. The identification of yeast genes necessary for autophagy, and related membrane trafficking events revolutionized the study of mitophagy in health and disease [69]. In brief, amino acid signals and insulin signals converge in turning on the mammalian target of rapamycin (mTOR), which suppresses autophagy. Amino acids also suppress ERK1/2 signaling. Loss of insulin signals, loss of amino acids, or direct inhibition of mTOR then serve to derepress autophagy induction, while 5′ adenosine monophosphate-activated protein kinase (AMPK) senses low energy to turn on autophagy. Beclin 1-Vps34-mediated changes in lipid composition are needed to define the phagophore and nucleate the membrane deposition of ubiquitin-like proteins Atg12 and LC3 in response to deprivation of growth factors or nutrients. Because beclin 1 can be found in several competing protein complexes [34–36], beclin 1-dependence has been proposed to serve a potential rheostat role in fine tuning levels of autophagy.

3.2. Mitophagy Regulation during Nutrient-Deprivation-Induced Autophagy. Starvation-induced autophagy is traditionally thought of as a nonselective bulk degradation process, with nonselective or bystander engulfment of mitochondria. However, yeast studies suggest a degree of mitochondrial recognition even in this process. The clearance of presumably undamaged mitochondria during nitrogen starvation requires the presence of an outer mitochondrial membrane protein Uth1p, [70], which does not have a clear mammalian homolog. Efficient mitochondrial autophagy in stationary-phase yeast are also regulated by an intermembrane space protein Aup1p [71]. Yeast cells grown in lactate undergo mitochondrial autophagy. Recently, the mitochondrial protein Atg32 was identified as a yeast mediator of selective mitophagy [72]. Atg 32 binds to Atg11, a known adaptor protein for selective autophagy in yeast. This system recruits mitochondria to autophagosomes, but does not directly regulate macroautophagy induction itself.

3.3. Beclin 1-Independent Injury-Induced Autophagy. Mitophagy is induced in neuronal cells and primary neurons injured with MPP+. Interestingly, in this system, autophagy induction proceeds even in the presence of PI3K inhibitors or siRNA knockdown of beclin 1 [10]. PI3K inhibitors are also unable to inhibit the selective clearance of photodamaged mitochondria in hepatocytes [73]. Beclin 1-independent mitophagy would no longer be negatively regulated by Bcl2 or rubicon, and thus, is more likely to allow a harmful level of autophagy activation. While the mechanism of beclin 1-independent autophagy has not been defined, it could reflect

alternative enzymatic means of increasing localized membrane concentrations of phosphatidylinositol 3-phosphate (PI(3)P) [38]. Alternatively, there could be other pathways of nucleation in which membrane changes mediated by ROS or kinase activation can substitute for PI(3)P in recruiting Atg18–Atg2 [74] or Atg16L to membranes.

Beclin 1-independent autophagy has been described in several model systems. As inhibition of autophagy is protective in these models [10, 48, 75–77], these observations support the concept of harmful overactivation of autophagy. It remains to be determined whether this is accidental, or forms part of a programmed cell death pathway.

3.4. Quality Control Autophagy and Cargo Regulation. Other variations from classic rapamycin-induced autophagy are beginning to emerge. Both basal autophagy and injury-induced autophagy play roles in organellar and protein quality control. A key feature of quality control-related autophagy relates to the ability of the cellular autophagy machinery to selectively remove damaged proteins and organelles while sparing their normal counterparts.

Interestingly, studies of quality control autophagy reveal requirements for HDAC6 and actin remodeling for maturation and completion of autophagy [78]. HDAC6-dependent retrograde transport of autophagy substrates and mediators to the perinuclear region is necessary for degradation of aggregated huntingtin [79], and selective transport may represent one mechanism of cargo enrichment. For protein aggregates, another mechanism of cargo recruitment involves direct adaptor protein interactions mediated by p62, which bridges ubiquitin on the cargo with LC3 on the autophagic membrane [80, 81]. Other adapter proteins that have been identified include NBR1, which can cooperate with p62 [82], and Nix, which binds to GABARAP-L1 [83]. While specific proteins may differ between yeast and mammals, the general concept of cargo receptors and adapter proteins that link into the autophagy machinery represents a rapidly emerging area of research.

3.5. Depolarization-Induced Mitophagy. One of the exciting developments in mitophagy regulation is the use of chemically depolarized mitochondria to dissect proteins needed for mitochondrial clearance. With nutrient deprivation, depolarization of mitochondria can occur after sequestration by GFP-LC3 [84], preceding their entry into acidic lysosomal compartments in rat hepatocytes [85]. Live imaging studies also show that mitotracker-labeled mitochondria disappear within 8 minutes of entering lysotracker-stained lysosomes in hepatocytes [86]. Thus, the observation of "mitophagosomes" is a transient event, most readily observed when elicited by synchronized chemical insults, or with inhibition of autophagosome maturation [11, 87].

The past year has witnessed significant advances in delineating mechanism(s) by which depolarization promotes mitophagy, subsequent to the original observation that the ubiquitin ligase parkin translocates to mitochondria in FCCP/CCCP-treated cells to mediate their clearance [18]. Of note is the observation that parkin functions only in the cargo recruitment step of mitophagy, but other mechanisms involving Nix mediate the induction of autophagy by depolarization [8]. Subsequent discussion will focus on surface changes on depolarized mitochondria that could mediate their autophagic recruitment in the depolarization model.

Depolarization inhibits membrane potential-dependent proteolytic processing of PINK1 [88]. This, in turn, causes full-length PINK1 to accumulate at the surface of mitochondria [62, 63], which is necessary for stable and global association of parkin with mitochondria in the FCCP/CCCP treated cells. From here, several mechanisms have been described that could specify parkin-bearing mitochondria for mitophagy. Parkin ubiquitination of mitofusins serve to promote fission [60, 89], which is necessary for mitophagy [87]. Parkin has also been reported to polyubiquitinate voltage-dependent anion-selective channel protein 1 (VDAC1) [90], which may explain PINK1-parkin-dependent perinuclear aggregation of mitochondria [19, 91]. VDAC1 interacts with a dynein light chain [92], although it is unknown whether or not ubiquitination modulates this. PINK1 may also interact directly with LC3 [59]. Finally, the p62 adaptor discussed above is recruited to parkin-ubiquitinated mitochondria, although its role in aggregation versus mitophagy recruitment is controversial [90, 93].

3.6. Alternative Mechanisms Relating to Mitophagy. Mitophagy can be initiated prior to depolarization of the mitochondrial membrane potential in several model systems. In starvation-induced mitophagy in hepatocytes, mitochondria remain polarized until after they are encircled by GFP-LC3 [84]. Likewise, mitochondrial depolarization occurs downstream of autophagosome formation in reticulocytes during Nix-dependent developmental mitophagy [94]. Indeed, depolarization-independent, Nix-dependent mitophagy may involve direct interactions of Nix with an LC3 homolog GABARAP [83], indicating alternative signaling for mitochondria recognition during autophagosome formation.

Parkin also compensates for PINK1 deficiency [95, 96]. While this could be mediated by nonmitophagy-related mechanisms, increased parkin expression causes increased autophagy in PINK1-deficient cells [7], and parkin-mediated protection from cell death is substantially diminished by RNAi knockdown of autophagy mediators [9]. Whether or not stable parkin recruitment to mitochondria is necessary for its compensatory effects is unknown, but hydrogen peroxide can recruit parkin to mitochondria through a mechanism not requiring mitochondrial targeting of PINK1 [97]. Finally, a parkin mutant that is deficient in translocating to mitochondria can be rescued in this function by the DnaJ/Hsp40 chaperone HSJ1a [98]. Besides ubiquitination, phosphorylation or changes in lipid composition could conceivably also trigger loss of mitochondria [99].

It is reasonable to assume that these mechanisms would allow for selective removal of irreversibly damaged mitochondria while sparing normal mitochondria, but this has not yet been robustly demonstrated. FCCP- or CCCP-treated cell lines exhibit rather global changes of parkin

translocation, perinuclear aggregation, and clearance. Likewise, selective autophagy of mitochondria with mtDNA mutations is inferred from studies showing that fibroblasts from patients with mitochondrial DNA diseases exhibit higher rates of mitophagy upon amino acid deprivation than those containing normal mitochondria [100], but selective autophagic engagement of individual mitochondria with high burdens of mtDNA mutations was not assessed. Cells expressing mitochondrial DNA deletions exhibit elevated transcripts for Atg proteins [101], and thus generalized elevations in autophagic capacity may also result in greater mitophagy for stochastic reasons. Live imaging studies such as those demonstrating that relatively depolarized mitochondria show decreased fusion [102], or preferential retrograde axonal transport [103], could be used to further establish selective recognition of abnormal mitochondria on an organelle-by-organelle basis.

4. Summary

Autophagy has emerged as a central response observed in multiple models of Parkinsonian neurodegeneration. In several chronic models, autophagy induction plays beneficial roles in clearing protein aggregates or damaged mitochondria. Autophagy can also play a harmful role in neurons subjected to acute injury such as ischemia-reperfusion or neurotoxin treatment. As understanding of mechanisms underlying autophagy and mitophagy develops, it will be interesting to determine whether distinct regulatory inputs to the core autophagy machinery underlies differences in the degree and outcome of autophagy or mitophagy induction. Just as pathways are emerging that show differences between autophagy induced for quality control and in response to trophic/nutrient deprivation, variations on the depolarization-initiated PINK1-parkin pathway are likely to emerge. Cell type specific mechanisms must also be considered, as parkin translocation is not observed in CCCP-treated cortical and striatal/midbrain neurons [104]. Redundancy in mechanisms that underlie the removal of damaged mitochondria may account for the relatively minor symptoms observed in knockout mice and form the basis for future therapies to heighten neuroprotective responses in PD patients.

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Research Article

PINK1-Interacting Proteins: Proteomic Analysis of Overexpressed PINK1

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Recent publications suggest that the Parkinson's disease- (PD-) related PINK1/Parkin pathway promotes elimination of dysfunctional mitochondria by autophagy. We used tandem affinity purification (TAP), SDS-PAGE, and mass spectrometry as a first step towards identification of possible substrates for PINK1. The cellular abundance of selected identified interactors was investigated by Western blotting. Furthermore, one candidate gene was sequenced in 46 patients with atypical PD. In addition to two known binding partners (HSP90, CDC37), 12 proteins were identified using the TAP assay; four of which are mitochondrially localized (GRP75, HSP60, LRPPRC, and TUFM). Western blot analysis showed no differences in cellular abundance of these proteins comparing *PINK1* mutant and control fibroblasts. When sequencing *LRPPRC*, four exonic synonymous changes and 20 polymorphisms in noncoding regions were detected. Our study provides a list of putative PINK1 binding partners, confirming previously described interactions, but also introducing novel mitochondrial proteins as potential components of the PINK1/Parkin mitophagy pathway.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder with three cardinal manifestations: tremor, rigidity, and bradykinesia. In about 25% of all PD patients, at least one additional affected family member can be found, likely pointing to a direct, genetic cause of the disease. To date, eight genes have been confirmed to be associated with PD [1].

Besides the mutational analysis of PD-linked genes, the search for potential interactions between the protein products of these genes has recently gained increasing importance. Since the identification of the second monogenetic PD gene product Parkin, a common pathway leading to dopaminergic neurodegeneration has been proposed. To date, several studies reported such connections.

First, Shimura et al. hypothesized that Parkin plays a role in the coregulation of Alpha-synuclein (SNCA). The group identified a protein complex in normal human brain that included the E3 ubiquitin ligase Parkin, UBCH7 as its associated E2 ubiquitin-conjugating enzyme, and a novel form of SNCA as its substrate [2]. Later, an early-onset PD patient with a heterozygous missense mutation in both the *DJ-1* and the *PTEN-induced putative kinase 1* (*PINK1*) gene has been described. Additionally, overexpression of *DJ-1* and *PINK1* in SHSY-5Y cells revealed that the wildtype as well as the mutant forms of both proteins interact, and DJ-1 stabilized PINK1 [3]. Furthermore, *Drosophila pink1* and *parkin* loss-of-function mutants showed a similar mitochondrial phenotype. Since the *pink1*-related abnormalities could be rescued by *parkin* overexpression but not vice versa, it was suggested that pink1 acts upstream of parkin in a common pathway [4–6]. Recent studies provided evidence that the PINK1/Parkin pathway promotes mitochondrial fission as an initial step of mitophagy by ubiquitination of Mitofusins [7–9].

Still, only the minority of hereditary forms of PD can be explained by a mutation in one of the nine PD-associated

genes [10]. Therefore, this research focuses not only on proteins known to be involved in PD but also on novel interactors.

In the present study, we employed tandem affinity purification (TAP) to isolate proteins that are directly associated with PINK1. Using this approach, we aimed at a better characterization of the PINK1/Parkin mitophagy pathway.

2. Material and Methods

2.1. Patients. All patients underwent a standardized neurological examination performed by a movement disorders specialist. For sequencing of one of the candidate genes, *Leucine-rich PPR motif-containing* (*LRPPRC*), 46 patients with atypical Parkinsonism including features of dementia, depression, or rapid disease progression were included in the present study. After obtaining informed consent, we collected blood from all patients for DNA extraction according to a published protocol [11].

Furthermore, fibroblasts from three PD patients with a homozygous p.Q456X PINK1 mutation and from two age-matched mutation-negative healthy controls were included in the study. Clinical features of these mutation carriers are described elsewhere [12, 13].

2.2. Tissue Culture. HEK cells and fibroblasts were cultured in Dulbecco's modified Eagle's medium (PAA Laboratories) and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cells were maintained at 37°C in a saturated humidity atmosphere containing 5% CO_2. Passage numbers <10 were used for all experiments. To inhibit the mitochondrial membrane potential, fibroblasts were treated with the potassium ionophore valinomycin (1 μM, Sigma).

2.3. Protein Isolation by TAP and Mass Spectrometry (MS). The InterPlay Mammalian TAP System was employed according to the manufacturer's (Stratagene) protocol. In brief, human control RNA was extracted using the RNeasy Mini Kit (Qiagen) and *PINK1* cDNA was synthesized using Superscript II Reverse Transcriptase in combination with oligo dT primers (Invitrogen). Full-length *PINK1* cDNA was cloned into the pCTAP expression vector, which encodes two tandem affinity tags (a streptavidin binding peptide and a calmodulin binding peptide) after its multiple cloning site. Next, 10^8 HEK cells were transiently transfected with the pCTAP-*PINK1* vector by means of the $Ca^{2+}PO_4$ method [14]. After 24 h, cells were harvested and resuspended in lysis buffer supplemented with protease inhibitors (Sigma). Cells underwent three rounds of freeze-thawing, cell debris was pelleted by centrifugation at 16,000× g for 10 min, and the supernatant collected. Next, 2 mM EDTA, 10 mM β-mercaptoethanol, and the InterPlay streptavidin resin were added to the cell lysate and incubated at 4°C while rotating for 2 h. The resin was collected by centrifugation at 1,500× g for 5 min, washed twice, and incubated in biotin-containing buffer for 30 min at 4°C to elute the bound protein complexes. To further purify the protein complexes, calmodulin resin and a calcium-containing buffer were added to the supernatant. After 2 h of incubation at 4°C, the resin was collected (centrifugation at 1,500× g for 5 min) and washed twice. From the calmodulin resin, protein complexes were eluted by adding EDTA-containing buffer and incubation for 30 min at 4°C on a rotator. The resulting eluate was concentrated by means of trichloroacetic precipitation and subsequently resuspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH7.6, 150 mM NaCl, 1% DOC, and 1% NP-40) with protease and phosphatase inhibitors (Roche Diagnostics). Next, purified proteins were resolved by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized on the gel by silver staining as published [15]. In a control experiment, the pull-down approach was performed with beads only. Bands which appeared on the gel from the PINK1 overexpression experiment but not the control experiment were excised and sent for MS analysis to the Taplin Biological Mass Spectrometry Facility, Harvard Medical School, Boston, USA. According to the quality requirements of the facility, a protein can only be considered as interactor if two or more peptides match the respective protein in the Swiss-Prot database.

2.4. Knock-down Approach. For *PINK1* and *LRPPRC* knock-down, Hs_PINK1_4_HP (Qiagen) and LRPPRC UTR (complementary to the segment of the 3′UTR region of LRPPRC [16]) siRNAs were used with a final concentration of 50 nM. Scrambled siRNA (Silencer negative control 1 siRNA [Ambion]) with no known mammalian homology served as negative control (final concentration 50 nM). For transfection, the Nucleofector Device (Lonza) was used.

2.5. Mitochondrial Preparation. Mitochondria were isolated from fibroblasts as previously described [17]. In brief, cells were harvested and homogenized in buffer containing 250 mM sucrose, 10 mM Tris, and 1 mM EDTA, pH7.4. After that, nuclei and unbroken cells were removed by centrifugation at 1,500× g for 20 min. The supernatant containing intact mitochondria was transferred into a new tube and centrifuged at 12,000× g for 10 min. The resulting supernatant (cytosolic fraction) was transferred into another new tube and the mitochondria-enriched pellet (mitochondrial fraction) was dissolved in RIPA buffer containing protease and phosphatase inhibitors (Roche Diagnostics). Cytoplasmic fractions were concentrated by using Centricon YM-10 devices (Millipore) according to the manufacturer's instructions.

2.6. Protein Extraction. Proteins were extracted using RIPA buffer containing 0.1% SDS. Cells or mitochondria-enriched pellets were dissolved in the appropriate amount of buffer and incubated on ice for 30 min. After that, the lysates were centrifuged at 16,000× g for 20 min at 4°C. The supernatant was transferred into a new tube for further processing.

2.7. Western Blot Analysis. SDS-PAGE was performed using NuPAGE 4%–12% Bis-Tris gels (Invitrogen). After electrophoresis, proteins were transferred to the nitrocellulose

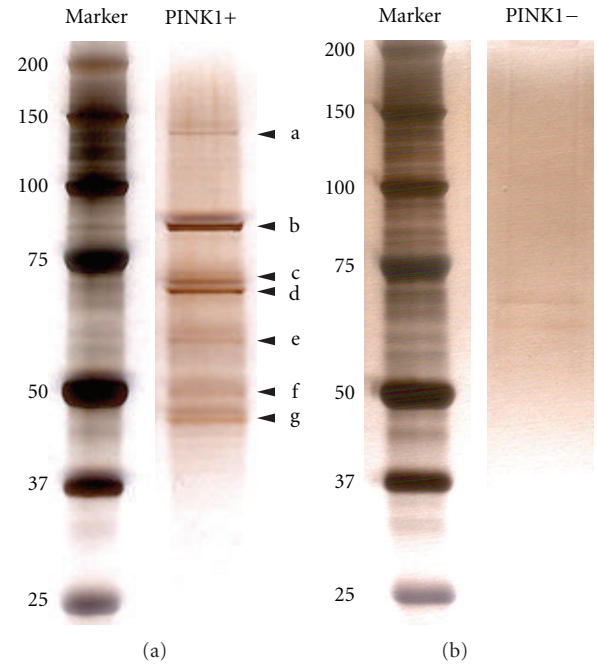


Figure 1: SDS-PAGE result after TAP with overexpressed PINK1. (a) A TAP approach was performed with HEK cells overexpressing *PINK1* (PINK1+). Purified proteins were resolved by SDS-PAGE and visualized on the gel by silver staining. Protein bands which were excised from the gel are marked by an arrow head (a–g). (b) In a control experiment, the pull-down approach was performed with beads only (PINK1−). A molecular weight marker was used to estimate the size of the detected protein bands.

membrane (Protran) and probed with antibodies raised against β-actin (Sigma), Mortalin (GRP75, Abcam), Heat shock 60 kDa protein (HSP60, Cell Signalling), Mitochondrially encoded cytochrome c oxidase I (MT-CO1, MitoSciences), Voltage-dependent anion channel 1 (VDAC1, Abcam) and Elongation factor TU (TUFM, Abcam). An antibody against LRPPRC was kindly provided by Professor S. Piñol-Roma, Brookdale Department of Molecular, Cell and Developmental Biology, Mount Sinai School of Medicine, New York, USA [18].

2.8. Mutational Screening. All 38 coding *LRPPRC* exons and flanking intronic regions were sequenced on an ABI 3100 Genetic Analyzer. Primers and PCR conditions are summarized in Supplementary Table 1 available online at doi:10.4061/2011/153979.

3. Results and Discussion

3.1. New PINK1 Interactors. Using a TAP approach with overexpressed PINK1 (PINK1+), or empty beads (PINK1−) we identified interactors of the target protein. The resulting TAP eluates were resolved by SDS-PAGE. Silver staining was used to visualize protein bands on the PAGE gels. Seven bands were excised from the PINK1+ gel (Figure 1(a), a–g) which were not detectable on the PINK1− gel (Figure 1(b)) and analyzed by MS (Table 1).

A total of 14 proteins were identified which met the requirement of having two or more peptides matched to it by the database-searching program. Out of those, seven are predominantly found in the cytoplasm (Heat shock 90 kDa proteins alpha and beta, Heat shock 70 kDa proteins 1, 2, 8, and 1-like, and Hsp90 cochaperone Cdc37), two are components of microtubuli (Tubulin alpha-1C chain and Tubulin alpha-3C/D chain), one is associated with the endoplasmic reticulum (78 kDa Glucose-regulated protein) and four are mitochondrially localized (GRP75, HSP60, LRPPRC, and TUFM) (for details on subcellular localization see: http://expasy.org/sprot/). Of note, also PINK1 itself was identified by MS in most of the analyzed bands on the PAGE gel (Figure 1(a), b–g).

To our knowledge, only the interaction between the HSP90/CDC37 chaperone system and PINK1 has been described so far [19, 20]. CDC37 is a molecular cochaperone that functions with HSP90 to promote folding of kinases [21]. With respect to PINK1, the chaperone system was found to influence the protein's subcellular distribution. The authors of this study proposed that the HSP90/CDC37/PINK1 complex is destined for a translocation that leads to PINK1 processing, whereas in the absence of HSP90 in the complex, PINK1 might be attached to mitochondria as full-length precursor [19].

Given that PINK1 was reported to be associated with mitochondria [17, 22], we focused primarily on the mitochondrial proteins GRP75, HSP60, LRPPRC, and TUFM in the ensuing experiments.

3.2. Cellular Abundance of GRP75, HSP60, LRPPRC, and TUFM. First, we determined the quality of the noncommercially available LRPPRC antibody by means of a knock-down

Table 1: Potential interactors of PINK1.

Swiss-Prot accession no.	Gene	Protein name	Subcellular localization	No. of unique peptides	Sequence coverage in %	Band on PAGE gel	Previous report of interaction
P42704	*LRPPRC*	Leucine-rich PPR motif-containing protein	Mitochondrion	2	1.8	a	none
P07900	*HSP90A*	Heat shock 90 kDa protein alpha	Cytoplasm	27	28.5	b	Weihofen et al. [19]
P08238	*HSP90B*	Heat shock 90 kDa protein beta	Cytoplasm	18	21.9	b	Weihofen et al. [19]
P38646	*GRP75*	75 kDa glucose-regulated protein/Mortalin	Mitochondrion, cytoplasm	6	10.8	c	none
P11021	*GRP78*	78 kDa glucose-regulated protein	Endoplasmic reticulum	15	28.7	c	none
P08107	*HSPA1*	Heat shock 70 kDa protein 1	Cytoplasm, organelles	10	17.3	c	none
P34931	*HSPA1L*	Heat shock 70 kDa protein 1-like	Cytoplasm, organelles	8	13.7	c	none
P54652	*HSPA2*	Heat shock 70 kDa protein 2	Cytoplasm, organelles	4	7.2	c	none
P11142	*HSPA8*	Heat shock 70 kDa protein 8	Cytoplasm	7	12.7	c	none
P10809	*HSP60*	Heat shock 60 kDa protein	Mitochondrion	7	16.2	d	none
Q9BQE3	*TUBA1C*	Tubulin alpha-1C chain	Microtubule	2	5.3	f	none
Q13748	*TUBA3C*	Tubulin alpha-3C/D chain	Microtubule	3	10.7	f	none
Q16543	*CDC37*	Hsp90 cochaperone Cdc37	Cytoplasm	10	22.2	g	Weihofen et al. [19]
P49411	*TUFM*	Elongation factor Tu	Mitochondrion	3	8.0	g	none

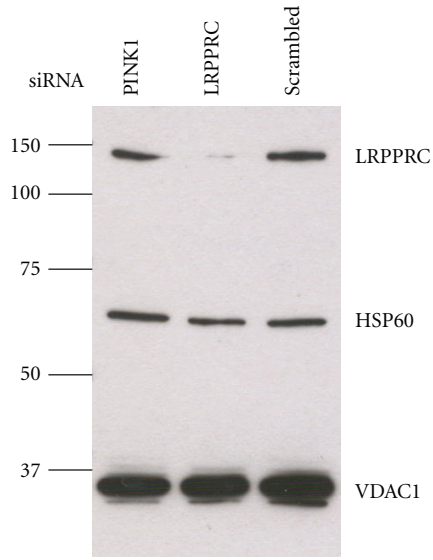


Figure 2: Specificity of an anti-LRPPRC antibody. Fibroblasts were incubated with *PINK1* siRNA, *LRPPRC* siRNA, or scrambled siRNA for 24 h. Whole cell lysates were analyzed by Western blotting with an antibody against LRPPRC. LRPPRC levels decreased only when *LRPPRC* siRNA was employed, confirming the specificity of the anti-LRPPRC antibody used in our study. The mitochondrial markers HSP60 and VDAC1 served as loading controls. HSP60: Heat shock 60 kDa protein; LRPPRC: Leucine-rich PPR motif-containing protein; VDAC1: Voltage-dependent anion channel 1.

approach. This experiment showed a drop in LRPPRC levels when siRNA against *LRPPRC* was employed but not when scrambled siRNA was used, confirming the specificity of the antibody. LRPPRC protein levels were also not affected by a *PINK1* knockdown (Figure 2).

Next, the abundance of GRP75, HSP60, LRPPRC, and TUFM was investigated in mitochondrial fractions from control and *PINK1*-mutant fibroblasts. This experiment revealed comparable levels of GRP75, HSP60, and LRPPRC in both groups (Figures 3(a) and 3(b)-left half). The abundance of TUFM was variable in all investigated samples showing no clear trend when comparing mutants and controls (Figure 3(a)).

Furthermore, we tested the quality of the GRP75 antibody by investigating GRP75 in the cytosolic fraction under valinomycin stress conditions. GRP75 is a mitochondrial matrix chaperone, synthesized as a 679-amino acid preprotein, which contains a 51-residue N-terminal mitochondrial targeting sequence (MTS). After the membrane potential-dependent import into mitochondria, it is cleaved into the mature protein which is ~5.5 kDa shorter than the preprotein [23–25]. When treating cells with the mitochondrial membrane potential inhibitor valinomycin, the full-length form of GRP75 (MTS-GRP75) was detected in the cytosolic fractions from mutants and controls. Consequently, we considered the anti-GRP75 antibody as specific. In both groups, the levels of MTS-GRP75 were comparable (Figure 3(b)-right half). The abundance of the processed form of GRP75 in the cytosolic fraction was likely due to contamination with the mitochondrial fraction, although previous studies have shown that GRP75 can also be cytosolically localized [26].

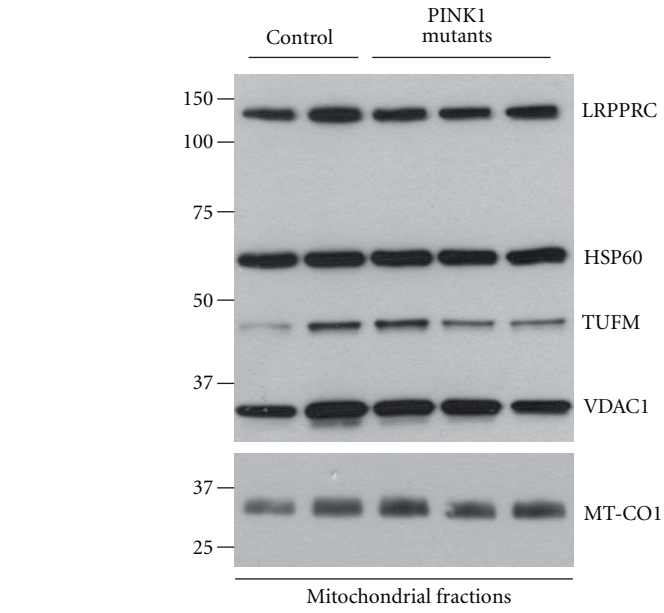
Though our Western blot results are not supporting a direct link between PINK1 and any of the detected mitochondrial proteins, it should be noted that GRP75, HSP60, and LRPPRC have been identified by proteomic analysis as potential interactors of Parkin earlier [27]. Furthermore, their molecular functions render them interesting targets in the context of PD.

GRP75 serves as a major mitochondrial molecular chaperone and plays a key role in the import and partitioning of nuclear-encoded proteins within the two mitochondrial membranes and the matrix [28–30]. Furthermore, GRP75 seems to function in the management of oxidative stress via the PD-associated protein DJ-1. Mutations in DJ-1 were found to weaken the protein's interaction with GRP75 [31, 32]. GRP75 has also been described as an antiapoptotic agent. By binding of the transcription regulator p53, GRP75 prevents the formation of the proapoptotic p53/Bcl-xL/Bcl-2 complex [33, 34]. Furthermore, putative mutations in *GRP75* were suggested to contribute to the risk of developing PD [35] and a decrease in GRP75 expression was detected in PD patient brains compared to controls [36].

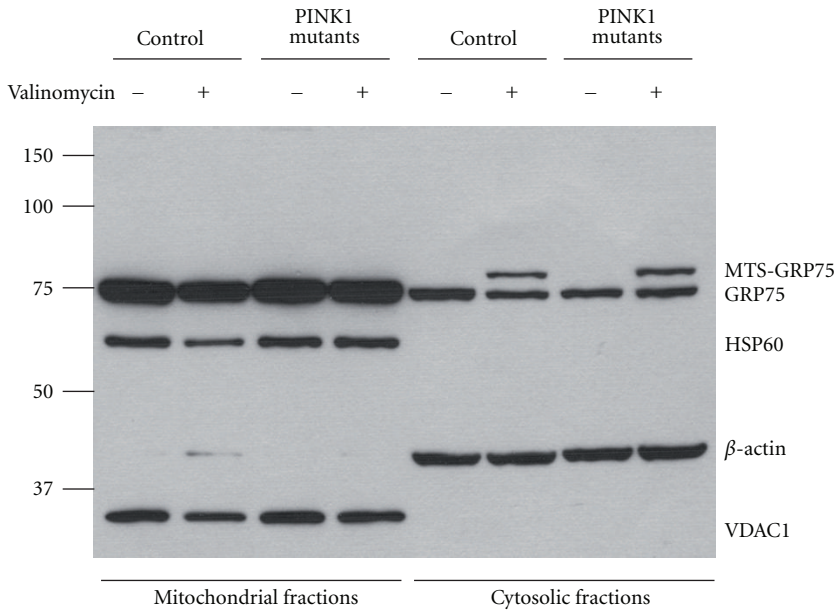
HSP60 is a mitochondrial chaperone responsible for the transport of nuclear-encoded proteins via the mitochondrial membranes and their refolding in the matrix [37, 38] and has been linked to the pathogenesis of Alzheimer's disease. Apparently, HSP60 provides protection against intracellular β-amyloid stress through maintenance of mitochondrial respiratory complex IV activity [39]. Complex IV deficiency in turn, has been implicated in PD [40, 41] opening the possibility for a role of HSP60 in the pathogenesis of the disease.

LRPPRC has been linked to cytochrome C oxidase deficiency. Mutations in the gene lower *MT-CO1* and *MT-CO3* mRNA levels and, in turn, impair complex IV assembly [42, 43]. Recent functional studies further strengthen the link between LRPPRC and mitochondrial RNA metabolism [44, 45]. However, when we compared MT-CO1 protein levels in fibroblasts from *PINK1* mutants and controls, no differences were observed (Figure 3(a)). Furthermore, LRPPRC was identified as a component of the PGC-1α complex which itself is also linked to energy homeostasis in the cell [46]. Like in the case of HSP60, LRPPRC's impact on the respiratory chain offers a potential connection with PD.

TUFM is part of the translational apparatus of mitochondria. During protein biosynthesis, it mediates the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes [47]. Additional described functions of TUFM comprise recognition and translocation of cotranslationally damaged proteins to the proteasome [48], rearrangement of cytoskeletal components [49, 50], and regulation of cell survival [51]. Mutations in the *TUFM* gene cause combined oxidative phosphorylation deficiency type 4 due to decreased mitochondrial protein synthesis [52]. Interestingly, however, there is also a report connecting TUFM and PD, where TUFM was found to co-immunoprecipitate with Leucin-rich repeat kinase 2 which is encoded by the PARK8 gene *LRRK2*. Coincubation with recombinant TUFM reduced the kinase



(a)



(b)

Figure 3: Cellular abundance of potential mitochondrial PINK1-interacting proteins. Mitochondrial and cytosolic fractions from fibroblasts were analyzed by Western blotting using antibodies against HSP60, LRPPRC, TUFM, MT-CO1, and GRP75. (a) The mitochondrial localization of LRPPRC and TUFM was confirmed, and no differences in their cellular abundance were detected when comparing *PINK1* mutants and controls. Furthermore, the level of LRPPRC-associated MT-CO1 was not altered in *PINK1* mutants. (b) In the mitochondrial fractions, the abundance of (processed) GRP75 was comparable in *PINK1* mutants and controls under basal and valinomycin stress conditions (1 μM for 24 h). In the cytosol, an additional band representative of accumulation of nonprocessed MTS-GRP75 was detected when cells were treated with the mitochondrial membrane inhibitor valinomycin. Due to a possible contamination of the cytosolic fraction with mitochondria and/or partially cytosolic localization of GRP75, also the processed form of the protein is apparent in this fraction. The mitochondrial marker VDAC1 and the cytosolic marker β-actin served as loading controls. GRP75: 75 kDa glucose-regulated protein; HSP60: Heat shock 60 kDa protein; LRPPRC: Leucine-rich PPR motif-containing protein; MT-CO1: Mitochondrially encoded cytochrome c oxidase I; MTS-GRP75: GRP75 with mitochondrial targeting sequence; TUFM: Elongation factor Tu; VDAC1: Voltage-dependent anion channel 1.

Table 2: Allelic frequencies of sequence variations identified in *LRPPRC*.

Gene position	DNA variation	NCBI no.	AF PD	AF DB	Database*
5′UTR	c.-45G>A	rs11124961	7.6%	1.4%	pilot.1.CEU
Exon 2	c.246G>A (p.Q82Q)	rs6741066	66.7%	65.5%	HapMap-CEU
Intron 3	IVS3-132C>G	rs6721144	6.8%	13.3%	HapMap-CEU
Intron 6	IVS6-70T>C	rs17031786	14.4%	13.8%	HapMap-CEU
Exon 9	c.1068A>G (p.Q356Q)	rs4953042	16.3%	19.2%	HapMap-CEU
Intron 9	IVS9+30A>G	rs7593842	15.2%	12.7%	HapMap-CEU
Intron 13	IVS13+28T>C	rs62135104	9.5%	1.5%	pilot.1.CEU
Intron 15	IVS15+11C>G	rs58811869	7.8%	13.9%	pilot.1.CEU
Intron 17	IVS17-28T>G	rs72877186	15.2%	15.3%	pilot.1.CEU
Intron 20	IVS20-40A>C	rs7594526	42.4%	47.5%	HapMap-CEU
Intron 22	IVS22+27T>G	rs28394191	43.5%	40.3%	pilot.1.CEU
Exon 23	c.2481A>G (p.P827P)	rs115993634	1.1%	none	none
Intron 27	IVS27+26C>T	rs4952694	51.1%	53.0%	AoD_Caucasian
Intron 27	IVS27-38A>G	none	2.2%	none	none
Intron 28	IVS28+21C>A	rs7568481	43.5%	47.4%	HapMap-CEU
Intron 30	IVS30+97T>C	rs17424482	8.7%	3.7%	HapMap-CEU
Intron 32	IVS32-3C>T	rs35113761	6.5%	none	none
Intron 35	IVS35+14C>T	rs3795859	15.2%	15.0%	HapMap-CEU
Intron 35	IVS35+15C>T	rs76850904	8.7%	none	none
Intron 36	IVS36-42G>C	none	1.1%	none	none
Exon 37	c.4023T>C (p.Y1341Y)	none	1.1%	none	none
Intron 37	IVS37+37G>A	rs2955280	51.1%	53.4%	HapMap-CEU
3′UTR	*399G>A	none	2.3%	none	none
3′UTR	*556A>T	rs1136998	7.6%	8.3%	HapMap-CEU

Note: AF: allelic frequency, DB: database, PD: Parkinson's disease and *Only studies based on European populations included.

activity of LRRK2, whereas the GTPase activity remained unchanged [53].

3.3. LRPPRC Mutational Screen. Among the identified PINK1 interactors, LRPPRC is the only protein which is unequivocally linked to a neurodegenerative disorder. Mutations in *LRPPRC* are the cause of the French-Canadian type of Leigh syndrome (LSFC). LSFC patients suffer from progressive focal necrotizing lesions of the brainstem, basal ganglia, and cerebellum, accompanied by capillary proliferation. Besides metabolic acidosis, clinical features include generalized developmental delay, cerebellar signs, and a striking paucity of facial and limb movement, as well as hypomimia [46]. Given the presence of Parkinsonian signs in LSFC patients, we decided to sequence the 38 exons and flanking intronic regions of *LRPPRC* in 46 patients with atypical PD with early onset and/or rapid disease progression and dementia. This mutational screen revealed 24 substitutions; four of which have not yet been reported in any database (Table 2). Four synonymous variations were detected in the coding region (c.246G>A [p.Q82Q], c.1068A>G [p.Q356Q], c.2481A>G [p.P827P], c.4023T>C [p.Y1341Y]). Seventeen changes were found in introns, one in the 5′UTR and two in the 3′UTR. The frequencies of most substitutions in our sample were similar to those reported in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/) for studies based on populations of European origin, such as pilot.1.CEU, HapMap-CEU, and AoD_Caucasian. Interestingly, frequencies of SNPs c.-45G>A, IVS13+28T>C and IVS30+97T>C, were markedly higher than those reported in the databases. The significance of this finding needs to be investigated in a larger sample. The screening techniques used here allow, however, only for the identification of qualitative sequence changes. Therefore, although no single nucleotide changes of likely pathogenic relevance have been found in the *LRPPRC* gene, gene dosage variations cannot be excluded.

4. Conclusions

In the current study, TAP technology was employed for the first time to identify PINK1-associated proteins. These experiments resulted in a list of 14 putative PINK1 binding partners, confirming two reported interactions (HSP90 and CDC37), but also introducing four novel mitochondrially localized proteins (GRP75, HSP60, LRPPRC, or TUFM) as potential components of the PINK1/Parkin mitophagy pathway. Although preliminary results from protein expression and DNA sequencing analyses do not strengthen a link between the PINK1/Parkin pathway and any of these interactors, it cannot be excluded that their connection

with the pathway may be more complex. Additional protein function studies, for instance under mitochondrial stress conditions, will be needed to fully characterize this potential link. In addition, future perspectives include association studies with SNPs in all identified genes in a larger PD patient sample.

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Review Article

Mitochondrial Dysfunction in Parkinson's Disease

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Parkinson's disease (PD) is a progressive, neurodegenerative condition that has increasingly been linked with mitochondrial dysfunction and inhibition of the electron transport chain. This inhibition leads to the generation of reactive oxygen species and depletion of cellular energy levels, which can consequently cause cellular damage and death mediated by oxidative stress and excitotoxicity. A number of genes that have been shown to have links with inherited forms of PD encode mitochondrial proteins or proteins implicated in mitochondrial dysfunction, supporting the central involvement of mitochondria in PD. This involvement is corroborated by reports that environmental toxins that inhibit the mitochondrial respiratory chain have been shown to be associated with PD. This paper aims to illustrate the considerable body of evidence linking mitochondrial dysfunction with neuronal cell death in the substantia nigra pars compacta (SNpc) of PD patients and to highlight the important need for further research in this area.

1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative condition characterised by deterioration of the dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc) [1, 2]. The exact mechanism by which SNpc cell death in PD occurs is poorly understood, but several lines of evidence implicate mitochondrial dysfunction as a possible primary cause due to the central role of mitochondria in energy production, along with oxidative stress, ubiquitin system impairment and excitotoxicity, all of which may be interlinked [3–5]. Mitochondrial dysfunction with complex I deficiency and impaired electron transfer in the substantia nigra in PD have been reported [3, 4]. Moreover, mutations in several mitochondrial proteins have been associated with familial forms of PD [5–7], as well as the presence of deletions in mitochondrial DNA identified in aging controls and PD substantia nigra [8]. This paper discusses the possible mechanisms relating mitochondrial impairment to cell death in PD.

2. Mitochondria

Mitochondria are found in virtually all eukaryotic cells and function to generate cellular energy in the form of adenosine triphosphate (ATP) by oxidative phosphorylation and are thought to be derived evolutionarily from the fusion of prokaryotic and eukaryotic organisms [9, 10]. They are also involved in regulation of cell death via apoptosis, calcium homeostasis, haem biosynthesis, and the formation and export of iron-sulphur (Fe-S) clusters, that have been reviewed in detail elsewhere [11–13], and function in the control of cell division and growth.

Structurally, mitochondria are composed of a double lipid bilayer with a phospholipid outer membrane and an inner membrane which surrounds the intracompartmental matrix (Figure 1). The space between the two membranes is important in, and contains the major units of, oxidative phosphorylation [9]. Mitochondria are unique amongst cellular organelles in that they have their own, circular, double-stranded, DNA (mtDNA) which is inherited almost

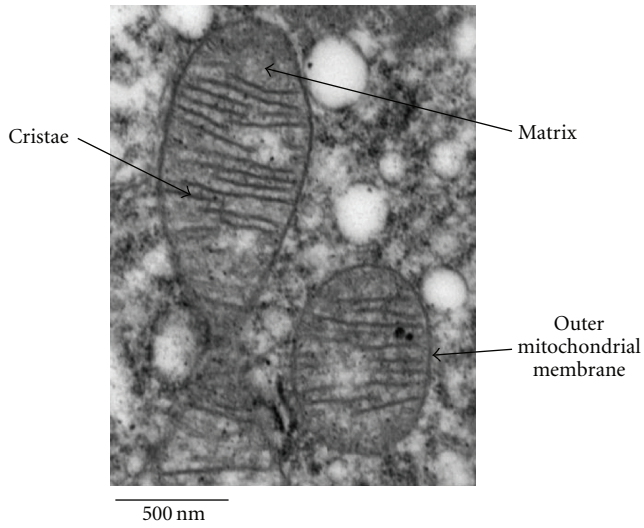

Figure 1: An electron micrograph to show mitochondrial ultrastructure. Reproduced with the kind permission of Tracy Davey, EM Research Services, Newcastle University.

exclusively down the maternal line and codes for 37 mitochondrial genes, 13 of which translate to proteins involved in oxidative phosphorylation [10, 14]. The remaining genes encode transfer (22) and ribosomal (2) RNA allowing the mitochondria to generate their own proteins [10]. Although mitochondria have the ability to produce proteins, the vast majority of proteins that function within the mitochondria, including those involved in DNA transcription, translation, and repair, are encoded by nuclear DNA and are transported into the mitochondria from the cytosol [10]. As mtDNA is located in the mitochondria in close proximity to the electron transport chain it is more susceptible to damage from free radicals generated during oxidative phosphorylation [15]. This damage may lead to mutations that have been linked with PD [13, 16] and will be discussed later.

2.1. Electron Transport Chain. The electron transport chain is composed of five complexes including an ATP-synthase located in the inner mitochondrial membrane. The function of the chain is to generate cellular energy in the form of ATP. This is accomplished by the transport of electrons between complexes causing proton (H^+ ions) movement from the matrix to the intermembrane space generating a proton concentration gradient used by ATP-synthase to produce ATP (Figure 2).

Whilst electron transport is a highly efficient process in mitochondria [17], during the process of oxidative phosphorylation, electrons can leak from the chain, specifically from CI [18] and CIII [19], and react with oxygen to form superoxide ($\cdot O_2^-$). Under normal physiological conditions this $\cdot O_2^-$ production occurs at relatively low levels, with approximately 1% of the mitochondrial electron flow forming $\cdot O_2^-$ [20], and is removed by mitochondrial antioxidants such as manganese superoxide dismutase (MnSOD) which converts $\cdot O_2^-$ to H_2O_2 which is then converted to H_2O by glutathione (GSH). Given the high activity of the respiratory chain under even normal circumstances, the small leakage of electrons in mitochondria is still a major source of $\cdot O_2^-$ within many eukaryotic cells [21]. It is thought that dysfunction in this process leading to an increase in $\cdot O_2^-$ production could be one of the main drivers of cell death in the SNpc in PD [22, 23].

2.2. Electron Transport Chain Dysfunction in PD. As neurons have a considerable energy need and are also highly equipped with mitochondria they are extremely sensitive to mitochondrial dysfunction. Several neurological disorders are associated with mitochondrial dysfunction and demonstrate enhanced production of free-radical species [16, 24]. The first line of evidence for a link between mitochondrial dysfunction and PD came from the description of Complex I (CI) deficiency in the postmortem SNpc of PD patients and has been suggested to be one of the fundamental causes of PD [4, 25]. This CI deficiency was also seen in the frontal cortex in PD [26], and in peripheral tissues such as platelets [27] and skeletal muscle [28] suggesting that there is a global reduction in mitochondrial CI activity in PD. This defect may be due to oxidative damage to CI and misassembly, since this is a feature of isolated PD brain mitochondria [3]. Incidental Lewy body disease (ILBD) which is considered by some to be a preclinical indicator of PD, has been shown to have an intermediate level of CI activity in the SNpc between healthy and PD patients [22] which further supports the theory of mitochondrial dysfunction. This inhibition of CI can lead to the degeneration of affected neurons by a number of mechanisms such as increased oxidative stress and excitotoxicity [23] which will be described below.

A decrease in the function of Complex III has also been reported in the lymphocytes and platelets of PD patients [27, 29]. A link between impairment of mitochondrial CIII assembly, an increase in free radical-production, and PD has also been identified [30]. This increase in free radical release may be due to the increased leakage of electrons from CIII (as explained below). Alternatively the inhibition of CIII assembly causes a severe reduction in the levels of functional CI in mitochondria [31] which could lead to an increase in free-radical production through CI deficiency. In addition, the CI and II electron acceptor ubiquinone has also shown to be reduced in the mitochondria of PD patients [32] and loss of DA neurons in aged mice treated with the Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was attenuated by ubiquinone [33], providing more evidence for the involvement of mitochondrial dysfunction in PD.

3. Mitochondrial Generation of Reactive Oxygen Species in PD

Complex I or III inhibition causes an increase in the release of electrons from the transport chain into the mitochondrial matrix which then react with oxygen to form reactive oxygen species (ROS) such as $\cdot O_2^-$, hydroxyl radicals ($\cdot OH$) and nitric oxide ($NO\cdot$). This increase in the normal electron leakage occurs by blockage of electron movement along the chain to the next acceptor molecule. An example of this is

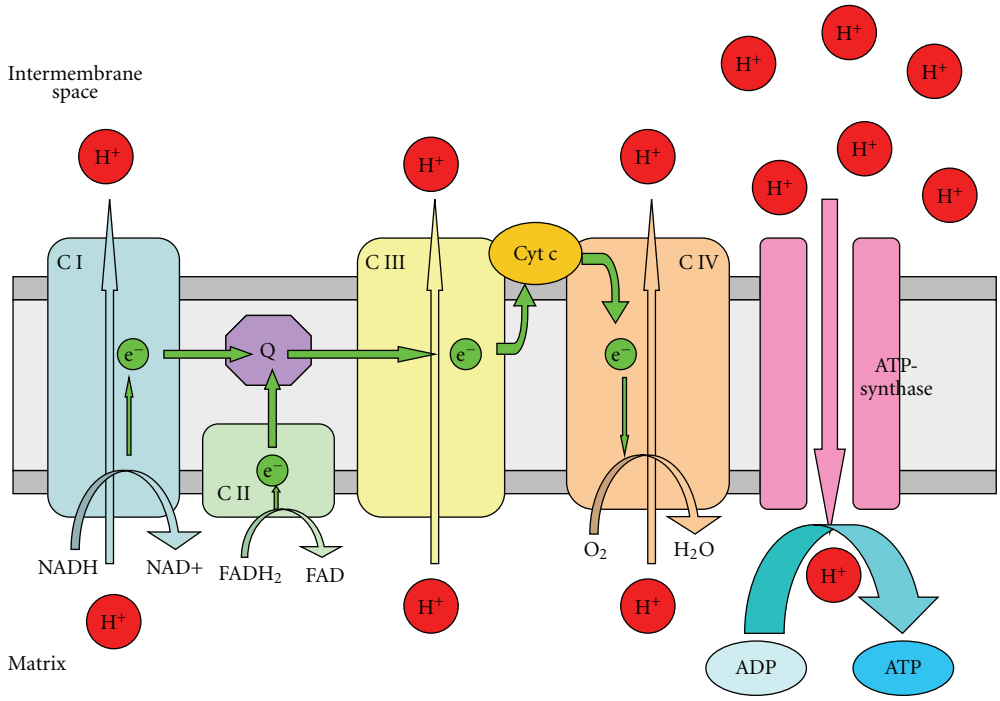

Figure 2: Mitochondrial electron transport chain: schematic representation of the mitochondrial electron transport chain involved in oxidative phosphorylation. CI and II (Complexes I and II) transport electrons (e^-s) generated by the conversion of NADH to NAD+ (CI) or $FADH_2$ to FAD (CII) through Q (ubiquinone), CIII, Cyt c (cytochrome c) and finally CIV, which uses an e^- to convert O_2 to H_2O. During electron transfer, CI, II, and IV pump protons (H^+s) from the mitochondrial matrix into the intermembrane space generating a H^+ concentration gradient that drives the formation of ATP from ADP by ATP-synthase (Complex V).

the finding that blockade of the electron accepting capability of the ubiquinone binding site of Complex I leads to an increased production of electron radicals in rat skeletal muscle mitochondria [34]. The ROS formed can act as signalling molecules by causing lipid peroxidation or promote excitotoxicity, all leading to modification of proteins and eventual cell death. The main areas in which ROS cause damage in the cell include oxidative DNA damage, lipid peroxidation, and protein oxidation and nitration. The ·OH radical has been shown to react with the double bond of DNA bases leading to damaging DNA lesions [35] and an impairment of normal cellular function. There is evidence linking this mechanism of cell death to PD in the finding of increased levels of 8-hydroxyguanosine, produced by oxidative damage to DNA, in the SNpc of PD patients [36, 37]. Peroxynitrite (formation discussed below) also increases DNA single-strand breaks [38] which cause activation of poly (ADP-ribose) polymerase (PARP) which in turn disrupts the mitochondrial respiratory chain and ATP production via depletion of intracellular NAD+ stores, so exacerbating the original mitochondrial dysfunction [39].

3.1. ROS-Mediated Protein Damage. Amongst the most common mechanisms of protein damage caused by ROS is oxidation to form carbonyl groups on proteins and nitration by peroxynitrite. Reactive ROS can readily oxidise amino acids on various cellular proteins to form carbonyl groups, which can disrupt the physiological function of the affected protein and lead to cytotoxic protein aggregates, activation of cell death pathways, and impairment of neuroprotective pathways [40]. An increase in these carbonyl groups has been reported in the SNpc and in the basal ganglia and prefrontal cortex of PD patients [41] suggesting a role in the disease which is widespread throughout the brain. Peroxynitrite formed by the reaction of ROS with nitric oxide and nitrates tyrosine residues on proteins [42], damaging them and leading to cell death. These nitrotyrosine residues are present in Lewy bodies in PD neurons signifying the possibility that protein nitration may contribute to the pathology of PD [43]. Peroxynitrite [44] and other ROS [45] can also oxidise sulphydryl groups on glutathione, leading to depletion of antioxidant defences, and other thiol containing cofactors disrupting various cellular processes and structures. The final link between PD and mitochondrially generated ROS through protein damage comes with the discovery of nitrated and oxidatively damaged misfolded proteins that have strong genetic links with the disease such as α-synuclein, DJ-1, Parkin and PINK1 [46–51] suggesting that these disease-associated proteins are major targets of free-radical damage in sporadic forms of PD.

Lipid peroxidation is another of the main types of cellular damage caused by ROS occurring when ROS react with hydrogen in the lipid leading to the formation of a lipid radical. This radical can then form a further lipid radical via an intermediary by reacting with a hydrogen atom leading to a chain reaction and breakdown of the lipid. In the cell

membrane, phospholipids are particularly susceptible to this damage due to their polyunsaturated nature, leading to damage to cell and organelle membranes and severe cellular dysfunction. There have been strong links between increased lipid peroxidation and PD with increased levels shown to be present and cause cell death in the nigral cells of PD patients [36, 37], suggesting a role for it in the mechanisms of neuronal death.

All of these mechanisms of oxidative stress can feed back to the mitochondria via a series of signalling pathways and cause further exacerbation and mitochondrial dysfunction by damaging mtDNA. In this way, ROS-driven mitochondrial dysfunction acts to propagate itself via a feedback loop leading to accelerated cellular damage and death and contributing to PD.

This oxidative damage could be exacerbated in PD by the discovery of reduced antioxidant defences in patients with the disease. The main line of evidence regards reduced GSH, which has been found to be selectively decreased in the SNpc of patients with PD [52], leading to a decline in cellular capability to inactivate H_2O_2 and peroxynitrite. This is thought to be an early event in PD pathology since lower levels of reduced GSH in incidental LB disease are of the same magnitude as those found in PD [53]. There have not been consistent reports of changes in the levels of most of the other major antioxidant systems; however, cases of an increase in mitochondrially located MnSOD in PD patients have been described [54, 55]. This supports a link between mitochondrial dysfunction generated superoxide and PD since MnSOD is the main $\cdot O_2^-$ scavenging system in mitochondria.

Since there are elevated markers of oxidative damage in the brains of PD patients, including lipid peroxidation [36] protein damage [41, 43], and oxidative DNA damage [36, 37], oxidative stress is an established event in PD. This evidence combined with the PD-like effects of known Complex I inhibitors such as MPTP [56] and rotenone [57] and an increase in the activity of neutralising superoxide dismutase (SOD), particularly the mitochondrially localised MnSOD, being found in neurodegenerative disease patients [58] points to a strong link between ROS, possibly caused by mitochondrial dysfunction and PD.

3.2. Involvement of Iron in Oxidative Stress. Iron may also be involved in the production and propagation of oxidative stress in the mitochondria of DA neurons in PD. Iron is intrinsically linked with mitochondrial function and particularly the brain, since iron is an integral component of all respiratory chain complexes and the uptake of iron by the brain is linked with mitochondrial energy demands [59]. Increased iron levels in the brain have long been associated with PD [60–63] although this increase may be an effect of the normal pathological clearance of iron that occurs during cell death that occurs in PD. More recently, a specific increase in intracellular iron has been observed in SNpc neurones in PD patients [46] suggesting an increase in the cellular accumulation of iron in the disease. Cellular iron normally enters into neurones via the transferrin receptor system and evidence suggests that there is a reduction of this system in PD [64, 65] although this may relate more to the loss of SNpc neurones in PD [47]. This iron may also be taken up into the mitochondria in the SNpc via the transferrin receptor 2 and increased levels of ferritin have been reported in the mitochondria of SNpc DA neurons of PD patients and rats treated with rotenone [48]. ROS with a low oxidative potential, like $\cdot O_2^-$ generated via dysfunction of oxidative phosphorylation, can increase the release of reactive ferrous iron from this ferritin so leading to the creation of more ROS via the Fenton reaction [49] and increasing oxidative stress and cell damage. The evidence that abnormal iron homeostasis is present in PD is clear; however, the role of iron in PD as either an initiator or a consequence of pathology remains to be elucidated.

4. Involvement of Ca^{2+} in Mitochondrial Dysfunction and PD

4.1. Excitotoxicity. The cellular damage caused by electron transfer chain inhibition may contribute to neuronal excitotoxicity exacerbating neurotoxicity in PD. Several mechanisms for excitotoxicity in neurodegenerative conditions have been proposed [50, 51]. Excitotoxicity occurs when depolarisation of the neuronal cell membrane from −90 mV to between −60 and −30 mV leads to a decrease in the magnesium blockade of N-methyl-D-aspartate (NMDA) receptors. This, in turn, leads to NMDA receptor activation by latent levels of glutamate and causes an intracellular Ca^{2+} accumulation. This increase in Ca^{2+} is then thought to cause neurotoxicity by two main mechanisms. Firstly, Ca^{2+} causes an increase in intracellular NO via activation of nitric oxide synthase (NOS). The excess of NO in the cell can react with $\cdot O_2^-$ to form peroxynitrite [66], which can cause cell death by mechanisms similar to those caused by ROS and mentioned above. Besides the peroxynitrite, NO itself can lead to cell damage via nitrosylation of various proteins.

A second mechanism driven by intracellular Ca^{2+} increase causes toxicity in DA neurons by acting on mitochondria themselves. The Ca^{2+} influx is extensively accumulated in the mitochondria and leads to effects on mitochondrial membrane potential and ATP synthesis as well as generation of ROS [67] contributing to the oxidative damage discussed above. This also all feeds back causing further malfunction of the cell's Ca^{2+} homeostasis and additional cellular damage.

Mitochondrial dysfunction can lead to excitotoxicity and cause a reduction in cellular ATP levels, an increase in cellular Ca^{2+}, or a combination of both [23]. Inhibition of Complex I, and consequently ATP generation, lowers intracellular ATP, leading to partial neuronal depolarisation, due to a reduction in the activity of Na^+/K^+-ATPase. The Na^+/K^+-ATPase acts to maintain the resting membrane potential of the cell. A reduction in ATP levels will compromise this function leading to depolarisation and, therefore, excitotoxicity via over activation of NMDA receptors. Intracellular Ca^{2+} can be increased by two methods, that is, either directly by mitochondrial impairment or by over activity of NMDA receptors. Mitochondria can take up Ca^{2+} from the cytosol via an uniporter transporter or a transient "rapid mode",

both of which rely on the mitochondrial membrane potential, reviewed by [68]. The ROS generated by mitochondrial respiratory chain dysfunction can damage the mitochondrial membranes and disrupt this mechanism of Ca^{2+} uptake and storage, thereby raising intracellular Ca^{2+} levels and exacerbating the excitotoxicity. Sherer et al. showed that this could be a viable mechanism for cell death in PD by inhibiting Complex I in SH-SY5Y cells and showing a disruption in the mitochondrial membrane potential leading to an increased susceptibility to calcium overload [69]. It has also been shown that there is an increase in glutamate activity linked to excitotoxicity in the SNpc of mice treated with the Parkinsonian toxin MPTP, which may be linked to apoptosis and autophagy [70]. This evidence suggests that mitochondrially derived or driven excitotoxicity could be a major contributory factor in PD.

4.2. Nonexcitotoxic Involvement of Ca^{2+}. Further to being integrally involved in mitochondrially generated excitotoxic cell death in PD, Ca^{2+} has been implicated in other mechanisms of cell death in the disease that may involve compromise of the role of mitochondria as one of the major intracellular Ca^{2+} stores. Sheehan et al. reported that mitochondria from PD patients showed lower sequestration of calcium than age-matched controls suggesting a role for Ca^{2+} homeostasis dysfunction in PD [71]. This would lead to higher intracellular Ca^{2+} levels, which has been shown to amplify free-radical production [72] and, therefore, an increase in ROS. An increase in cytosolic Ca^{2+} due to mitochondrial dysfunction has also been suggested to activate calpains and so raise levels of toxic α-synuclein proposing another link with PD [73], a discovery supported by the protective effect of calpain inhibition in an MPTP model of PD [74].

A role for Ca^{2+} and mitochondria has also been suggested in a mechanism of increased susceptibility to cell death specific to SNpc DA neurons and, therefore, relevant for PD. SNpc DA neurons are atypical in the brain in that they have self-generated pacemaker activity [75] mediated by Cav1.3, a rare L-type Ca^{2+} channel [60]. This activity means that that the Cav1.3 channels are open for a higher proportion of time than Ca^{2+} channels in other neurons leading to an increased ATP usage in the cells to pump Ca^{2+} across the membrane up steep concentration gradients [61]. This elevated need for ATP would lead to an increase in the activity of the electron transport chain, therefore increasing ROS production so exacerbating any mitochondrial dysfunction and making SNpc DA neurons more prone to cell death.

5. Dopamine Metabolism and Mitochondrial Dysfunction in PD

Dopamine metabolism generated oxidative stress has long been implicated in PD [40]. However, more recently, it has been suggested that ROS or reactive quinones produced by oxidation of dopamine, either spontaneously or by monoamine oxidase (MAO), may have a inhibitory effect on the proteins of the mitochondrial respiration chain [76–78]. Khan et al. showed that dopamine inhibits Complexes I and IV, most probably through the actions of the dopamine-generated quinones rather than through ROS [62]. MAO-A is bound to the outer mitochondrial membrane and can oxidise dopamine to form the metabolite 3,4 dihydroxyphenylacetic acid (DOPAC), which can itself, or through oxidation to quinones, locally inhibit Complexes I and IV [78, 79] although a contradictory report suggests dopamine but not DOPAC inhibits the electron transport chain [63]. A recent study has shown that dopamine itself, rather than any oxidation products can directly inhibit Complex I [80] however, more work would need to be carried out to confirm this as there is no corroborating evidence.

These links between mitochondrial dysfunction and dopamine are supported by reports that the PD neurotoxins and Complex I inhibitors MPTP and rotenone increase dopamine oxidation and turnover [64, 65] although whether this is indirectly due to the ROS generating effects of the toxins is not clear. This hypothesis could offer an explanation as to why DA are more susceptible than other neurons to toxin or mutation mediated mitochondrial dysfunction PD, as they are already under a higher level of oxidative stress due to dopamine metabolism generated electron transfer chain inhibition.

6. Genetic Links between Mitochondrial Dysfunction and PD

Studies of families who suffer from inherited forms of PD have identified a number of genes encoding mitochondrial proteins or proteins implicated in mitochondrial dysfunction [46–51, 81].

6.1. α-Synuclein. The α-synuclein gene encodes a small protein of the synuclein family that is localised to nerve terminals although the physiological function of α-synuclein is unclear. The first link between α-synuclein and PD came with the discovery of mutations in the α-synuclein gene in PD patients [48, 49, 51]. This evidence was supported by the discovery that aggregates of α-synuclein are the major components of LBs [82]. Although there are many theories on the mechanism by which mutant α-synuclein causes neuronal cell death in PD, it has recently been reported that there is an association between α-synuclein and mitochondrial dysfunction [83, 84]. Mutant α-synuclein is targeted to and accumulates in the inner mitochondrial membrane (IMM) and can cause Complex I impairment and an increase in ROS, possibly leading to cell death [85].

6.2. Parkin, PINK1, and DJ-1. Parkin, PTEN-induced putative kinase 1 (PINK1), and DJ-1 are genes that code for proteins that are crucially involved in mitochondrial function and resistance to oxidative stress and have been linked with PD. The Parkin gene encodes an E3 ubiquitin ligase involved in the ubiquitin-proteasomal system, marking proteins for degradation by the proteasome. Mutations in Parkin have been linked to autosomal recessive juvenile parkinsonism [86]. Parkin has also been shown to interact with and promote degradation of α-synuclein [87]; therefore,

a loss of function Parkin mutation could contribute to the α-synuclein driven cell death mentioned above. It has been suggested that Parkin may also be involved in mitochondrial function and protection from mitochondrially generated ROS. Recent work in Parkin null mice has shown a reduction in subunits of Complex I and IV and reduced function in the mitochondrial respiratory chain along with increased oxidative stress in brain tissue [88]. This is supported by reduced mitochondrial Complex I activity in Parkinsonian patients with Parkin mutations [89] and the increased age-dependent or rotenone (a Complex I inhibitory toxin) induced DA neuronal degeneration and mitochondrial abnormalities in Parkin mutant drosophila [90]. Moreover, a recently generated zebrafish model of Parkin deficiency showed increased sensitivity of Parkin mutants to proteotoxic stress with no manifestation of dopaminergic neuron loss or affected mitochondrial morphology or function [91].

PTEN-induced putative kinase 1 (PINK1) is a serine/threonine kinase located in the inner mitochondrial membrane [92] which functions to protect neurons against various types of cell stress. Mutations in PINK1 are found to be associated with a autosomal recessive form of PD [93]. This pathogenesis may be associated with the loss of kinase activity as PINK1 is known to protect against cell death only if the kinase function is intact [94]. Several independent groups reported a role of PINK1 in mitochondrial integrity by regulating the mitochondrial fission machinery [95], which might have an impact on dopaminergic synapses and contribute to DA neuron degeneration [96–98]. Knockdown of PINK1 has been shown to induce mitochondrial oxidative stress, mitochondrial fragmentation, and autophagy and dysregulate calcium homeostasis in SH-SY5Y cells [76, 98]. Moreover, there is evidence of interplay between PINK1 and Parkin, since Parkin can reduce mitochondrial dysfunction caused by down-regulation of PINK1 [77]. Furthermore, it has been shown recently that both PINK1 and Parkin are involved in removal of damaged mitochondria. The proposed model suggests that in response to mitochondrial depolarization PINK1 accumulates on the mitochondrial membrane and recruits Parkin to promote mitophagy [78, 79]. Therefore, the pathology of mutant PINK1 and/or Parkin in Parkinsonism may arise from the inability to remove dysfunctional mitochondria [99].

The DJ-1 gene encodes a widely expressed protein found in neurons and glia in all central nervous system (CNS) regions [100]. Bonifati first identified an association between DJ-1 and autosomal recessive early-onset PD [46, 101], with numerous further familial mutations in DJ-1 being identified [102]. Reports have shown that DJ-1 is present in mitochondria and protects against oxidative neuronal death [103], DJ-1 deficient mice are more susceptible to MPTP [81] and DJ-1 knockdown in a neuroblastoma cell line renders the cells vulnerable to oxidative stress [104]. Finally, a critical role for DJ-1 in mitochondrial function is shown by a rescue effect of DJ-1 on neuronal cell death caused by PINK1 but not Parkin deletion [83]. Moreover, loss of DJ-1 leads to mitochondrial fragmentation, impaired dynamics, induced oxidative stress, and autophagy [84, 105]. In conclusion, DJ-1 protein has been observed to have pleiotropic functions (see [106] for further review), with an antioxidative role to be the most consistent finding. This may provide a link to PD whereby a loss of function mutation of DJ-1 causes an increase in mitochondrial dysfunction and oxidative damage leading to nigral neuron cell death.

6.3. Other Nuclear Mutations. Omi/Htra2 is a mitochondrially located stress protective serine protease which has been linked to neurodegeneration [107, 108] with loss of function mutations being seen in some PD patients [109]. Although certain studies report no clear genetic association between the Omi/HtrA2 gene sequence variations and PD [110], there is evidence of mitochondrial pathology caused by loss of the Omi/HtrA2 protein function [111]. Omi/Htra2 has also been shown to have links with Pink1 in protecting against oxidative stress [112] and interacting in a prosurvival pathway [113, 114]. Therefore, mutations in this mitochondrially targeted gene may predispose cells to damage via oxidative stress generated from mitochondrial dysfunction.

Leucine-rich repeat kinase 2 (LRRK2) is a large (280 kDa) protein with kinase activities. Mutations in LRRK2 were first linked with autosomal dominant PD by Zimprich et al. in 2004 [115] and represents the most significant cause of autosomal dominant PD. LRRK2 has been shown to be localised to the outer mitochondrial membrane [116, 117] providing an association between mutations in this protein in PD and mitochondrial function. LRRK2 has also been shown to be linked with mitochondria by the finding that Drosophila with LRRK2 mutations show increased susceptibility to the Complex I inhibitor and mitochondrial toxin rotenone and increased protection against DA neuron degeneration by the mitochondrially protective protein Parkin [118].

A loss of function mutation in the lysosomal type 5 P-type ATPase, ATP13A2, has been shown to have links with a hereditary form of autosomal recessive early onset PD with dementia [119]. Gitler et al. reported that functional ATP13A2 can protect against α-synuclein overexpression induced toxicity and that knockdown of ATP13A2, enhances α-synuclein misfolding in neuronal models of PD [120]. With α-synuclein shown to be toxic to Complex I [85], loss of function mutations in ATP13A2, and therefore impaired α-synuclein clearance, could be a link to mitochondrial dysfunction in PD.

PLA2G6 is a calcium-independent phospholipase A2 linked to infantile neuroaxonal dystrophy [121] and neurodegeneration with brain iron accumulation [101] that has recently been implicated in PD [122, 123]. PLA2G6 have been shown to locate to [124], and have a protective role against oxidative stress in [125], pancreatic β-cell mitochondria providing a possible link between loss of function mutations in this protein and mitochondrial dysfunction in PD. How, for example, PLA2G6 regulates iron, a major mitochondrial cofactor is not clear.

6.4. mtDNA Mutations. Besides nuclear DNA mutations, there have been links between mtDNA mutations and PD. Bender et al. [8] and Kraytsberg et al. [126] showed high levels of mtDNA deletions in neurons in the SNpc of PD

Table 1: Summary of mutations and links to mitochondrial dysfunction and PD.

Mutation	Link to mitochondria	Links with PD
α-synuclein	Targeted to IMM and causes CI inhibition	(i) Major component of LBs (ii) Mutation linked with PD
Parkin	Suggested role in mitochondrial function and antioxidant protection	Mutation linked to autosomal recessive juvenile PD
PINK1	Interaction with Parkin	Loss of kinase mutation linked to familial PD
DJ-1	(i) Antioxidant present in mitochondria (ii) Rescues mitochondria from PINK1 deletion	Mutation linked with numerous cases of familial PD
Omi/Hrta2	(i) Mitochondrial location (ii) Interaction with PINK1	Loss of function mutations found in PD patients
LRRK2	(i) Protects against mitochondrial CI toxin rotenone (ii) Increases Parkin mediated cell protection (iii) Located in outer mitochondrial membrane	Mutation linked with autosomal dominant PD
ATP13A2	Leads to lysosomal dysfunction and build up of α-synuclein which is toxic to CI	Mutation linked with hereditary form of PD
PLA2G6	(i) Located in mitochondria (ii) Protect mitochondria against oxidative stress	Mutations linked to neurodegeneration and recently PD
TFAM	(i) Regulates transcription of mtDNA (ii) Knockout mouse has respiratory chain deficiency	(i) Knockout mouse develops PD phenotype (ii) Mutations in some variants give increased risk of PD
POLG1	(i) Involved in the synthesis and regulation of mtDNA (ii) Linked to respiratory chain deficiency in PD	Mutation linked with PD

sufferers. It has been hypothesised that these pathologic DNA rearrangements are not primary drivers of the disease but may be caused by oxidative stress generated during mitochondrial dysfunction, and this may further exacerbate cellular damage [127]. The mitochondrial transcriptional factor A (TFAM) regulates transcription of mtDNA and was linked to PD by the finding that TFAM knockout mice (MitoPark mice) had reduced mtDNA expression and respiratory chain deficiency in SNpc DA neurons, which lead to a Parkinsonian phenotype [128]. Although some studies showed that TFAM mutations do not significantly increase the risk of PD [129, 130], an investigation into the influence of TFAM variants on PD depending on mtDNA haplogroup found certain variants increased the chances of developing PD [131], suggesting a possible role for mtDNA, in some instances, and respiratory chain dysfunction in PD. Mitochondrial DNA polymerase γ 1 (POLG1) is an enzyme involved in the synthesis and regulation of mtDNA and has been shown to have links with PD, including reduced activity of mitochondrial respiratory chain complexes [132, 133]. The involvement of mutations of this protein in PD suggests a role for dysregulation of mtDNA in mitochondrial dysfunction in the disease. However, whether or not there is a common hereditary role for POLG1 in PD needs further study, since a large-scale study does not support this hypothesis [134] (Table 1).

7. Mitochondria and Lewy Body Formation

The histological hallmark of PD is the presence of proteinaceous intraneuronal inclusions termed Lewy bodies (LBs) along with the presence of Lewy neurites (LNs) within neuronal dendrites and axons [135, 136]. Dysfunction of protein metabolism appears to be an important factor in LB formation and the associated neurodegeneration (see for further review [137]), but the significance of these aggregates is still a subject of debate. It is unclear if LBs are pathogenic and mechanistically cause neuronal death [138] or rather the actual function of LBs is neuroprotective by sequestering unwanted, potentially toxic proteins [139–141]. The latter hypothesis is supported clinically by evidence, particularly from autosomal recessive juvenile Parkinson's disease due to Parkin mutation demonstrating that neurodegeneration can occur without the presence of LBs in both apparently sporadic and familial forms of PD [142, 143]. LBs are also reported in cognitively intact individuals over 65 years [144] although this may indicate a prodromal phase of PD prior to clinical presentation.

LBs are typically found in brainstem nuclei and in limbic and neocortical regions in PD and Dementia with Lewy bodies patients (DLB), and additionally, they can be identified in autonomic ganglia in the periphery [145]. There are two morphological types of LBs: brainstem (classic) and cortical Lewy bodies. Classic Lewy bodies are intracytoplasmic eosinophylic inclusions that consist of a dense core surrounded by a pale halo [135]. They are spherical in shape however, a recent report of Kanazawa et al. showed also convoluted LBs and their continuity with LNs, which may suggest evolution from LNs to LBs [146]. Typically, classic LBs are seen in dopaminergic neurons of the substantia nigra and noradrenergic neurons of the locus coeruleus although they were originally described in neurons of the basal forebrain [147]. Cortical LBs are less well-defined structures compared to classic LBs and are

without the halo and are predominantly located in limbic areas of the brain, such as the amygdala, entorhinal, insular, and cingulate cortices [145]. Ultrastructurally, both classic and cortical LBs are composed of filamentous, insoluble in SDS, material resembling neurofilament [148]. Electron microscope examination of Lewy bodies demonstrates that the core contains dense granular material, whereas the outer halo is composed of radiating filaments of 7–20 nm in diameter [135]. Staging of severity of the pathology of PD and DLB based on the distribution of LBs in the brain have been put forward and are used in the classification of the neuropathology of PD and DLB [149].

Immunohistochemical staining and proteomic analyses have deciphered the complexities of the molecular composition of Lewy bodies and Lewy neurites. α-synuclein [135, 150], ubiquitin [151], and neurofilaments [152] appear to be the major components, with α-synuclein representing the most prominent and consistent marker of LB/LN. More recently triple immunolabeling for these epitopes has revealed a three-layered internal structure of LBs and LNs [146]. These primary molecular constituents are stratified into concentric layers with ubiquitin staining in the center, surrounded by α-synuclein and neurofilament on the periphery, which is consistent with previous observations [153].

A large number of mitochondria have been observed in early-stage LBs [132, 154]. Mitochondrial accumulation has also been found in nigral Lewy and pale bodies (possibly precursors of LBs [133]) and cortical LBs but not in classical LBs in PD and in a mouse 26S proteasomal knockout model [132]. This may suggest direct involvement of mitochondria in early stages of LB formation. According to the aggresome-related model of LB formation, mitochondria may be sequestered to the inclusion bodies in order to facilitate the removal of unwanted proteins (see [140] for extensive review). LBs also contain components of the ubiquitin-proteasome system although unlike functional aggresomes, they fail to degrade abnormal proteins but sequester them to delay neuronal death [153].

Proteomic analysis of mitochondrial protein composition of the substantia nigra of mice treated with MPTP compared to controls showed significant changes in numerous protein expression. Of these proteins, DJ-1 protein levels were significantly increased in the toxin-treated mice and also colocalised with α-synuclein in LB-like inclusions in the remaining nigral neurons. Moreover, DJ-1 was present in the halo of classical LBs in nigral tissue of PD patients [155]. Parkin, PINK1, and Omi/HtrA2 have also been localized to the LBs in cases with PD [156–159]. These proteins have been shown to interact and are also involved in proteasomal functions [160, 161]. Thus, there is a potential link between mitochondrial and proteasomal functions, which may influence α-synuclein biology. The interplay may involve oxidative stress and ATP production [161]; however, a wider impact of these cellular pathologies may be speculated. In addition, α-synuclein has been shown to affect both proteasome and mitochondria [162, 163] therefore, a vicious cycle of mitochondrial and proteasomal dysfunction and α-synuclein aggregation in LBs may exist (see for review [164]).

8. Neurotoxins That Link Mitochondrial Dysfunction and PD

There is extensive evidence that PD can be caused by neurotoxins, specifically MPTP [165], rotenone [166], paraquat [167], diquat [168], and 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) [169, 170]. These compounds are thought to act via various mechanisms, but all by causing mitochondrial dysfunction.

8.1. MPTP. The first toxin that linked mitochondrial Complex I inhibition and PD was MPTP (Figure 3). MPTP is produced as a byproduct of the synthesis of a meperidine analogue with heroin-like properties [171]. Langston et al. described in 1983 that users of meperidine reported striking Parkinsonian symptoms and related this to the presence of MPTP [172]. It has also been shown to closely reproduce the DA degeneration and symptoms of PD in various animal experimental models [173, 174] and has been the most widely used toxin in animal models of PD [175]. MPTP readily crosses the blood brain barrier and is converted to the toxic 1-methyl-4-phenyl-2,3-dihydropyridium ion (MPP+) (Figure 3) by monoamine oxidase B in astrocytes [176] and is then taken up into DA neurons by DAT seen as a reduction in MPTP toxicity in DAT deficient mice [177]. MPP+ is taken up into mitochondria via passive transport due to the large mitochondrial transmembrane gradient [178], where MPP+ inhibits mitochondrial Complex I [179]. This inhibition of Complex I leads to cell death via energy deficits [180], free radical and ROS generation [181], and possibly excitotoxicity [182]. In an MPTP mouse model of PD, α-synuclein is nitrated [183], providing another link between MPTP and PD. However, despite all of the evidence of links between MPTP and PD, there are differences between MPTP models of PD and idiopathic PD with variations in disease progression, an acute onset, and the lack of typical LB formation [184].

8.2. Rotenone. Rotenone (Figure 3) is a widely used pesticide and naturally occurring neurotoxin that has been found to have links to PD [166]. Rotenone is highly lipophilic and able to easily cross the blood brain barrier and enter neuronal cells and intracellular organelles, such as mitochondria, without the aid of transporters. Rotenone specifically blocks the ubiquinone binding site of Complex I, preventing the transport of electrons from Complex I to ubiquinone leading to the release of free radicals into the mitochondrial matrix and ROS formation [34]. This evidence that rotenone is a specific inhibitor of Complex I, which in turn leads to ROS production and oxidative stress, can also cause PD-like behavioural symptoms such as akinesia and rigidity in rats [185]. Rotenone administration has been shown to oxidatively modify DJ-1 and cause α-synuclein aggregation, which are effects linked to PD and localised to the DA neurons of the SNpc [186]. Betarbet et al. reported LB-like ubiquitin and α-synuclein containing cytoplasmic inclusions in the brains of rotenone treated rats [57]. This evidence, taken together, presents a strong link between rotenone exposure, mitochondrial dysfunction and PD. However,

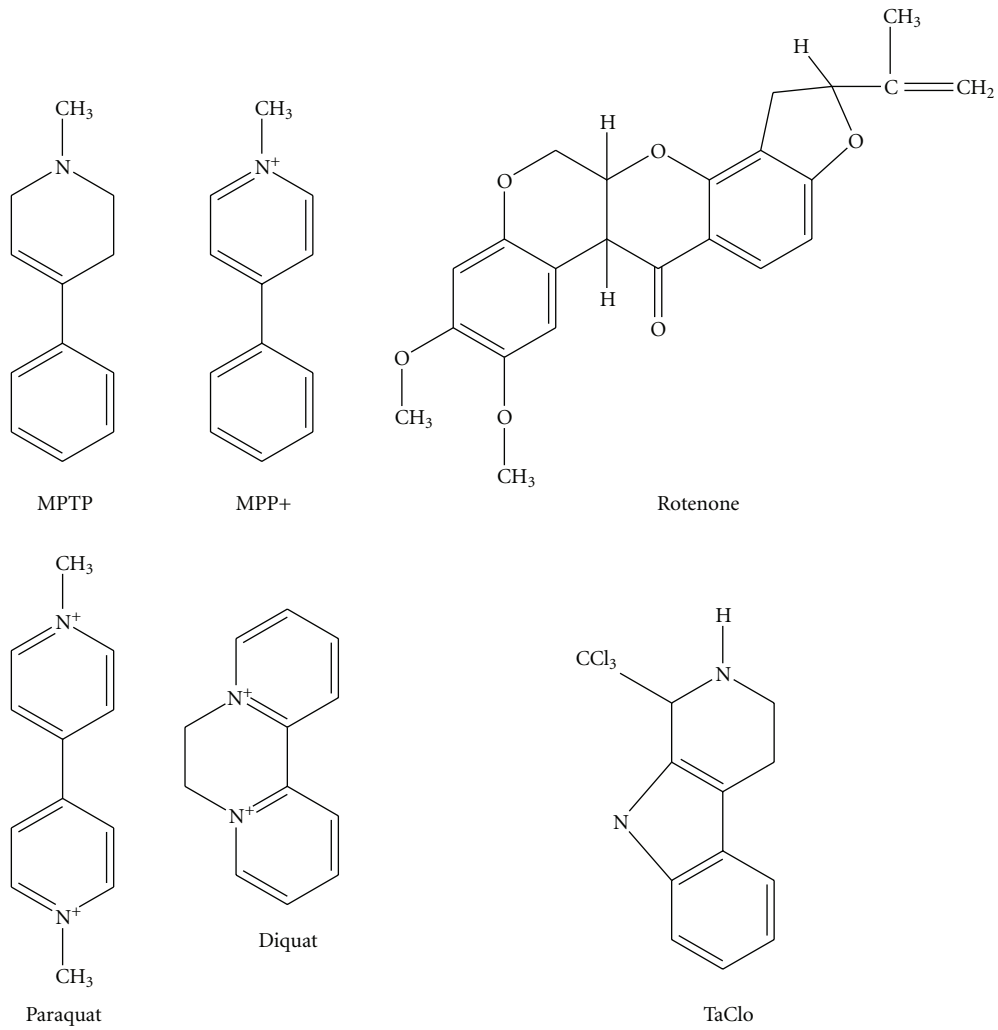

Figure 3: Structures of the PD-linked neurotoxins MPTP/MPP+, Rotenone, Paraquat, Diquat, and TaClo.

there is also evidence to suggest rotenone causes damage to neurons in the striatum but not SNpc [187], suggesting that it may not be an entirely accurate model of PD.

8.3. Paraquat and Diquat. Paraquat and diquat are widely used herbicides shown to have links with PD [167, 168]. They are hydrophilic compounds and, therefore, do not readily cross the blood brain barrier and the mechanism by which they enter the brain is unclear although uptake by a neutral amino acid [188] or polyamine [189] transporters has been suggested. Paraquat and diquat have very similar structures to MPTP and MPP+ (Figure 3), suggesting a similar toxic mechanism. However, unlike rotenone and MPP+, paraquat does not inhibit Complex I and is not taken up by DAT, suggesting an alternative mechanism of cell death [190]. A toxic mechanism is described where paraquat is reduced to the paraquat radical by Complex I causing accelerated lipid peroxidation [191, 192]. An alternative theory whereby paraquat is reduced to the paraquat radical by Complex II has also been reported [193]. A final hypothesis for the formation of the paraquat radical is that paraquat is reduced by NADPH-cytochrome p450 reductase [194] or NADPH-cytochrome c reductase [195] in the cell. Whichever complex or enzyme is involved, the paraquat radical can react with oxygen to form $\cdot O_2^-$ and cause oxidative stress and mitochondrial dysfunction [192]. This mechanism of cell death may target the DA neurons specifically because of their constant state of oxidative stress leaving them more vulnerable than other cells. It has also been shown that paraquat can lead to an increase in both α-synuclein levels and aggregation [196]. Although the exact mechanism has yet to be elucidated, mitochondrially derived paraquat radical, combined with its effects on α-synuclein, indicate paraquat toxicity as a possible mitochondrially mediated form of PD. Diquat also generates oxygen radicals in rat brain microsomes [197]; a discovery that, when coupled with its similarity in structure and properties with paraquat, suggests a possible similar role for diquat in mitochondrial dysfunction in PD.

8.4. TaClo. β-carbolines such as TaClo have structures similar to that of MPTP (Figure 3) and may be neurotoxic

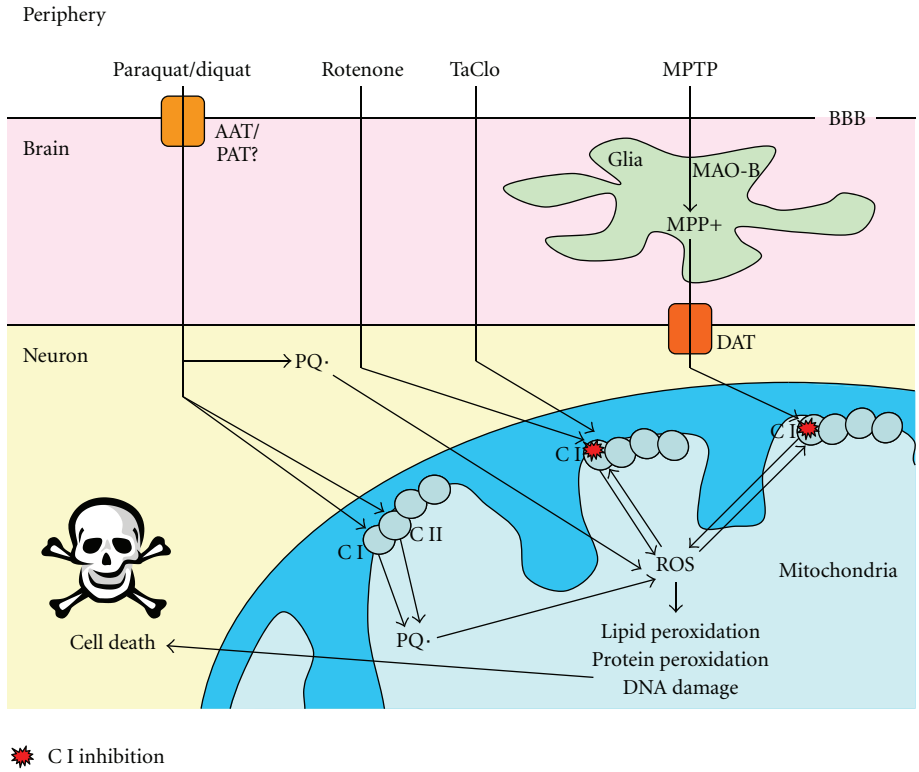

Figure 4: Mechanisms of mitochondrial dysfunction-mediated cell death generated by neurotoxins: Paraquat crosses the blood brain barrier (BBB) by an as yet unclear mechanism, possibly via an amino acid transporter (AAT)/polyamine transporter (PAT), where it enters the cell and is spontaneously reduced to the paraquat radical PQ· or reduced by Complex I or II (CI or II) and can then form reactive oxygen species (ROS). Rotenone and TaClo enter neurons and can cause CI inhibition which leads to the production of ROS. MPTP enters the brain, where it is converted to MPP+ in glial cells by monoamine oxidase-B (MAO-B) which is transported into neurons by the dopamine transporter (DAT). Once in the cell, MPP+ also causes CI inhibition and generation of ROS. ROS formed by these environmental toxins can then exacerbate the CI inhibition as well as causing lipid and protein peroxidation, DNA damage, and, ultimately, cell death.

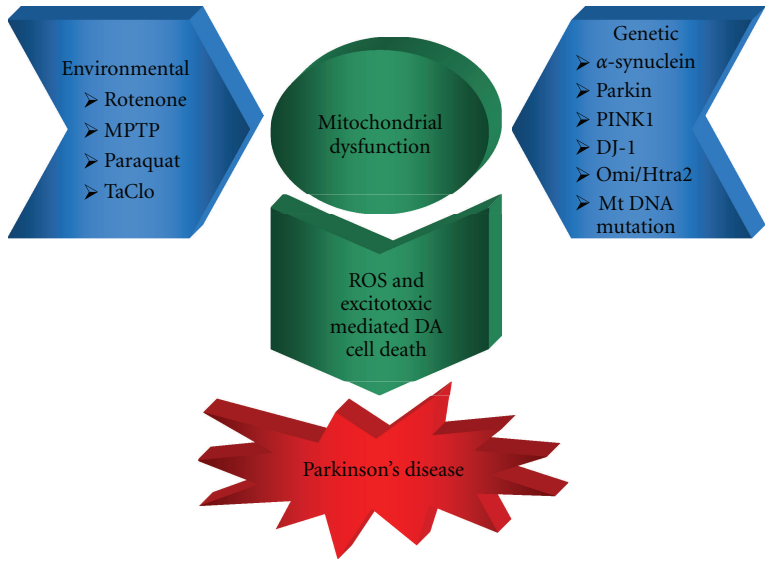

Figure 5: Schematic representation of mitochondrial dysfunction in Parkinson's disease: environmental and genetic factors combine to cause mitochondrial dysfunction leading to ROS- and excitotoxic mediated DA cell death and Parkinson's disease.

for DA neurons and lead to a PD-like syndrome [169, 170]. Trichloroethylene (TCE) is an industrial solvent used as a degreasing agent and in dry cleaning. TCE is a major environmental contaminant in the air, the water system and soil, and there is, therefore, exposure at low levels to various groups in the population. It has been discovered that TCE can be converted to the β-carboline TaClo in man [198]. TaClo can cross the blood brain barrier following intraperitoneal injection in the rat [199]. Rausch et al. found that micromolar concentrations of TaClo caused up to 50% cell death in primary C57/B16 mouse DA mesencephalon cultures [200]. This is supported by *in vivo* studies showing a reduction in DA metabolism [201], with a progressive reduction in locomotion and increase in apomorphine-induced rotations in behavioural tests [202–204] and the discovery of a strong inhibition of mitochondrial Complex I *in vitro* [205]. Taken together, these data present a strong case for the neurotoxic properties of TaClo against DA neurons. Interestingly, further work showed that N-methyl-TaClo is a more potent inhibitor of mitochondrial Complex I and more neurotoxic than TaClo itself [206]. This evidence suggests that TaClo or another related toxin could cause PD with mitochondrial dysfunction playing a central role. The mechanisms of environmental toxin induced neuronal cell death are summarised in Figure 4.

However, this evidence of mitochondrial Complex I inhibition leading to neuronal cell death in PD following toxin exposure requires further investigation following the findings of Choi et al. that midbrain neurons without Complex I activity were still susceptible to cell death following rotenone, MPTP, or paraquat treatment [207]. This suggests that mitochondrial dysfunction could either be occurring parallel to another nonmitochondrial cell death mechanism or as a secondary effect driven by some other cellular stress or damage such as toxic protein accumulation due to ubiquitin-proteasome system impairment [208], inflammation [209], or DNA damage [210].

9. Conclusions

This paper demonstrates a considerable body of evidence linking mitochondrial dysfunction, specifically respiratory chain inhibition, with neuronal cell death in the SNpc of PD patients. Many of the mutations related with PD have been shown to involve mitochondrial proteins or proteins linked to mitochondrial dysfunction. Furthermore, neurotoxins that can cause a PD-like syndrome are strong inhibitors of the mitochondrial electron transfer chain (see Figure 5). However, the exact mechanisms of cell death in sporadic PD are still unclear and it has not been conclusively proved whether mitochondrial dysfunction is a primary driver of cellular stress and damage in the disease or a secondary consequence of another insult.

Further investigation should be carried out into how genes implicated in PD, such as UCH-L1 [211], as well as those mentioned in this paper, may affect mitochondrial function and how mutations in these genes could lead to mitochondrial defects. Although slightly out of the scope of this paper, it would also be of interest to explore how reported crosstalk between mitochondria and endoplasmic reticulum [212] may be involved in cell death control, the processing of proteins and Ca^{2+} homeostasis, and how any deficits in these systems could impact on PD pathology.

Abbreviations

AAT: Amino acid transporter
ATP: Adenosine triphosphate
CNS: Central nervous system
DA: Dopaminergic
DAT: Dopamine transporter
DLB: Dementia with Lewy bodies
e^-: Electron
GSH: Glutathione
H^+: Proton
H_2O_2: Hydrogen peroxide
ILBD: Incidental Lewy body disease
LB: Lewy body
LN: Lewy Neurites
LRRK2: Leucine-rich repeat kinase 2
MnSOD: Manganese superoxide dismutase
MAO: Monoamine oxidase
MPP+: 1-methyl-4phenyl-2,3-dihydropyridium ion
mtDNA: Mitochondrial DNA
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA: N-methyl-D-aspartate
NO·: Nitric oxide radical
NOS: Nitric oxide synthase
$\cdot O_2^-$: Superoxide radical
·OH: Hydroxide radical
PARP: poly(ADP-ribose) polymerase
PAT: Polyamine transporter
PD: Parkinson's disease
POLG1: Mitochondrial DNA polymerase γ 1
ROS: Reactive oxygen species
SNpc: Substantia nigra pars compacta
TaClo: 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline
TCE: Trichloroethylene
TFAM: Mitochondrial transcriptional factor A.

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Review Article

The Nrf2/ARE Pathway: A Promising Target to Counteract Mitochondrial Dysfunction in Parkinson's Disease

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Mitochondrial dysfunction is a prominent feature of various neurodegenerative diseases as strict regulation of integrated mitochondrial functions is essential for neuronal signaling, plasticity, and transmitter release. Many lines of evidence suggest that mitochondrial dysfunction plays a central role in the pathogenesis of Parkinson's disease (PD). Several PD-associated genes interface with mitochondrial dynamics regulating the structure and function of the mitochondrial network. Mitochondrial dysfunction can induce neuron death through a plethora of mechanisms. Both mitochondrial dysfunction and neuroinflammation, a common denominator of PD, lead to an increased production of reactive oxygen species, which are detrimental to neurons. The transcription factor nuclear factor E2-related factor 2 (Nrf2, NFE2L2) is an emerging target to counteract mitochondrial dysfunction and its consequences in PD. Nrf2 activates the antioxidant response element (ARE) pathway, including a battery of cytoprotective genes such as antioxidants and anti-inflammatory genes and several transcription factors involved in mitochondrial biogenesis. Here, the current knowledge about the role of mitochondrial dysfunction in PD, Nrf2/ARE stress-response mechanisms, and the evidence for specific links between this pathway and PD are summarized. The neuroprotection of nigral dopaminergic neurons by the activation of Nrf2 through several inducers in PD is also emphasized as a promising therapeutic approach.

1. Introduction

Parkinson's disease (PD) is a prevalent and progressive neurodegenerative incurable movement disorder. The disease is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of proteinaceous deposits within neuronal perikarya (Lewy bodies) and processes (Lewy neurites). These deposits are composed of α-synuclein, ubiquitin, neurofilaments, and molecular chaperones [1]. Nigral dopaminergic neuron death leads to a deficiency of the neurotransmitter dopamine in the striatum and consequent dysregulation of basal ganglia circuitries accounting for motor symptoms of bradykinesia, hypokinesia, progressive rigidity, resting tremor, and postural instability. Lewy body deposition is also associated with nonmotor features such as autonomic dysfunction, sleep disturbances, depression, and cognitive impairment [1, 2]. A major risk factor is aging, as in other neurodegenerative diseases, although people with the familial monogenic forms of the disease can present before 45 years of age [1]. Since the sporadic and monogenic forms of PD share important clinical, pathological, and biochemical features, notably the progressive demise of dopaminergic neurons in the substantia nigra (SN), inherited mutations underlying familial forms have provided insight into the molecular mechanisms of disease pathogenesis [1, 3]. Oxidative stress, neuroinflammation, mitochondrial dysfunction, aberrant protein aggregation, excitotoxicity, and alterations in the autophagic-lysosomal pathway are implicated in the development and progression of PD [4–6]. PD also seems to have a mitochondrial component, so events that would modulate normal mitochondrial functions may compromise neuronal survival.

Nuclear factor E2-related factor 2 (Nrf2, NFE2L2) is a master regulator that induces a battery of cytoprotective genes including antioxidative enzymes, anti-inflammatory

mediators, the proteasome, and several transcription factors involved in mitochondrial biogenesis. Thus, it may be a promising target to counteract mitochondrial dysfunction and its consequences in PD. Following its nuclear translocation, Nrf2 binds the antioxidant response elements (AREs) in the promoter region of its target genes. Several lines of evidence including in vivo Nrf2-deficient mouse studies, postmortem studies of PD brains, and genetic association studies of patients indicate a link between Nrf2 dysregulation and PD pathogenesis. Endogenous responses to upregulate Nrf2 and mitochondrial biogenesis in PD may be insufficient to prevent the progression of neurodegeneration. Thus, further activation of the Nrf2/ARE system using exogenous inducers may be a plausible therapeutic approach for PD.

This paper provides a brief overview of mitochondrial dynamics, mitochondrial dysfunction in PD, and the Nrf2/ARE system. In addition, recent studies indicating a link between Nrf2 and PD are summarized. Finally, recent efforts employing inducers of Nrf2 activity to provide neuroprotection of nigral dopaminergic neurons in PD are emphasized.

2. The Functions, Biogenesis, and Dysfunction of Mitochondria

Mitochondria are membrane-enclosed organelles, which uniquely contain genetic material independent of nuclear DNA. These 1–10 μm long cellular structures contain their own genome, which encodes tRNAs, rRNAs and 13 mitochondrial proteins [7, 8], while other components of the mitochondrial respiratory chain are encoded by the nuclear genome [9]. Multiple copies of the circular DNA are present in each mitochondrion. In humans, the size of the mitochondrial genome is 16,569 bp and encodes for 2 ribosomal RNA molecules (16S and 12S rRNA) and 22 transfer RNA molecules [9, 10].

The structure of a mitochondrion includes outer membrane, intermembrane space, inner membrane, and matrix [8]. The topography of the inner membrane is complex and includes the electron transport system complexes, the adenosine triphosphate (ATP) synthetase complex, and transport proteins [11]. Mitochondria produce most of the cell's energy in the form of ATP through oxidative phosphorylation (OXPHOS). Depending on this process, there are five intramembrane complexes and two mobile electron carriers, called coenzyme Q and cytochrome *c* [12]. Cytochrome *c,* which is an essential component of the electron transport chain, is located in the outer face of the inner membrane (intermembrane space) of the mitochondrion [13]. Cristae are structures that are formed by the folding of the inner membrane and provide increased surface area for chemical reactions, which take place in mitochondria [14].

Mitochondrial biogenesis is a process that involves interaction between the genetic systems of the organelle and the nucleus. During this process, mitochondria are newly formed in the cell. Many different signals can activate mitochondrial biogenesis. The main regulators of mitochondrial biogenesis include the peroxisome proliferator-activated receptor gamma coactivator (PGC) family of transcriptional activators, which consists of PGC-1α, PGC-1β, and PGC-related coactivator (PRC) [15]. PGC-1α plays a role in the activation of nuclear respiratory factor 2 and together they coactivate nuclear respiratory factor 1. Consequently, nuclear respiratory factor 1 activates Tfam, which is important for mitochondrial DNA (mtDNA) transcription, translation, and repair. Thus, PGC-1 family coactivators act as mediators between the environment and the transcriptional machinery regulating the biogenesis of mitochondria [16].

Mitochondria also have roles in signaling, cellular differentiation, cell growth and cell death [17]. Dysfunction of the mitochondria is implicated in primary mitochondrial disorders, cardiac dysfunction, and the aging process. As one of the main sources of reactive oxygen species (ROS), these organelles can themselves be affected by oxidative damage, potentially membrane permeability transition, affecting the aging process and disease pathogenesis [18]. Oxidative damage to mitochondrial macromolecules can also transform into an apoptosis mechanism, which is the process of programmed cell death. Thus, many apoptotic responses occur in mitochondria which are altered in electron transport, the loss of membrane potential, the changes in oxidation-reduction potential, the release of caspase regulators, and the attendance of pro- and anti-apoptotic Bcl-2 family proteins. Therefore, it is plausible that the effects of mitochondrial disruptions can play a role in human aging processes and degenerative diseases such as PD [19]. Sometimes, mitochondria containing a mutated genome are removed from the cell by mitophagy, a term for autophagy of the mitochondria [20].

3. Mitochondrial Dysfunction in Parkinson's Disease

The combination of mitochondrial dysfunction and increased oxidative stress are assumed to assist in the pathogenesis of PD [21]. Many lines of evidence suggest that mitochondrial dysfunction plays a central role in the pathogenesis of PD. The first observation in the early 1980s was that an inhibitor of complex I of the electron transport chain can induce parkinsonism. Moreover, mitochondria are targeted for the actions of parkinsonian neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and its metabolite 1-methyl-4-phenylpyridinium (MPP^+), 6-hydroxydopamine (6-OHDA), rotenone, and paraquat [22, 23].

Deficiency in mitochondrial respiratory chain complex I and cristae disruption have been consistently described in PD [24]. It has been known that mitochondrial respiratory complex I (NADH-quinone oxidoreductase) activity declines in the SNpc of PD patients [25, 26]. Moreover, the first transgenic mouse model for complex I deficiency has recently been generated. However, in this model, ATP levels were at the normal level, and oxygen consumption was not affected in NADH:ubiquinone oxidoreductase iron-sulfur protein 4 (NDUFS4) knocked-out mice. Furthermore, ROS formation in the neuronal cultures derived from those mice did not increase as was expected. It is clear that more in vivo

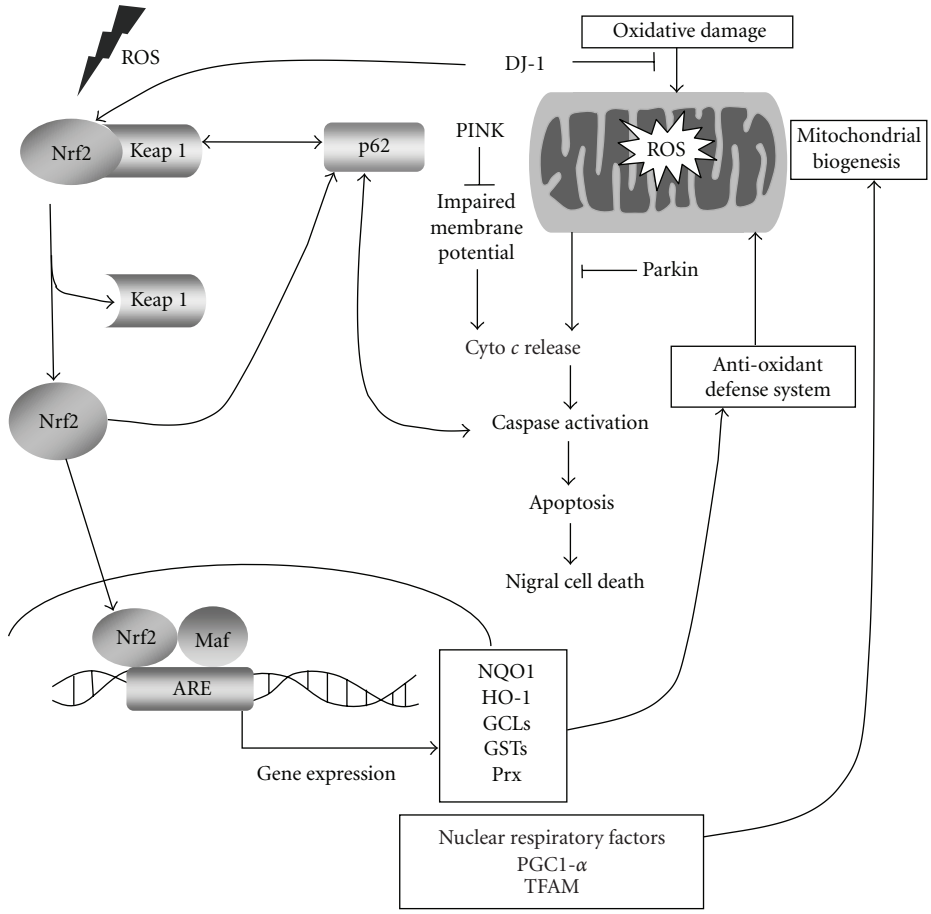

Figure 1: The role of the Nrf2 pathway in PD pathogenesis. In the case of oxidative damage, mitochondria produce increased amounts of ROS. Then, ROS activate Nrf2 bound to Keap1 in the cytoplasm, and it translocates into the nucleus to transactivate the transcription of ARE-bearing genes, which in turn activates the antioxidant defense system and mitochondrial biogenesis. In this pathway, PD-related genes are also involved. DJ-1 is found to inhibit oxidative damage in mitochondria. Another PD-related gene is PINK, which prevents impaired membrane potential of mitochondria and prevents apoptosis by counteracting cytochrome *c* release that leads to apoptosis of nigral cells. Lastly, Parkin was found to inhibit cytochrome *c* release that leads to caspase activation and apoptosis of nigral cells. Ubiquitin-interacting p62 also has a role in Nrf2 activation. It normally plays a role in transportation of ubiquitinated proteins to autophagosome. p62 was found to be interacting with Keap1 and transports it for autophagic degradation and provides indirect activation of Nrf2. p62 also has ARE in its promoter region which creates a positive feedback loop between Nrf2 and p62.

models are needed in order to explain the role of complex I deficiency in PD [27].

As in vivo evidence of mitochondrial dysfunction in PD, Hattingen et al. used a combination of anatomical magnetic resonance imaging and phosphorus and proton magnetic resonance spectroscopic imaging. They reported the presence of mitochondrial dysfunction of nigrostriatal dopaminergic neurons (see Figure 1). They also suggested that mitochondrial dysfunction phenomenon occurs in the early stages of PD pathogenesis [28].

mtDNA has an increased susceptibility to mutations because of less efficient DNA-repair mechanisms and the absence of protective histones. There are several clinical studies indicating a link between PD and specific mtDNA point mutations [29]. There is also a link between mtDNA and neurodegeneration, which was verified by genetic mouse models. Mitochondrial transcription factor A (Tfam) is a gene encoding the Tfam protein, which plays a role in promoters within the D-loop region of mtDNA and provides regulation of the transcription and replication [30]. Both nuclear respiratory factor 1 and nuclear respiratory factor 2 can adjust the expression of the Tfam gene by attaching to consensus-binding sites. When a conditional Tfam knockout mouse in midbrain dopaminergic neurons was generated, decreased mtDNA expression, respiratory chain deficiency, and neuronal cell death leading to staggered L-dopa-responsive impairment of motor functions were observed [31].

Remarkably, recent studies indicate that several PD-associated genes, directly or indirectly, impinge on mitochondrial integrity, thereby providing a specific link to the mitochondrial dysfunction observed in sporadic PD [3, 32]. The protein products of PD genes, including alpha-synuclein, Parkin, PTEN-induced kinase 1 (PINK1),

DJ-1, leucine-rich repeat kinase 2 (LRRK2) and HTR2A are localized to the mitochondria under certain conditions [33]. Functional studies in animal and cellular model systems have shown that PINK1 and Parkin play important roles in maintaining mitochondrial integrity and regulate mitochondrial morphology [3]. Loss-of-function mutations in the genes for these proteins are the primary cause of early-onset autosomal recessive forms of PD [1]. Parkin is an E3 ubiquitin-protein ligase, and impaired proteasome function has been described in sporadic PD. Parkin, therefore, promotes the degradation of dysfunctional mitochondria in cell culture conditions [34]. PINK1 represents the only kinase known to exhibit a canonical N-terminal mitochondrial localization signal [35]. These proteins are involved in mitophagy, the degradation process of terminally dysfunctional mitochondria in the lysosome [3, 32, 36]. Mutations in LRRK2 cause autosomal-dominant familial PD. This kinase modulates vulnerability to mitochondrial dysfunction induced by neurotoxins [37]. Furthermore, DJ-1 localizes to mitochondria during oxidative stress, where it exhibits peroxiredoxin-like activity [38]. Mutations in DJ-1 render animals and cultured cells more susceptible to oxidative stress and mitochondrial toxins implicated in sporadic PD, lending support to the hypothesis that some PD cases may be caused by gene-environmental factor interactions [39, 40]. A small proportion of alpha-synuclein is imported into mitochondria, where it accumulates in the brains of PD patients and may impair respiratory complex I activity [41]. Alpha-synuclein binds to mitochondria and leads to mitochondrial fragmentation [42].

4. The Nrf2/ARE Pathway

In pathology of neurodegenerative disorders, the generation of ROS may be harmful affecting proteins, lipids, and nucleic acids [45]. ROS itself regulates redox homeostasis activating a group of genes and signal transduction pathways [46]. The Nrf2 pathway is one of the pathways that respond to ROS by activating the transcription of phase II detoxification enzymes [47]. It was first identified by Moi et al. in 1994, as controlling the expression of β-globin gene [47]. It belongs to the cap and collar family of transcription factors having a distinct basic leucine-zipper motif [48]. Nrf2 is found in the cytosol bound to its inhibitor kelch-like ECH-associated protein (Keap1). When redox balance is tipped toward the oxidative side, Nrf2 translocates into the nucleus and activates the transcription of ARE-containing genes [49]. During this activation, Keap1, which is sensitive to electrophilic and oxidative stimuli, regulates Nrf2 modification, holding Nrf2 in the cytosol along with actin filaments [50]. Unless it is activated, Nrf2 is ubiquitinated by the E3-ubiquitin ligase-like domain of Keap1, followed by 26S proteasomal degradation [50]. Therefore, Keap1 regulates Nrf2 negatively, by promoting its sequestration and degradation.

Nrf2 is regulated by interactions between the conserved motifs DLG and ETGE within the Neh2 domain of Nrf2, the domain which regulates cellular stress responses, and the DGR region on Keap1. All of these domains provide correct positioning of Nrf2 for ubiquitination. When ROS accumulation increases in the cell, the binding to the DLG motif weakens; thus, ubiquitination is prevented [51–53]. In this way, degradation of Nrf2 decreases and stability of Nrf2 increases [43]. Extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways were shown to regulate Nrf2 transcriptional activity with an unknown mechanism. ERK and JNK appear to regulate positively the Nrf2 pathway after ARE-inducing substances, whereas p38 MAPK was reported to regulate Nrf2 both positively and negatively [43, 54]. ERK, JNK, and p38 MAPK act by phosphorylating N-terminal serine residues on Nrf2, which results as a response to electrophiles and oxidative stress [55]. Moreover, one of main signaling pathways, cytoprotective phosphotidylinositol-3 kinase (PI3K), which regulates maintenance of cellular homeostasis, was shown to regulate hemeoxygenase-1 (HO-1) under inflammatory stress [56]. Thus, it is proposed that the PI3K/Akt signaling pathway acts upstream of Nrf2 signaling. There are different upstream mechanisms, other than MAPK and PI3K/Akt, which are pancreatic endoplasmic reticulum kinase (PERK) and protein kinase C (PKC). In the case of redox imbalance, ROS can induce endoplasmic reticulum (ER) stress resulting in improper folding of proteins. There are different mechanisms to deal with this, one of which is PERK. Cullinan et al. found that Nrf2-target genes are activated in ER stress proposing PERK as the activator of Nrf2 [57]. Another Nrf2 mediator is PKC, which was first found by Huang et al. in 2000. As a result of inhibition experiments, they proposed that PKC is an upstream activator of Nrf2 signaling [58].

There are two hypotheses to explain the mechanism of Nrf2 translocation into the nucleus. According to the first hypothesis, there is nuclear localization signal in its basic region and two nuclear export signals in its leucine zipper and transactivation domain in order to provide Nrf2 nuclear translocation. In normal conditions, the nuclear localization signal is balanced by the nuclear export signal, and the sequestration of Nrf2 occurs in the cytosol. However, in stress conditions, the redox sensitive nuclear export signal found in the transactivation domain is disrupted. Thus, the nuclear localization signal drives translocation into the nucleus [59, 60]. However, this hypothesis fails to explain constitutive transcription of drug-metabolizing enzymes under normal conditions. For this reason, an alternative hypothesis was proposed. In this second hypothesis, Nrf2 is constitutively expressed and translocates into the nucleus. Its regulator and Keap1 enter into the nucleus by the CRM1/exportin pathway for removal and degradation of Nrf2, and after degradation it gets back into the cytosol. Consequently, under oxidative stress conditions, Nrf2 is expressed at the normal rate; however, its degradation rate decreases. In this way, its nuclear accumulation and transcriptional activity are enhanced [61, 62]. In addition to the above hypotheses, a novel ubiquitin-binding protein, p62 (sequestosome 1, SQSTM1), was recently shown to activate Nrf2. Studies suggest that p62 interacts with the Keap1-binding site of Nrf2, resulting in activation and triggering of downstream events of the Nrf2 pathway [63, 64].

In cells, there are mechanisms to deal with oxidative stress, including phase II detoxification enzymes, which

Table 1: Nrf2 target genes.

Group	Gene symbol	Gene name	Function
Glutathione homeostasis	GSH	γ-glutamyl-cysteinyl-glycine	Maintains redox homeostasis during oxidative stress
	GST	Glutathione-S-transferase	Cellular detoxification
	Gcl	Glutamate cysteine ligase	Catalyzes glutathione synthesis
	Gcs	Glutamate cysteine synthetase	Catalyzes glutathione synthesis
	GS	Glutathione synthetase	Catalyzes glutathione synthesis
	GPx	Glutathione Peroxidase	Catalyzes reduction of H_2O_2 or organic hyperoxides to water
	GR	Glutathione Reductase	Catalyzes reduction of oxidized GSSH to GSH
Drug metabolism	NQO-1	NAD(P)H quinone oxidoreductase-1	Catalyzes two-electron reduction of quinones
	Ugt	UDP-glucuronosyltransferases	Catalyzes endogenous and exogenous substances with glucuronic acid
	mEH	Microsomal epoxide hydrolase	Inactivates epoxides converting to vicinal dihydrodiol
Stress response proteins/iron metabolism	Ferritin	Ferritin	Iron binding protein having role in iron oxidative stress
	HO-1	Heme oxygenase-1	Catalyzes oxidative cleavage of Fe-protoporphyrin-IX
Excretion/transporter	Mdr	Multi-drug resistance protein	Drug efflux pump for xenobiotic compounds
	Mrp	Multidrug resistance associated protein	Multispecific organic anion transporter
Other genes metabolism	G6PDH	Glucose-6-phosphate dehydrogenase[a]	Glycolysis
	Taldo	Transaldolase[a]	Pentose phosphate pathway enzyme
	Tkt	Transketolase[a]	Channeling of excess glucose phosphates to glycolysis
Immune system	Pafah	PAF acetylhydrolase[a]	Catalyzes the degradation of platelet-activating factor to biologically inactive products
	Ptgs2	Prostaglandin-endoperoxide synthase 2[a]	Prostaglandin biosynthesis
	Dig	Dithiolethione-inducible gene[a]	Inhibition of chemically induced tumorigenesis
	Tac2	Tachykinin 2[a]	Peptide neurotransmitter
Calcium homeostasis	Calb1	Calbindin-28K[b]	Calcium binding protein
	Syt1	Synaptotagmin-1[b]	Synaptic transmission
	Hpca	Hippocalcin[b]	Calcium binding protein
	S100A1	S100 calcium binding protein A1[b]	Calcium binding protein

Table 1: Continued.

Group	Gene symbol	Gene name	Function
Growth factor	Ngfg	Nerve growth factor-γ[b]	Growth factor for neuron survival
	FGF-13	Fibroblast growth factor-13[b]	Nervous system development and function
	FGF-14	Fibroblast growth factor-14[b]	Nervous system development and function
	BDNF	Brain-derived neurotrophic factor[b]	Growth factor for neuron survival
Intracellular signaling	nGEF	Neuronal GEF[b]	Intracellular signaling networks
	Prkcb	Protein Kinase C-β[b]	Signal transduction
	Gng3	G-protein-γ3[b]	Signal transduction
	Adm	Adrenomedullin[b]	Adrenal development
	Crh	Corticotropin-releasing hormone[b]	Hormone released in response to stress
Neurotransmission/channel	Clcn	Chloride channel[b]	Ion channel protein
	Gabr1	GABA-A receptor-1[b]	Neurotransmitter receptor
	Gabrg3	GABA-A receptor, gamma 3[b]	Neurotransmitter receptor
	Gabbr1	GABA-B receptor-1[b]	Neurotransmitter receptor

Nrf2 activates a battery of ARE-driven genes, also known as classic Nrf2-target genes, which are classified into 4 groups as glutathione homeostasis, drug metabolism, stress-response protein/iron metabolism, and excretion/transporter [43]. In addition to classic genes, microarray experiments revealed cell-type specific target genes. From (a) primary astrocyte and (b) primary neuronal cultures, novel targets were identified [44]. They also are stated as other genes.

have ARE in its promoter or enhancer regions. According to genetic analyses, the ARE sequence is found in many genes, which play important roles in gene regulation [65, 66]. Nrf2 also interacts with the AP-1 family proteins Jun, Fos, and Maf. Maf binds to the maf recognition element (MARE), which is very similar to the core sequence of ARE [67–70]. However, maf proteins are reported to regulate ARE-containing genes negatively [69]. Furthermore, some maf genes, MafF, and MafG modulate the expression of ARE-containing genes [48]. Overexpression studies on Jun revealed that Jun acts as a positive regulator of the transcription of ARE-regulated genes. Moreover, according to the studies which unravel the relationship between Fos and Jun, it was concluded that Fos acts as negative regulator, in an opposite fashion to Jun [71].

Genes transcribed after Nrf2 activation are called the "Nrf2 regulon." This regulon performs several cellular functions, such as drug metabolism, ROS scavenging, glutathione homeostasis, efflux transport pathways, and activation of stress response proteins, which are vital for redox homeostasis [61, 72]. In the last decade, the role of Nrf2 in disease pathogenesis and toxic insults was shown to be protective, and it is confirmed to be a multiorgan protector [44]. The Nrf2 regulon genes can be classified into various classes based on their functions: glutathione homeostasis, drug metabolism, stress-response protein or iron metabolism and excretion/transporter. The functions of each gene group and genes are summarized in Table 1. In some studies, microarray analysis of the Nrf2 regulon revealed more genes than classical ARE-containing genes by comparing expression patterns of neurons and astrocytes, which are indicated as other genes in Table 1 [44]. In addition to the genes listed in Table 1, we performed *in silico* transcription factor binding analysis using the JASPAR database by searching for ARE sequence in the promoter [73]. Novel Nrf2 targets were obtained, such as nuclear respiratory factor-1, Tfam, and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1A). Nuclear respiratory factor-1 was experimentally validated to be a target gene for Nrf2 activation, which leads to mitochondrial biogenesis. In this way, apoptosis and necrosis can be prevented [74]. Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a protein that has many beneficial effects including anti-inflammation in multiple tissues. Cho et al. first showed the regulation of PPAP-γ by Nrf2. They showed antioxidant effects of both Nrf2 and PPAR-γ, and they suggested that a PPAR-γ agonist can be used to combat oxidative damage [75].

5. The Nrf2/ARE Pathway and Parkinson's Disease

Several lines of evidence suggest the involvement of the Nrf2/ARE pathway in the pathogenesis of PD. The first evidence came from a postmortem study that examined the expression and localization of Nrf2 in susceptible neuron populations in AD and PD brain tissues [76]. Ramsey et al. showed that in neurodegenerative diseases, Nrf2 expression is altered in both neurons and astrocytes. As compared with age-matched normal controls, nuclear Nrf2 staining was decreased in hippocampal CA1 neurons and surrounding glia in AD brains, whereas nuclear localization of Nrf2 was induced in SN in PD brains, even though the response

appeared insufficient to protect neurons from degeneration. Consistent with the findings of the study by Ramsay et al., increased glutathione was found in PD patients, probably reflecting a response to oxidative damage [77]. Another study by Spencer et al. found decreased glutathione levels [78]. Since Nrf2 regulates several enzymes involved in glutathione synthesis, increased Nrf2 in surviving PD nigral neurons could be interpreted as an appropriate neuronal response to oxidative stimuli, even though the response appears insufficient to protect neurons from neurodegeneration. Similarly, in the MPTP model of PD, ARE-dependent gene expression was decreased in striatum whereas it increased in SN [79]. Because subcellular trafficking is critical to the activity of the Nrf2/ARE pathway, Ramsey et al. concluded that disrupted or insufficient ARE responses likely occur downstream of Nrf2 nuclear localization in PD [76]. Additional downstream mechanisms may interfere with ARE transactivation despite nuclear stabilization of Nrf2. Further investigation is needed to understand why Nrf2 nuclear translocation is not sufficient to prevent oxidative stress or ongoing neurodegeneration in SN. Ramsey et al. did not report increased nuclear Nrf2 staining in surrounding glia in SN in postmortem brains from PD individuals and questioned the necessity of astroglial Nrf2 activation in neuroprotection. In reduced in vitro cell culture systems, increased neuronal Nrf2 activation protects neurons from the oxidative insult induced by parkinsonian neurotoxins such as paraquat, 6-OHDA, MPP^+, and rotenone even in the absence of astroglia [80–88].

Primary cell culture systems used in these studies typically utilize either neuronal cell lines or dissociated cells. Nevertheless, it is likely that increased Nrf2 activity in both neurons and glia contributes to neuronal survival in disease states. The glia-enriched and mixed neuron-glia culture studies by Shih et al. suggest that in vitro neuroprotection against glutamate toxicity is not limited to increased neuronal Nrf2 activation, because activation of glial Nrf2 system also protects cultured neurons from insult [89]. A microarray analysis used to evaluate potential glial versus neuron-specific contributions to the neuroprotective effects of ARE activation and Nrf2 dependence showed that Nrf2 induction activates the expression of different genes in cultured astrocytes as compared with cultured cortical neurons. These results suggest that Nrf2-dependent genetic changes alter neuron-glia interactions resulting in neuroprotection [90]. The unique combination of cells found within organotypic nigrostriatal cocultures provides an ideal system by which we can examine this relationship between neurons and glia [91]. The neuroprotective effects of in vitro Nrf2 activation were also demonstrated in such organotypic nigrostriatal cocultures although the astroglial component was not studied [91]. All neurodegenerative diseases have different causes and mechanisms including progression, age of onset quality and severity of symptoms, survival after onset and cell population affected. Nrf2 was found to be regulated differently in neurons versus astrocytes [90]. Therefore, Ramsey et al. proposed that Nrf2 might regulate different gene products in various neuronal subpopulations (i.e., hippocampal neurons versus nigral neurons) [76]. Further studies are needed to explore how Nrf2 localization is regulated in a cell-type-specific manner, how it differs in various cellular populations and neuronal subpopulations, and how these differences may contribute to neuronal protection.

The link between the Nrf2/ARE pathway and PD was also studied using in vivo neurotoxin-based animal models of PD [84, 92]. Nrf2 knockout mice display increased vulnerability to 6-OHDA, and the induction of the Nrf2/ARE pathway by transplantation of astrocytes overexpressing Nrf2 can protect against 6-OHDA-induced damage in mouse brain [84]. Genetic deficiency of Nrf2 increases MPTP sensitivity in mice [79]. Furthermore, Nrf2 expression restricted to astrocytes is sufficient to protect against MPTP in transgenic mice with Nrf2 under control of the astrocyte-specific promoter for the glial fibrillary acidic protein (GFAP)-Nrf2. These results show that astroglial modulation of the Nrf2/ARE pathway also plays a pivotal role in neuroprotection in vivo. According to the results of a very recent study based on the MPTP model, Nrf2 also modulates toxin-mediated activation of microglia, another glial cell type in the CNS [93]. Dopaminergic neurodegeneration and microglial activation induced by chronic injection of MPTP were more severe in Nrf2 knockout mice than in wild-type mice. Characteristic of classical microglial activation, the levels of proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) and the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were increased, while anti-inflammatory markers attributable to alternative microglial activation were decreased [94]. These results demonstrate a crucial role of Nrf2 in the modulation of microglial dynamics [93]. There may be crosstalk between glial cell subpopulations characterized by the modulation of the activation status of microglia by astroglia [95]. The astrocytes, the most abundant cells in the brain, can secrete one or more factors capable of modulating microglial activation by regulating microglial Nrf2 activation. Treatment of microglia with astrocyte culture-conditioned media induces the nuclear translocation of Nrf2 and enhanced HO-1 promoter activity in an ARE-dependent manner and increases the expression and activity of HO-1 in microglia. Furthermore, treatment with astrocyte culture-conditioned media suppresses Interferon-gamma- (IFN-γ-)induced ROS production, leading to reduced iNOS expression and nitric oxide (NO) release [95]. However, as demonstrated by a very recent in vivo study, HO-1 may not be involved in the neuroprotection elicited by Nrf2 in experimental parkinsonism [92]. Deficiency of HO-1 does not protect or enhance sensitivity to neuronal death, whereas Nrf2-knockout mice showed exacerbated gliosis and dopaminergic nigrostriatal degeneration in the MPTP model of PD [96, 97].

Genetic evidence indicating a link between Nrf2/ARE pathway and PD came from a recent study in which a complete haplotype analysis of the NFE2L2 and Keap1 genes in relation to the risk of PD was performed in two independent case-control materials [98]. A protective NFE2L2 haplotype was found in both European case-control materials. The molecular consequence of this haplotype may be increased efficiency in the Keap1-Nrf2-ARE response to

oxidative stress and thereby higher capacity to withstand endogenous or environmental risk factors for PD. Genetic variation in Keap1 did not show any associations. These results together with recent preclinical data provide another link between oxidative stress and the pathogenesis of PD and support NFE2L2 as a novel susceptibility gene for PD [98].

The interaction of Nrf2 with a parkinsonian gene, DJ-1 (PARK7), may provide another link between Nrf2 function and PD. A recently discovered function of DJ-1 is to stabilize Nrf2 by preventing its interaction with Keap1 and Nrf2's subsequent ubiquitination [99]. Furthermore, protein expression of Nrf2 significantly decreases in paraquat-treated DJ-1-deficient mice [100]. However, the findings of the study by Gan et al. using primary mouse embryonic fibroblasts were not confirmed in primary cortical neuronal and astrocyte cultures in vitro and ARE-driven human placental alkaline phosphatase (hPAP; ARE-hPAP) transgenic reporter mice in vivo [101]. Nrf2 activation, Nrf2-dependent gene induction, and Nrf2-mediated neuroprotection do not appear to be dependent on the presence of DJ-1 in the brain or in primary cultures derived from the brain. The most probable explanation for this discrepancy is the different cell types used in the two studies, suggesting that the relationship between DJ-1 and Nrf2 is cell-type specific. Neuroprotection by DJ-1 was confirmed in the second study [101].

Studies examining the Nrf2-activating effects of PD drugs support the link between the Nrf2/ARE pathway and PD. Apomorphine (Apo) is a drug used for clinical therapy of PD. It is a dopamine D(1)/D(2) receptor agonist and has scavenger and protective effects in ROS-induced cell death. Hara et al. reported that Apo enhances protection in 6-OHDA in vitro PD model in SH-SY5Y neuroblastoma cells. In that study, the involvement of the Nrf2 pathway in Apo-enhanced protection was also investigated. They observed nuclear translocation of Nrf2 into the nucleus and induced the expression of HO-1 gene in a dose-dependent fashion. On the other hand, cotreatment of Apo with antioxidant N-acetylcysteine suppressed the induction of HO-1 expression. Thus, Apo acts by producing intracellular ROS and activating the Nrf2 pathway to promote neuroprotective effects [81]. In addition to Apo, there is another cytoprotective drug, Deprenyl (Selegiline), that is, a promising candidate for neuroprotection, but its mode of action was unknown. However, Nakaso et al. reported a novel mechanism for the mode of action of Deprenyl, which involves PI3K and Nrf2 downstream oxidative-stress-related proteins. It increased the expression levels of HO-1, PrxI, TrxI, TrxRxI, gamma-GCS, and p62/A170, induced Nrf2 nuclear accumulation, and increased the strength of Nrf2 binding to ARE site. Nrf2 activation led to the induction of PI3K-controlled antioxidant molecules, and TrkB was identified as the upstream element of the PI3K/Nrf2 mechanism. Thus, protective effects of Deprenyl depend on PI3K-Nrf2 activation, which switches the antioxidant mechanism leading to cytoprotection [63]. The latest clinically used PD drug is bromocriptine, which is a dopamine agonist. Not only does it normally improve motor deficits by dopamine D2 receptor activation, but it also has neuroprotective and antioxidant activities. As reported by Lim et al., bromocriptine upregulates NAD(P)H quinone oxidoreductase-1 (NQO1) expression and increases its activity, thus leading to the protection of PC12 rat adrenal pheochromocytoma cells against oxidative damage. Bromocriptine also increased Nrf2 expression and nuclear translocation. It is known that bromocriptine leads to cytoprotection and antioxidant effects via the PI3K/Akt pathway, but independent from dopamine receptor activation. Also, dopamine receptor D2 antagonist does not affect the cytoprotective effect of bromocriptine and Nrf2-ARE activation by bromocriptine in dopamine D2 receptor expressing and nonexpressing cells. Consequently, NQO1 is a novel therapeutic target for PD, which can be upregulated by PI3K and Nrf2 activation [102].

Specific links between Nrf2 function and PD have been revealed. The activity of Nrf2 decreases with aging [103, 104], the major risk factor for the development and progression of PD [105]. Dysregulation of the Nrf2/ARE pathway is seen in PD. Nrf2 activators including known antiparkinsonian drugs (deprenyl and apomorphine [63, 81]) protect dopaminergic neurons against parkinsonian neurotoxins both in vitro and in vivo. Deficiency of Nrf2 aggravates experimental parkinsonism. Finally, apart from the stringent link between the Nrf2/ARE pathway and pathophysiological processes involved in PD such as oxidative stress, neuroinflammation, and ER stress, Nrf2 has the potential to interact with several molecules implicated in mitochondrial biogenesis (nuclear respiratory factors, PGC-1α, Tfam) and p62 which plays a role in mitophagy and the ubiquitin proteasome system (UPS) implicated in the pathogenesis of PD.

6. Nrf2/ARE Pathway Activation as a Novel Therapeutic Approach to Mitochondrial Dysfunction in Parkinson's Disease

As evidenced from postmortem tissue analyses and genetic analyses in humans and pathological studies in animal models, mitochondrial dysfunction plays a major role in PD, which leads to oxidative stress, DNA damage, and altered mitochondrial morphology and physiology. Therefore, therapeutic agents targeted to the mitochondria are thought to be promising tools for PD patients, such as agents targeting energy metabolism, mitochondria-targeted antioxidants, and more promising drugs that target the ARE/Nrf2/Keap1 pathway.

The Nrf2 pathway plays a role in redox homeostasis in cells. The main functions of Nrf2 were described in the previous part of this paper. The Nrf2 pathway is activated by ROS accumulation in cells; however, for therapuetic purposes, it is found to be activated by synthetic triterpenoids (TP), which are analogs of olenolic acid. They function as inhibitors of oxidative stress and cellular inflammatory processes and were shown to be protective in cancer models [106]. Moreover, the use of dietary compounds, synthetic chemicals, and xenobiotics decreases the incidence of diseases. For many decades, chemical substances from plants, called phytochemicals, have been shown to have chemopreventive activities, and most of them are suggested as Nrf2 inducers [107]. The potent chemopreventive compounds that induce Nrf2 can

be listed as sulforaphane from cruciferous vegetables [108], curcumin [109], epigallocatechin-3-gallate from green tea [110], resveratrol from grape [111], caffeic acid phenethyl ester [109], wasabi [112], cafestol, kahweol [113], cinnamon based compounds [114], zerumbone [115], garlic organosulfur compounds [116], lycopene [117], carnosol [118], and avicins [119]. Novel compounds have been continuously discovered.

TPs are a group of Nrf2-activating compounds. Recently, novel Nrf2-activating, synthetic TP, CDDO methylamide (CDDO-MA), was tested by Yang et al. for neuroprotective effects in 3-nitropropionic acid (3-NP) rat and MPTP mouse models. CDDO-MA showed significant protection against both models and *tert*-butylhydroxyperoxide-induced ROS degeneration [120]. In addition to protective effects, CDDO-MA upregulated the expression of genes involved in mitochondria biogenesis, glutathione synthesis, and antioxidant mechanisms. Thus, this compound was thought to be useful for the treatment of neurodegenerative diseases.

Other neuroprotective agents that induce the expression of hemeoxygenase-1 are electrophilic neurite outgrowth-promoting prostaglandin (NEPP) compounds [121]. NEPP compounds are cyclopentenone prostaglandin derivatives and function as neurotrophic factors. According to a report by Satoh et al., NEPPs were preferentially taken up into neurons, where they bind to Keap1 in a thiol-dependent fashion. They showed that NEPPs are neuroprotective in a glutamate excitotoxicity model in vitro and in a stroke model in vivo. They suggest that NEPPs activate the Keap1/Nrf2 pathway to modulate the prevention of excitotoxicity; therefore, they can be used as therapeutics in stroke and neurodegenerative diseases [122].

Shih et al. showed that dietary administration of Nrf2 inducer (*tert*-Butylhydroxyquinone) reduced susceptibility to 3-NP and increased striatal and cortical gamma-glutamyl-cysteinyl- glycine (GSH) levels in Nrf2-expressing mice but not in Nrf2−/−. These results suggested that Nrf2 inducers to be dietary administration may be alternative drugs for the treatment of neurodegenerative disorders [123].

Neuroprotective agents such as erythropoietin (Epo), which is known to pass the blood brain barrier [124], also exert Nrf2-activating effects. Epo provides neuroprotection in MPTP- and 6-OHDA-induced toxin models of PD [125, 126]. Recently, Epo has been found to activate Nrf2 in SH-SY5Y cells [127]. Epo induced Nrf2 translocation to the nucleus and upregulated the expression of the HO-1 gene. Also, MAPK and PI3K inhibitors caused decreased translocation of Nrf2 caused by Epo. These results suggest that Epo activates the Nrf2 pathway and the PI3K and MAPK pathways as well. It is interesting that Epo, which is reported as neuroprotective in PD animal models, triggers neuronal Nrf2 translocation. Thus, the neuroprotective effects of Epo can be mediated by Nrf2.

7. Conclusions

Mitochondrial dysfunction plays a central role in the pathogenesis of PD. For this reason, new therapeutic strategies in PD targeting the restoration of mitochondrial dysfunction are being developed. The failure of antioxidant therapy strategies alone in clinical trials has encouraged efforts to find novel approaches strengthening endogenous antioxidant defense systems and inducing mitochondrial biogenesis and anti-inflammatory responses [123]. In this regard, Nrf2, a master regulator of the induction of a battery of genes affecting several cytoprotective systems, may be a promising target to counteract mitochondrial dysfunction and its consequences in PD. Thus, exogenous Nrf2 inducers should be tested for therapeutic potential in PD.

Abbrevation

MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+:	1-methyl-4-phenylpyridinium
3-NP:	3-nitropropionic acid
6-OHDA:	6-hydroxydopamine
ATP:	Adenosine triphosphate
ARE:	Antioxidant response element
Apo:	Apomorphine
hPAP; ARE-hPAP:	ARE-driven human placental alkaline phosphatase
CDDO-MA:	CDDO methylamide
JNK:	c-Jun N-terminal protein kinase
COX-2:	Cyclooxygenase-2
Epo:	Erythropoietin
ERK:	Extracellular signal-regulated kinase
GSH:	Gamma-glutamiyl-cysteinyl- glycine
GFAP:	Glial fibrillary acidic protein
HO-1:	Hemeoxygenase-1
hPAP:	Human placental alkaline phosphatase
iNOS:	Inducible nitric oxide synthase
IFN-γ:	Interferon-gamma
IL-6:	Interleukin-6
Keap1:	Kelch-like ECH-associated protein
LRRK2:	Leucine-rich repeat kinase 2
MARE:	Maf recognition element
mtDNA:	Mitochondrial DNA
MAPK:	Mitogen-activated protein kinase
Tfam:	Mitochondrial transcription factor A
NDUFS4 gene:	NADH:ubiquinone oxidoreductase iron-sulfur protein 4
NADPH:	Nicotinamide adenine dinucleotide phosphate
NQO1:	NAD(P)H quinone oxidoreductase-1
NEPP:	Neurite outgrowth-promoting prostaglandin
NO:	Nitric oxide
Nrf2:	Nuclear factor E2-related factor 2
OXPHOS:	Oxidative phosphorylation
PD:	Parkinson's disease
PPAR-γ:	Peroxisome proliferator-activated receptor gamma
PGC:	Peroxisome proliferator-activated receptor gamma coactivator

PGC1A: Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PRC: PGC-related coactivator
PINK1: PTEN-induced kinase 1
ROS: Reactive oxygen species
SQSTM1: Sequestosome 1
SN: Substantia nigra
Snpc: Substantia nigra pars compacta
TP: Triterpenoids
TNF-α: Tumor necrosis factor-alpha
UPS: Ubiquitin proteasome system.

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Conflict of Interests

The authors declare no competing financial interests.

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Review Article

Mitochondrial Fusion/Fission, Transport and Autophagy in Parkinson's Disease: When Mitochondria Get Nasty

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Understanding the molecular basis of Parkinson's disease (PD) has proven to be a major challenge in the field of neurodegenerative diseases. Although several hypotheses have been proposed to explain the molecular mechanisms underlying the pathogenesis of PD, a growing body of evidence has highlighted the role of mitochondrial dysfunction and the disruption of the mechanisms of mitochondrial dynamics in PD and other parkinsonian disorders. In this paper, we comment on the recent advances in how changes in the mitochondrial function and mitochondrial dynamics (fusion/fission, transport, and clearance) contribute to neurodegeneration, specifically focusing on PD. We also evaluate the current controversies in those issues and discuss the role of fusion/fission dynamics in the mitochondrial lifecycle and maintenance. We propose that cellular demise and neurodegeneration in PD are due to the interplay between mitochondrial dysfunction, mitochondrial trafficking disruption, and impaired autophagic clearance.

1. Introduction: The Critical Role of Mitochondrial Dysfunction in PD

The intrinsic properties of mitochondria make them essential integrators of cellular functions. These organelles are critical as ATP suppliers, calcium buffers and transducers of intracellular signaling pathways which integrate programmed cell death. Therefore, mitochondrial function has a critical role in the brain physiology, where the impaired functioning of mitochondria has been implicated in several neurological disorders, like Parkinson's disease (PD).

PD is a chronically progressive, age-related neurodegenerative disease, clinically characterized by progressive resting tremor, rigidity, bradykinesia, gait disturbance, postural instability, and dementia. A major neuropathological hallmark of PD is the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and in other brainstem regions. A second neuropathological feature is the presence of intracytoplasmic inclusions (Lewy bodies, LBs) in surviving neurons, which comprise a dense core of different proteins as α-synuclein, parkin, ubiquitin, synphilin-1, tubulin, and other cytoskeletal proteins [1]. Over the last several decades, a growing body of evidence accumulated focusing on the crucial role of mitochondria and mitochondrial dysfunction in PD etiopathogenesis [2–6].

The most compelling evidence of mitochondrial dysfunction in PD emerged following the human accidental exposure to the synthetic meperidine analogue 1-methyl-4-phenyl-1,2,3,6-tetrahydrodropyridine (MPTP) which induced a parkinsonian syndrome through a decrease in complex I activity by its metabolite 1-methyl-4-phenylpyridinium (MPP^+) [7, 8].

Furthermore, mitochondrial association with idiopathic PD was first established when a mitochondrial NADH dehydrogenase (complex I) activity deficit was identified in the SNpc of postmortem PD patients brains [9] and in PD patients platelets [10]. Further evidence suggested a similar complex I deficiency in PD patients lymphocytes [11, 12], although few reports failed to demonstrate consistent

changes between PD and control cells [13]. To address the potential causes of complex I defect, that is, if it was due to an environmental toxin or to an alteration of mitochondrial or nuclear DNA, the cytoplasmic hybrid technique has been used. This approach consists on the transfer of sporadic PD subject platelet mitochondria to mtDNA-depleted cells, generating hybrid lines (cybrids). In this way, it is possible to follow the expression of the mitochondrial mutations and the effects of its heteroplasmy in a wild-type nuclear DNA environment. A stable decrease in complex I activity, increased reactive oxygen species (ROS) production, proton leak, and decreased maximum respiratory capacity were described in PD cybrids [14–17]. All of these features are consistent with the involvement of respiratory chain dysfunction in PD. In addition to mtDNA mutations, the products of familial PD-linked genes, including α-synuclein, parkin, DJ-1, PINK1 (PTEN-induced kinase 1), LRRK2 (Leucine-rich repeat kinase 2), were shown to locate in or interact with mitochondria under certain conditions [18, 19]. In addition, loss of OMI protease activity increases the susceptibility of mitochondria to induce the permeability transition [20]. Moreover, LRRK2 colocalizes with the mitochondrial outer membrane [21] and may regulate the response to mitochondrial inhibitors [22]. Other studies revealed many novel proteins with quantitative expression differences in PD when compared to controls. Those include subunits of complex I, mitochondrial creatine kinase, the chaperone mortalin (mthsp70/GRP75), and glutathione *s*-transferase pi, all proteins implicated in mitochondrial functions and cellular responses to oxidative stress [23, 24].

This body of evidence is suggestive that mitochondrial function is altered in PD. In this paper we will focus on mitochondrial dynamics, emphasizing the changes in mitochondrial motility and mitochondrial quality control mechanisms, critical to maintain mitochondrial function and cellular homeostasis. Moreover, we will also address the contributions of these topics to cellular demise associated to PD neurodegeneration and comment on the current controversies that exist on this issue.

2. Mitochondrial Dynamics and PD

2.1. Mitochondrial Dynamics. Mitochondria can appear as discrete tubules or interconnected networks in living cells [25]. This range on mitochondrial shaping and size profiles is continuous since the hundreds of mitochondria within a cell can undergo frequent cycles of fusion (the combination of two mitochondria into a single organelle) and fission (the separation of long, tubular mitochondria into two or more smaller parts) [26, 27]. This fusion/fission dynamics is very important in maintaining the functional integrity of mitochondria as the constituents of each network share solutes, metabolites, and proteins [28–30], as well as electrochemical gradient, making them electrically coupled [31, 32]. These mitochondrial networks characteristics suggest that fusion is a mechanism required for the proper respiratory activity and metabolic efficiency of mitochondria, as well as, for the complementation, stabilization, and protection of mitochondrial DNA (mtDNA) [33, 34]. Actually, the functionality of a damaged mitochondria can be complemented by fusion with a neighboring integral mitochondria and possibly be restored [26]. In addition, the transfer of mtDNA or whole mitochondria between cells can occur *in vitro* and rescue aerobic respiration in cells without functional mitochondria [35].

Both mitochondrial fission and fusion seem to be required to maintain mitochondrial function. However, the mechanisms differ. Fission occurs probably to protect function by facilitating equal segregation of mitochondria into daughter cells during cell division and to improve distribution of mitochondria along microtubules tracks. In addition, fission may also help to isolate segments of damaged mitochondria, promoting their clearance by macroautophagy as discussed below [36]. Besides to maintain normal mitochondrial functions, mitochondrial fusion, as well as mitochondrial fission have also been associated with cell death/survival mechanisms [37, 38].

Sophisticated genetic and biochemical studies in several organisms, ranging from *Drosophila melanogaster* and baker's yeast *Saccharomyces cerevisiae* to mammalian cells, have provided valuable inroads into understanding the biological processes of mitochondrial dynamics and greatly accelerated the identification and characterization of the major components of the fusion and fission machineries and their regulation [39–42]. The critical balance between mitochondrial dynamic systems is greatly maintained by a large group of conserved proteins, the dynamin-related GTPases.

2.2. Mitochondrial Fusion. Mitochondrial fusion involves mechanisms distinct or at least more complex from those that govern membrane fusion in the secretory pathway or other membrane-bound organelles. The fusion process can be divided in at least three events: docking, fusion of the outer membrane, and fusion of the inner membrane. Mitofusins Mfn1 and Mfn2 are engaged in mitochondria tethering and outer membrane fusion. Opa1 is involved in the outer membrane fusion step with the inner membrane contacts and can have a direct physical contribution to the fusion of inner membranes itself [43]. Moreover, it has been described that mutations in Opa1 and Mfn2 genes cause Kjer's disease/autosomal dominant optic atrophy and Charcot-Marie Tooth type 2A neuropathy in humans [44].

To date, several mechanisms, such as protein-protein interactions, posttranslational modifications, protein turnover and the lipid environment, have been proposed as regulators of mitochondrial fusion. Indeed, the modulation of the amount of Mfn protein regulates the extent of mitochondrial fusion. In addition, the ability of Mfn1 and 2 to oligomerize and hydrolyze GTP to promote the membrane rearrangements is another point of regulation of mitochondrial fusion [45]. The mechanism of inner membrane fusion is unknown, but findings indicate that two distinct isoforms of Opa1, resulting from proteolytic processing, are necessary for successful fusion events [46, 47].

Two mammalian members of proapoptotic Bcl-2 family members, Bax and Bak, induce mitochondrial fusion by

regulating the assembly and submitochondrial distribution of Mfn2. Intriguingly, their mitochondrial localization is required for apoptosis induction and for cell survival, pointing to an intimate connection of mitochondrial remodeling and programmed cell death [48].

2.3. Mitochondrial Fission. While the precise mechanism of mitochondrial fission in mammals is largely unknown, most insights concerning this mechanism have come from studies in yeast. It is thought that mitochondrial fission in mammals follows the same steps as in yeast: Drp1 is recruited to mitochondria, and constriction of the membranes takes place via direct or indirect interaction with hFis1 [49].

The major regulatory mechanisms that control mitochondrial fission seem to involve posttranslational modifications of Drp1 which determine its localization, dynamics, and activity. One of the posttranslational modifications that regulate mitochondrial division is ubiquitinylation. Drp1 ubiquitinylation seems to regulate the kinetics of Drp1 binding to the mitochondrial surface. Thus, ubiquitin conjugation might regulate the subcellular trafficking, assembly of Drp1, and influence the rate of mitochondrial division [50]. Mitochondrial fission is also regulated in part by phosphorylation. It was demonstrated that phosphorylation of Drp1 at serine 616 by the cyclin B-dependent kinase (CDK1) induces fragmentation of mitochondria during mitosis [51]. Unlike phosphorylation by CDK1, protein kinase A-(PKA, cAMP-dependent protein kinase-) dependent phosphorylation of a different serine residue (S637) can decrease Drp1 GTPase activity [52]. Thus, it seems that phosphorylation of Drp1 by different kinases at different amino acid residues causes opposite effects. Although the initial study indicate that Drp1 phosphorylation can modulate the frequency of mitochondrial division, it remains to be understood if the fission competent phospho-Drp1 (S616) is always phosphorylated by CDK1 or if this mechanism is only active during the cell cycle [39]. Moreover, dephosphorylation of S637 by the Ca^{2+}-dependent phosphatase calcineurin promotes Drp1 translocation to mitochondria and subsequently mitochondrial fission [53].

In addition to frequent cycles of fusion and fission in a "kiss and run" pattern, mitochondria are also turned over during the neuronal lifetime, being replaced throughout organelle biogenesis.

2.4. Mitochondrial Biogenesis. Mitochondria cannot be made *de novo.* The formation of new mitochondria, called mitochondrial biogenesis, encompasses all processes involved in maintenance and growth of these organelles, as well as the ones required for their division and segregation during the cell cycle. Thus, mitochondrial biogenesis is an extremely complex process that requires the synthesis, import, and incorporation of proteins and lipids to the existing mitochondrial reticulum, as well as the replication of the mtDNA. Although mitochondria have their own DNA, mitochondrial genome encodes only a small but essential group of 13 proteins, including hydrophobic proteins of the electron transport chain, as well as mitochondrial tRNAs and rRNA. The vast majority of the mitochondrial proteins are encoded by nuclear genes. Therefore, mitochondrial biogenesis requires the coordinated transcription of the large number of mitochondrial genes in the nucleus, as well as of the fewer but essential genes in mitochondria. This implies the cooperation of two genomes, which is a potential challenge to the neuronal cell. This could limit mitochondrial biogenesis to the cell body and require that all mitochondrial renewal in the distal axon occurs by axonal transport. However, there is also a reason to believe that mitochondrial biogenesis could occur within the axon. It is now well established that local protein synthesis occurs in axons [54–56] and is essential for axonal functions including direction [57–59], regeneration [56, 60–62], and maintenance of mitochondrial membrane polarization [63]. In addition, transcripts for nuclear-encoded mitochondrial proteins are among the species found in axons [64]. Moreover, mitochondria are found in close proximity to translation sites in the axon [65]. Several studies have revealed that components of the mitochondrial replication apparatus are located outside the perinuclear region in nonneuronal cells [66–70] and in SH-SY5Y neuron-like cells [70]. More recently, it was shown that mtDNA replication and mitochondrial fission and fusion occur in the distal axons of peripheral neurons in culture, showing that a portion of mitochondrial biogenesis, like protein biosynthesis, does occur in the axon at significant distances from the cell body [71].

2.5. The Relevance of Mitochondrial Dynamics for Neuronal Integrity. Neurons are highly specialized cells that undergo unique challenges in carrying out their important physiological functions. First, neurons are active cells and thus require large amounts of energy. Furthermore, some neurons have extremely long processes, with axons extending up to one meter in motor neurons. Thus, neurons must transmit energy across long distances. They are also long-lived postmitotic and highly interactive cells whose major function is communication. Therefore, neurons rely profoundly on the rapid and versatile distribution of mitochondrial activity and on mitochondrial biogenesis over the time and space [72]. In neurons, the mitochondrial fission/fusion machinery is intimately and critically involved in the formation of synapses and dendritic spines. Actually, both fusion and fission mechanisms contribute for the full-mitochondrial lifecycle and any disruption on their balance could change the steady-state distribution of mitochondrial span. Since mitochondria cannot be made *de novo*, the fission of preexisting organelles is essential for generation of the new mitochondria. Distressing fission or fusion mechanisms either by inhibiting expression of the fission protein Drp1 [73–75] or by overexpressing the fusion protein Mfn1 [76] has been shown to prevent mitochondria from distributing to synapses, leading to a loss of mitochondria from dendritic spines and, consequently, to a reduction of synapse formation. On the other hand, counteracting this process by overexpressing Drp1 and/or promoting its effects on dendritic mitochondria restores synapse formation [77]. In addition, mitochondrial fusion has been directly implicated in preventing the accumulation of damaged mtDNA [28, 78]. More recently, mitochondrial fission has also been shown

to be critical to mtDNA maintenance [79]. Accordingly, it seems that fission and fusion serve the same purposes along neurons: fission is the final step in mitochondrial duplication, whereas fusion dilutes errors in mtDNA, being both processes required to protect mitochondrial integrity and function [30, 80].

Thus, considering how much neurons depend on mitochondria, it should come as no surprise that there is a strong association between mitochondrial dysfunction and neurodegenerative diseases. However, key questions arise: why specific areas of the brain are differentially affected? and/or why only selective groups of neurons die in PD? What makes these specific groups of distinct neurons particularly susceptible to degeneration is not known yet. However, it is now well established that the degeneration of PD affects not only dopaminergic neurons, but many other neurons in the central nervous system, including populations in the brainstem, as well as in the subcortical and cortical regions [81]. Neurons of these areas have common features as they have long and thin axons, which have little or no myelination [82]. Neurons with these features are more vulnerable to degeneration and require high energy demands and so they are particularly dependent on suitable mitochondrial dynamics. In addition, it was recently reported that mitochondrial mass and size are correlated with cell size and that dopaminergic neurons in the SNpc, ventral tegmental area, and interfascicular nucleus have a significantly a smaller area of the cytoplasm occupied by mitochondria than in the neighboring nondopaminergic neurons. This suggests that the low mitochondria mass of the nigral dopaminergic neurons may contribute to their vulnerability to degeneration [83]. Moreover, mitochondrial content in axons, synapses, and dendrites (collectively known as neurites) plays a crucial role in regulating outgrowth and synaptic remodeling into adulthood [77, 84]. Alterations on neurites remodeling and plasticity due to aging and disease processes probably contribute to memory loss and neurodegeneration. In fact, the dynamic remodeling of preexisting mitochondria may play a more prominent role than biogenesis in regulating mitochondrial content in neuritis [75, 85].

3. Novel Implications of Mitochondrial Dynamics in PD Pathogenesis

3.1. Mitochondrial Motility: An On-Track Sliding-Dependent Cellular Process. Proper functioning of mitochondria depends on their intracellular location which is decisively governed by aspects of mitochondrial spatial arrangement and motility beyond fusion and fission. Those aspects are critically important when we look to cell polarity, such as in neurons [72], which require mitochondria at sites distant from the cell body. Transport of mitochondria and their positioning within neurons are microtubule-dependent and, in turn, transport on microtubules depends on the molecular motors kinesins and dyneins [86]. It was demonstrated that the dynein-dynactin motor complex interacts with Drp1 and recruits the protein to the mitochondrial surface [87]. In addition, disruption of F-actin also blocks translocation of Drp1 and, subsequently, mitochondrial fission [88]. Very recently, Milton and Miro were shown to localize to mitochondria in mammalian neurons and interact with each other *in vivo* in the brain [89, 90]. Miro has also been shown to bind directly to kinesin heavy chain KIF5 in a Ca^{2+}-sensitive manner [89, 90] and together with Milton (Miro-Milton complex) allows mitochondria to move along the microtubule network and supports on-demand distribution of mitochondria. In mammalian cells, manipulation of Miro1 was shown to dramatically affect mitochondrial morphology and these effects, in the particular case of fission, appear to involve Drp1 activation [91]. In Drosophila, loss of Miro-dependent transport pathway results in depletion of mitochondria in dendrites and axons, inducing neurotransmission defects during prolonged stimulation [92]. Furthermore, defects in both fusion and fission have been shown to impair mitochondrial movement. Apparently, the large interweave of highly connected mitochondria in fission-deficient cells prevents an efficient movement, particularly into thin locations such as neuronal processes [75, 77]. In fusion-deficient cells, the cause of decreased motility is less obvious. However, Mfn-deficient mitochondria display loss of directed movement, travelling in a manner reminiscent of Brownian motion [45]. In neurons lacking mitochondrial fusion, swollen and nucleoid-deficient mitochondria cluster at dendritic junctions and are unable to enter the distal, smaller diameter branches. Thus, both clustering of mitochondria in the cell body and the blockage of efficient entry into neurites may contribute to the lack of mitochondria in the axons and dendrites [37]. Collectively, pieces of evidence lead to the hypothesis that the effect of mitochondrial fusion and fission disturbances could secondarily impair motility and, on the other hand, transport defects affect mitochondrial shape.

3.2. Mitophagy: A Major Line of Defense against Mitochondrial Damage. As mentioned before, perturbations in mitochondrial dynamics, throughout fusion or fission alterations, can impair the energy provision by mitochondria in mammalian neurons. Nevertheless, cells have developed sophisticated systems to deal with the diverse challenges imposed on mitochondrial functional integrity. These systems could comprise a "multistep" mitochondrial quality control network that assists to the spatial segregation of damaged mitochondria. The first tier of quality control is provided by both molecular chaperones and the intracellular proteolytic system, which selectively remove excess and damaged proteins from mitochondria outer membrane. A second step in mitochondrial quality control could be mediated by fusion of damaged mitochondria with neighboring mitochondria [26]. However, severe injury of mitochondria impairs fusion and further activates fission-dependent fragmentation and sequestration by an autophagic process, termed mitophagy. Accumulating data suggests that mitochondrial dysfunction by itself triggers mitophagy [93]. In fact, it was demonstrated that mitochondria-derived ROS, at low concentrations, may act as signaling molecules and trigger mitophagy throughout redox regulation of Atg4, an essential cysteine protease in the autophagic pathway [94]. Similarly, Gomes and

Scorrano also provided evidence that the profission mitochondrial protein Fis1 induces mitochondrial fragmentation and enhances mitophagy. Nevertheless, these changes were correlated with mitochondrial dysfunction rather than with fragmentation [95]. As well, mitochondrial fission was also shown to be an important step for the autophagic clearance of depolarized or damaged mitochondria, since overexpression of Drp1 promotes mitophagy [36].

Additional studies showed the involvement of Parkin, PINK, and LRRK2 in the regulation of mitochondrial clearance and homeostasis. Parkin was found to be selectively recruited to dysfunctional mitochondria with low membrane potential in mammalian cells targeting mitochondria towards the autophagic-lysosomal pathway [96]. Moreover, translocation of Parkin to mitochondria is voltage dependent and does not depend on changes in pH or ATP levels [97]. Thus, these findings suggest that Parkin may act as a sensor for mitochondrial integrity and limit mitochondrial damage by acting in a pathway that identifies and eliminates damaged mitochondria from the mitochondrial network.

PINK1 and Parkin can cooperate in a common pathway that is involved in the protection of mitochondrial integrity and function. Indeed, it was reported that co-overexpression of Parkin and PINK1 collapses the normal tubular mitochondrial network into mitochondrial aggregates and/or large perinuclear clusters, many of which are associated to LC3-enriched autophagic vacuoles [98, 99]. These results suggest that both proteins are involved in the modulation of mitochondrial trafficking, especially to the perinuclear region, a subcellular area associated with autophagy-lysosomal degradation [98]. Moreover, PINK1 accumulation on mitochondria is both necessary and sufficient for Parkin recruitment to mitochondria [98, 100]. Recent studies have shown that PINK1 accumulates selectively on dysfunctional mitochondria and its kinase activity together with its mitochondrial sequence is a prerequisite to induce translocation of Parkin to depolarized mitochondria. Subsequently, Parkin mediates the poly-ubiquitylation of VDAC-1 (voltage-dependent anion channel 1) [101], Mfn1, and Mfn2 among other mitochondrial proteins [102]. Additionally, the autophagic adaptor p62/SQSTM1 is recruited to mitochondrial clusters and is essential for the clearance of mitochondria by mitophagy [101]. Taken together, these data provide a functional link between Parkin, PINK1 and mitophagy which is implicated in the pathogenesis of PD.

Moreover, the role of LRRK2 in regulating autophagy was also addressed. Interestingly, it was shown that LRRK2 specifically localizes to specific membrane subdomains and endosomal-autophagic structures, suggesting a functional relationship between LRRK2 and mitophagy [103]. Moreover, increased autophagic activity upon LRRK2 knockdown was observed, which indicates that LRRK2 may normally act as a negative regulator of autophagy [103]. Alternatively, LRRK2 regulation in neurite blunting and remodeling requires autophagy [104]. Thus, by impairing this pathway, mutations in Parkin, PINK1, and LRRK2 may alter autophagy-dependent mitochondrial turnover which, in turn, may cause the accumulation of defective mitochondria and, ultimately, neurodegeneration in PD. Conversely, Beclin 1-independent autophagy/mitophagy contributes to cell death elicited by the PD toxins MPP^+ and 6-OHDA [105], causing neurite retraction in cells expressing the G2019S PD-linked mutation in LRRK2 [105]. However, excessive or incomplete autophagy without suitable regenerative biogenesis, due to deficient retrograde trafficking of vesicles can lead to "autophagic stress" [106], which may ultimately lead to neuronal degeneration.

3.3. Failure of an Exquisite Network of Quality Control in PD?. An early study by de Mattos and coworkers [107] in brains from PD patients demonstrated mitochondrial tumefaction and deposits of amorphous substance into mitochondria and axons distinctively absent in the control patients. Consistent findings were further found by Trimmer and colleagues [108] in PD cybrids and in studies with the same model in our laboratory. Mitochondria in PD cybrid cells were found enlarged or swollen with disrupted cristae and a discontinuous outer membrane [16]. In contrast, sublethal concentrations of MPP^+ and rotenone, two mitochondrial complex I inhibitors that induce parkinsonian syndrome *in vivo*, were shown to promote Drp1-dependent mitochondrial fragmentation [109], to decrease mitochondrial mobility [110, 111] and to disrupt microtubule dynamics [112–114]. However, chronic low-dose exposure to rotenone and MPP^+ was shown to induce mitochondria swelling and decreased anterograde transport of mitochondria and vesicles, probably due to a reduction of ATP supply to molecular motors [110, 115].

Additional studies of mammalian PINK and parkin models have been suggestive of their involvement on the regulation of mitochondrial turnover, dynamics, and cellular homeostasis. The role for parkin was revealed using a *Drosophila parkin* null mutant or by overexpression of one pathogenic parkin mutation, showing severe mitochondrial pathology, reduced lifespan, and increased apoptosis [116, 117]. It was also found that functional parkin is necessary for proper mitochondrial organization and morphology throughout spermatid development in *Drosophila* [118]. Additionally, mitochondrial respiratory defects and morphological abnormalities have been reported in brains of *parkin*-knockout, *parkin*-mutant transgenic mice [119, 120], and in leukocytes from PD patients with parkin mutations [121, 122]. Primary fibroblasts from patients carrying mutations in parkin, or control fibroblasts treated with siRNA against parkin, revealed lower mitochondrial membrane potential, lower ATP levels, and increased susceptibility to rotenone toxicity [121]. The fibroblasts also exhibited mitochondrial morphological abnormalities, exhibiting mitochondria that were longer and more highly branched. Interestingly, there was a relationship between those mitochondrial deficits and increased length and branching, as well as between reduced complex I activity and mitochondrial branching. Thus, as parkin is not specifically located into mitochondria and must be translocated to this organelle, it is conceivable that its effects on mitochondrial morphology are tied into a larger pathway that mediates mitochondrial maintenance. Actually, within mitochondria, parkin mainly localizes in the inner membrane and matrix, where it has been shown

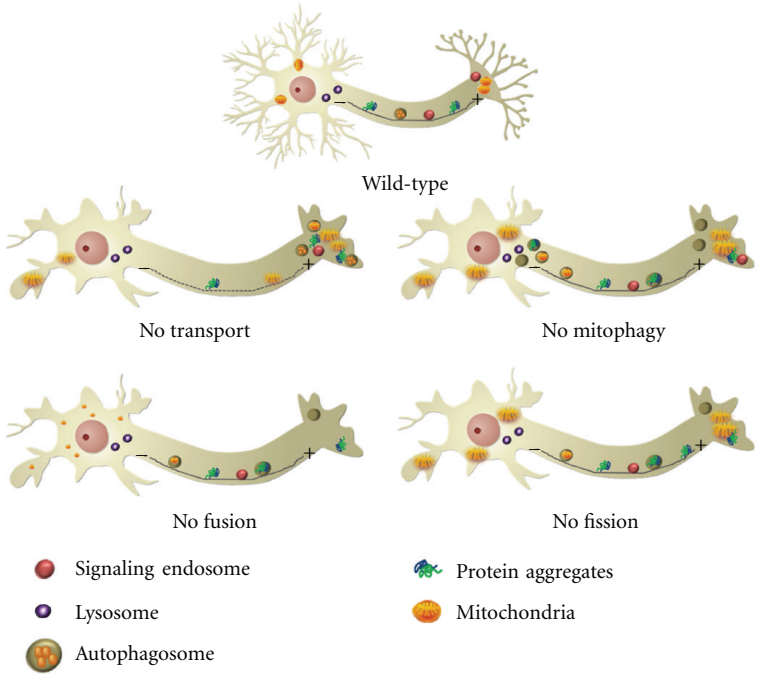

Figure 1: Potential susceptibility of neurons to mitochondrial dysfunction and impaired mitochondrial turnover. (*Wild type*) In healthy neurons, mitochondria, prosurvival signals associated to signaling endosomes, and autophagomes enclosing damaged organelles or protein aggregates are able to travel long distances from the cell periphery to perinuclear region in the cell body, where most lysosomes are concentrated. (*No transport*) Disruption of microtubule network and subsequent defects on retrograde transport prevent the proper distribution of mitochondria and the efficient transport of autophagy substrates towards lysosomes for degradation, which can lead to defects in energy supply and cargos clearance by autophagy. (*No mitophagy*) Blockage of autophagic activity seems to be responsible for the accumulation of damaged mitochondria, toxic protein products, aggregates, and leaking autophagic vesicles, all of which have a negative effect on neuronal functioning and survival, precipitating the "dying-back"-type of axonal degeneration. (*No fusion*) The absence of mitochondrial fusion may result in an accumulation of damaged mitochondria or decreased healthy mitochondria at the nerve terminal. Mitochondria secondarily have defects in motility that prevent proper distribution within the axon and in the periphery. (*No fission*) In the absence of mitochondrial fission, most of the mitochondrial population is extensively long and interconnected, and a subset shows ultrastructural defects and dysfunction. The large mitochondria clusters within dendrites are not efficiently transported and/or engulfed by autophagosomes towards cell body for lysosomal degradation. (MT: microtubule tracks oriented along the axon with plus (+) ends distal and (−) ends proximal to the cell body).

to enhance transcription and replication of mtDNA and to induce mitochondrial proliferation [123]. This effect seems to be mediated by the interaction of parkin with mitochondrial transcription factor A (TFAM), a protein that regulates mtDNA transcription by directly binding and coating mtDNA [123]. In PC12 cells differentiated into neurons by nerve growth factor, parkin was located in the outer mitochondrial membrane, where prevented ceramide-induced mitochondrial swelling, cytochrome c release, caspase activation, and apoptotic cell death [124]. This effect may be related to the above-mentioned ability of parkin to regulate mitochondrial morphology. Intriguingly, complex I inhibition with rotenone seems to induce the release of mitochondrial parkin to the cytosol [123]. In light of these results, complex I deficiency or loss-of-function mutations in parkin may impair the mitochondrial localization of parkin and thus abolish the boosting and beneficial effects of this protein at the mitochondria. Indeed, the protective effect of Parkin is abolished by PD-causing parkin mutations and proteasome inhibitors, indicating that it is mediated by its E3 ubiquitin ligase activity [124].

Regarding PINK, a significant decrease in mitochondrial respiration in the striatum of PINK1 knockout mice [125] was observed. Several groups also described mitochondrial abnormalities in pink *Drosophila* mutants, similar to those of parkin mutant *Drosophila* [126–128]. Moreover, the protective effect of PINK1 has also been confirmed in primary neuronal cultures and in an *in vivo* mouse model of PD [129]. In this study, PINK1 suppression by small interfering RNA in primary cortical neurons treated with MPP^+ promoted neuronal death, while overexpression of wild-type PINK1 protected these neurons against MPP^+ toxicity [129]. The protective effect of PINK1 was confirmed as being mediated by its kinase domain and was abolished in PD-related PINK1 mutants [129]. Adenoviral-mediated expression of PINK1 in mice SNpc protected dopaminergic neurons from MPTP-induced cell death, an effect that was abolished by expression of kinase-inactive PINK1 mutants [129]. Surprisingly, the mitochondrial targeting sequence and the mitochondrial localization of PINK1 were not necessary for its protective effect, either *in vitro* or *in vivo*, as demonstrated in a mutant PINK1 with impaired

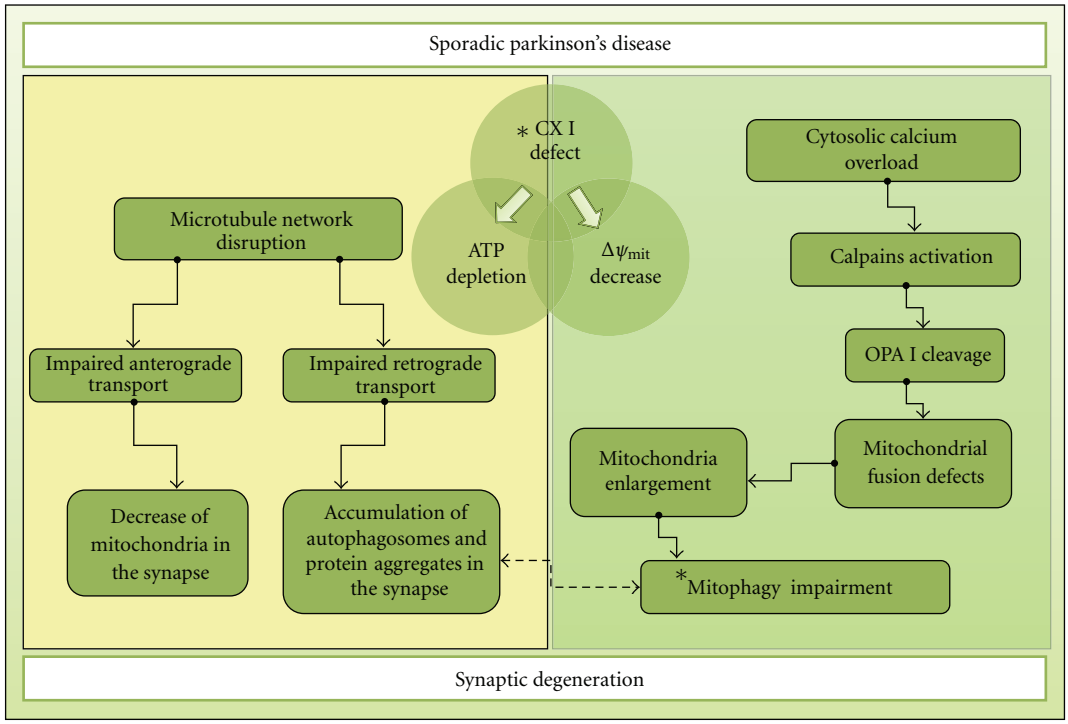

Figure 2: Rationale for the contribution of mitochondrial dysfunction to synaptic degeneration in sporadic PD. Mitochondrial dysfunction induced by a complex I defect leads to alterations in mitochondria-dependent metabolism (reduced ATP levels and decreased mitochondrial inner membrane potential). This bioenergetic failure seems to potentiate microtubule network breakdown. Subsequently, when the dynamics and functional integrity of microtubules are compromised, changes in anterograde and retrograde flux along the axon can be impaired. Cargos that are actively transported along the axon include mitochondria, autophagosomes, and proteins. Moreover, a decrease in mitochondrial membrane potential deregulates calcium homeostasis, which leads to the overactivation of calpains. Calpains are key regulators of mitochondrial fusion, since they impair Opa1 proper function. Alterations in fusion/fission events promote mitochondrial enlargement, which can impair their removal by mitophagy. Indeed, the accumulation of protein aggregates, autophagosomes, and enlarged deficient mitochondria in presynaptic *termini* is observed at early stages of PD. Our hypothesis implies that mitochondrial metabolism impairment could be responsible for synaptic degeneration in PD. (*Indicates that mutated or overexpressed α-synuclein could induce mitochondrial dysfunction and that loss-of-function of Parkin or/and PINK1 can deregulate mitochondrial mitophagy.)

mitochondrial localization [129]. These results indicate that cytoplasmic, rather than mitochondrial, kinase activity of PINK1 is critical for its protective effect. To reconcile these results with the known mitochondrial localization of PINK1, it has been reported that while PINK1 spans the outer mitochondrial membrane, with the N-terminal end inside the mitochondria, the C-terminal kinase domain of PINK1 actually faces the cytosol [130]. Very recently, it was also suggested that PINK1 may also be involved in mitochondrial transport. Weihofen and colleagues demonstrated that Miro and Milton overexpression reverted mitochondria pathology induced by the loss of functional PINK1 [131]. Moreover, PD-associated PINK1 mutations also compromise the selective degradation of depolarized mitochondria maybe due to a decreased physical binding of PINK1 to Parkin. In addition to an impaired PINK1 kinase activity, reduced binding of PINK1 to Parkin leads to failure in mitochondrial clearance, resulting in the accumulation of damaged mitochondria [132].

Both *Drosophila* and mammalian cells studies show that loss of PINK result in abnormalities in mitochondrial morphology, involving mitochondrial swelling accompanied by decreased or disorganized cristae [125, 133], which are remarkably similar to mitochondria in sporadic PD cybrid cell lines [16, 108, 134]. Interestingly, PINK and Parkin flies were shown to have comparable phenotypes. While parkin overexpression reverses the effects of PINK loss-of-function on mitochondrial morphology, the opposite was not observed, suggesting that parkin acts downstream of PINK [126, 128].

However, studies in PD patients fibroblasts carrying PINK mutations and in human HeLa, M17, and SH-SY5Y PINK knockdown cells showed an increase in fragmented mitochondria [135, 136]. These results may be correlated with the role of PINK in preventing mitochondrial oxidative stress and fission events. Indeed, an increase in mitochondrial fragmentation was reversed by overexpression of Parkin in HeLa and SH-SY5Y cells, which indicates a clearance of the damaged mitochondria.

Foremost, silencing PINK expression in dopaminergic SH-SY5Y cells resulted in progressive loss of mitochondrial function characterized by decreased mtDNA levels, impaired oxidative phosphorylation, and oxidative stress [137]. Nevertheless, in this study the decreases in mtDNA

and oxidative phosphorylation do not appear to be directly related to Parkin loss of function. Instead, loss of mtDNA can be suggested as the most probable cause of mitochondrial respiratory chain inhibition and consequent oxidative stress. This supports the notion that the combination of both mitochondrial dysfunction and perturbed PINK1 activity increases the susceptibility to oxidative stress or apoptosis as has been reported in brain cells [125, 138]. Besides, PINK knockdown cells show decreased phosphorylation of Drp1 at S637 through activation of calcineurin phosphatase activity [136]. While PINK overexpressing cells show Drp1 2D-gel mobility consistent with higher phosphorylation states [139]. Because Drp1 phosphorylation at S637 inhibits its ability to mediate fission [52], these results point to a role of PINK as a fission suppressor.

Even though PINK1/Parkin may regulate mitochondrial dynamics in some of the familial PD forms, it seems evident that mitochondrial physiology is the major determinant of mitochondria morphology in sporadic cases. Particularly, it is believed that the structural composition of mitochondria may determine their propensity for fragmentation and self-elimination, and that this may be influenced by the metabolic status within the cell. Accordingly, an RNAi screening for mitochondrial proteins in *Caenorhabditis elegans* demonstrated that the knockdown of more than 80% of mitochondrial genes lead to mitochondrial fragmentations and/or aggregation, showing that mitochondria morphology maintenance requires a huge number of proteins and not necessarily the specific action of mitochondrial fusion or fission machinery [140].

Furthermore, ultrastructural examination indeed revealed "autophagic stress" in melanized neurons of the SNpc in PD patients [141]. Moreover, accumulated autophagosomes have been observed in human PD nigral neurons [141], but not in nigral neurons during normal aging. Alterations in macroautophagy are also implicated in PD since its inhibition leads to *wt* α-synuclein accumulation, suggesting that this lysosomal pathway is also involved in normal α-synuclein turnover [142]. Very recently, it was also demonstrated that α-synuclein overexpression impairs macroautophagy in mammalian cells and in transgenic mice [143]. Interestingly, recent work in our laboratory has shown that cells with an mtDNA-mediated mitochondrial dysfunction have enhanced formation autophagic vacuoles but present decreased degradation ability, suggesting that the autophagic clearance is impaired in PD. We have also observed that macroautophagy inhibition increased α-synuclein oligomerization and prompts apoptosis (DM Arduíno, unpublished data).

4. Concluding Remarks and Perspectives

There is a growing body of evidence supporting that alterations in mitochondrial dynamics are implied in PD pathogenesis (Figure 1). However, there are still some controversies among the different models of disease, namely, concerning the mitochondrial morphology changes and the mechanisms that control mitochondrial shape and function. Common approaches, such as those modulating fusion/fission, are scarce to explain how mitochondrial abnormalities occur in certain disease states. Undoubtedly, it seems evident that cellular physiology is the major determinant of mitochondria function and morphology. In addition, mitochondrial fusion and fission are not isolated in the cell and other intrinsic cellular alterations, such as impaired vesicular trafficking and axonal transport, intracellular degradation systems and mitochondrial metabolism, have also been described in PD and could further alter mitochondrial biogenesis, turnover, and maintenance.

Taken in account the available data, we propose the mitochondrial cascade hypothesis to explain PD pathogenesis. Mitochondrial dysfunction induced by a complex I defect leads to alterations in mitochondrial-dependent metabolism (reduced ATP levels and decrease in mitochondrial membrane potential) [144]. This bioenergetic failure seems to play a role in microtubule network breakdown [145]. Subsequently, when microtubules dynamic and functional integrity are compromised, changes in anterograde and retrograde flux along the axon can lead to defects in the supply and clearance of mitochondria [146]. Moreover, a decrease in mitochondrial membrane potential potentiates an increase in cytosolic calcium which leads to calpains overactivation [147]. In addition, mitochondria with low inner membrane potential have a decrease in Opa1 levels and do not fuse [148]. Accordingly, calpain activation seems to mediate Opa1 loss of function [149]. Together, these findings point to an accumulation of enlarged mitochondria [16], that will impair efficient removal by autophagy. Our hypothesis implies that mitochondrial failure may be the initial event in sporadic PD, although it has a prominent role in some of PD familial forms (Figure 2).

Understanding this complex molecular network and indentifying the factors that control all of these interrelated mechanisms is currently a priority and a challenge for future studies.

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Review Article

Mitochondrial Dysfunction: The Road to Alpha-Synuclein Oligomerization in PD

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While the etiology of Parkinson's disease remains largely elusive, there is accumulating evidence suggesting that mitochondrial dysfunction occurs prior to the onset of symptoms in Parkinson's disease. Mitochondria are remarkably primed to play a vital role in neuronal cell survival since they are key regulators of energy metabolism (as ATP producers), of intracellular calcium homeostasis, of NAD^+/NADH ratio, and of endogenous reactive oxygen species production and programmed cell death. In this paper, we focus on mitochondrial dysfunction-mediated alpha-synuclein aggregation. We highlight some of the findings that provide proof of evidence for a mitochondrial metabolism control in Parkinson's disease, namely, mitochondrial regulation of microtubule-dependent cellular traffic and autophagic lysosomal pathway. The knowledge that microtubule alterations may lead to autophagic deficiency and may compromise the cellular degradation mechanisms that culminate in the progressive accumulation of aberrant protein aggregates shields new insights to the way we address Parkinson's disease. In line with this knowledge, an innovative window for new therapeutic strategies aimed to restore microtubule network may be unlocked.

1. Introduction

Parkinson's disease (PD) was first associated with the loss of the brown pigment neuromelanin from the substantia nigra. Later, it was postulated that the progressive loss of dopamine-producing cells in the substantia nigra pars compacta of the ventral midbrain caused PD symptomatology. In addition, PD is also associated with the presence of intracytoplasmatic inclusions known as Lewy Bodies (LBs), which are composed largely of alpha-synuclein (alpha-syn). The function of alpha-syn is still unclear, but its involvement in PD pathogenesis is further indicated by a subset of familial cases of PD that carry either a missense mutation in snca (alpha-syn) gene or have a duplication or triplication of alpha-syn locus [1–4].

To date, mutations in at least 16 PD-genetic loci (PARK) have been linked to the pathogenesis of PD [5]. The discovery of these genes in which mutations cause early or late-onset forms of PD has greatly accelerated research progress. These include pink-1, dj-1, parkin, lrrk2, uchl-1, omi/htra2, atp13a2, pla2g6, fbx07, and snca, as previously mentioned. OMI/HTRA2, PINK-1, DJ-1, LRRK2, and Parkin are either mitochondrial proteins or are associated with mitochondria and may be involved in pathways related to oxidative stress and free radical damage. Pink-1 encodes a putative serine/threonine kinase with a mitochondrial targeting sequence [6], and DJ-1 is a redox sensor which is involved in the response to oxidative stress [7]. Although Parkin is a putative E3 ligase in the ubiquitin proteasome system that localizes predominantly to the cytosol, it also associates with the mitochondrial outer membrane [8]. Most recently mutations affecting the mitochondrial serine protease OMI/HTRA2 have been linked to increased risk of PD [9]. Mice entirely lacking expression of omi/htra2 due to targeted deletion of its gene, Prss25, show a decrease in a population of neurons in the striatum and have a parkinsonian phenotype that leads to death of the mice around 30 days after birth [10]. In addition, loss of Omi protease

activity increases the susceptibility of mitochondria to induce the permeability transition pore [11]. LRRK2 is a kinase that colocalizes with the mitochondrial outer membrane [12] and may regulate the response to mitochondrial inhibitors [13].

PD etiopathogenesis includes genetic, epigenetic, and environmental factors. However, in sporadic cases of PD, in which ageing is the major risk factor, the initial event that causes the pathology is not clear. Nevertheless, it is known that several mechanisms are involved in the development of PD pathogenesis such as mitochondrial dysfunction, oxidative damage, autophagic alterations, proteasome impairment, microtubule network disruption, and protein aggregation. Efforts have been made in order to discover what triggers the pathogenesis of PD that culminates in the death of a specific group of neurons, dopaminergic neurons. Nowadays several studies highlight mitochondrial dysfunction as the leading event. In this paper, we will underscore mitochondrial-mediated mechanisms and their involvement in PD pathogenesis.

2. Parkinson's Disease: A Mitochondrial Disorder?

There is mounting evidence for mitochondria involvement in neurodegenerative disorders, including PD. Mitochondria play a fundamental role in energy metabolism; their primary function is to provide energy (in the form of ATP) for intracellular metabolic pathways. Mitochondrial electron transport chain deficiencies are thought to underlie defects in energy metabolism and have been implicated in the neurodegenerative process. Moreover, brain cells are highly dependent on the oxidative phosphorylation (OXPHOS) machinery in mitochondria, which involves the respiratory complexes [14].

The first evidence of mitochondrial dysfunction involvement in the pathogenesis of PD emerged following the observation that accidental exposure of drug abusers to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), an inhibitor of complex I (NADH/ubiquinone oxidoreductase) of the mitochondrial electron transport chain, induced parkinsonism [15]. Moreover, Parker and colleagues found a significant reduction of complex I activity in platelet mitochondria, purified from patients with idiopathic PD [16]. Evidence for dysfunctional mitochondrial metabolism in PD arises from studies performed in the substantia nigra of postmortem PD brain showing a deficient complex I activity [17, 18]. Additionally, a selective decrease in complex I subunits was demonstrated in PD brain [19]. This was corroborated by Keeney and coworkers that reported some abnormalities in the assembly and oxidation of complex I subunits in mitochondria from PD patients cortex [20]. Complex I deficiency has been also described in other PD tissues, namely, in peripheral cell models such as, platelets, lymphoblasts, muscle, and fibroblasts [21–26]. More recently, a specific complex I deficit was detected in highly purified mitochondria from the frontal cortex of PD patients [27]. To address mitochondrial involvement in the pathogenesis of PD, King and Attardi, in 1989, succeeded in repopulating human cells depleted from mtDNA, a Rho0 cell line, with mitochondrial genes from donor cells [28]. Human cell lines (SH-SY5Y neuroblastoma or NT2 teratocarcinoma cells) depleted of mtDNA were fused with either control or PD platelets that contain mtDNA, but no nuclear DNA. Several authors showed that these cybrids harbour a complex I defect [29–32] and have a decrease in ATP levels and loss of mitochondrial membrane potential [29]. Further support for mitochondrial involvement in PD comes from studies using toxins [33–35] like rotenone, paraquat, and maneb that appear to reproduce some of the features of PD in animal models. Although several of these toxins are mitochondrial, they induce nigrostriatal lesions motor and postural deficits by different mechanisms of action [36–44].

High levels of mtDNA deletions have been observed in dopaminergic neurons from the substantia nigra of postmortem human brains from aged individuals and idiopathic PD patients [45, 46]. mtDNA deletions in these neurons were correlated with a decrease in cytochrome c oxidase activity, one of the key enzymes of electron transport chain. This suggests that accumulation of mtDNA deletions in dopaminergic neurons from substantia nigra above a certain threshold can cause mitochondrial defects associated with PD. It is well established that mtDNA accumulates mutations during the ageing process which correlates with decline in mitochondrial function in late-onset PD. TFAM is a phylogenetically conserved protein and is imported to mitochondria which is essential for mtDNA maintenance and directly regulates mtDNA copy number and is absolutely required for transcription initiation at mtDNA promoters [47–49]. The most compelling evidence regarding TFAM involvement in PD was reported by Ekstrand and colleagues showing that the conditional and selective inactivation of both TFAM alleles in substantia nigra of transgenic mice induced respiratory chain deficiency in dopaminergic neurons leading to a Parkinsonism phenotype with adult onset of slowly progressive impairment of motor function accompanied by the formation of inclusions and neuronal death [50]. Taking into consideration the above findings, it was hypothesized that potentially functional polymorphic TFAM variants could influence PD risk depending on haplogroup background. However, Alvarez and coworkers study in 250 patients report no TFAM-mutations/polymorphisms that could contribute to the risk for PD [51, 52]. Other enzyme that regulates mtDNA replication and plays an important role in mtDNA maintenance is the mtDNA polymerase gamma 1 (POLG1). This enzyme is a nuclear-encoded protein involved in the synthesis, replication, and repair of mtDNA that is imported to mitochondria and localizes in the inner mitochondrial membrane. POLG1 mutations might impair mtDNA replication, which leads to gradual accumulation of multiple mtDNA deletions, resulting in respiratory chain dysfunction [53]. Luoma and colleagues have found that missense mutations of POLG1 cosegregate in patients with a phenotype that includes progressive external ophthalmoplegia and parkinsonism [54, 55]. Moreover, POLG1 mutations have also been described in case studies, in which parkinsonism was part of the clinical spectrum [56]. Deletion of functional ndufs4, a gene

encoding one of the subunits required for complete assembly and function of complex I, led to abolished complex I activity in midbrain mesencephalic cultures derived from ndufs4 knockout mice [57]. However, this deletion did not affect the survival of dopaminergic neurons in culture. Nonetheless, studies performed in rats that expressed an enzyme called "alternative NADH dehydrogenase" (Ndi1) in the substantia nigra, showed a reduced toxic effect of MPTP and rotenone [58, 59].

2.1. Oxidative Stress: ROS-Related Consequences. Mitochondrial OXPHOS is known to be the major source of ROS. Cells in normal conditions are able to cope with excessive ROS because they are endowed with robust endogenous antioxidant systems. However, when ROS production overwhelms the endogenous antioxidant systems, due to mitochondrial dysfunction, this can potentially damage various types of biomolecules, including proteins, lipids, and nucleic acids. Markers of oxidative stress, such as products of lipid peroxidation, protein oxidation, and oxidation of mtDNA and cytoplasmic RNA, are increased in postmortem samples of substantia nigra in PD brains as compared to controls [60–64]. In PD patient's postmortem substantia nigra, there is evidence for decreased levels of reduced glutathione and altered iron metabolism [65]. Furthermore, proteomic studies revealed that several mitochondrial and ROS scavenging proteins expressed in the substantia nigra of PD patients had oxidative modifications supporting a role of oxidative stress in PD [66]. It is strongly believed that proximity of mtDNA to ROS generation site, the lack of histones, and diminished capacity for DNA repair increase mitochondrial vulnerability to mutations and oxidative stress [67, 68]. It appears that substantia nigra is more vulnerable to impairments of complex I activity than other brain regions and peripheral blood cells, possibly due to increased levels of iron and to dopamine metabolism [69]. The intracellular auto-oxidation of dopamine generates $H_20_2$and dopamine-quinone species which will exert cytoxicity in dopaminergic neuronal cells [70, 71].

Several papers showed an involvement of oxidative stress in alpha-syn toxicity. Nitration and nitrosylation of proteins and especially of alpha-syn and parkin in PD have been documented [72–74]. Leong and coworkers demonstrated that methionine oxidation of dopamine generates soluble alpha-syn oligomers highlighting the potential role for oxidative stress in modulating alpha-syn aggregation [75]. Interestingly, studies performed in SH-SY5Y cells overexpressing alpha-syn A53T mutant or wild type and in isolated rat brain mitochondria showed that alpha-syn localizes at the mitochondrial membrane, and this interaction of alpha-syn with mitochondria causes oxidative modification of mitochondrial components [76].

The involvement of oxidative stress in PD is also corroborated by the fact that two of the PD-related genes, dj-1 and pink-1, have important roles in maintaining the balance between oxidative stress and antioxidant defenses. DJ-1 functions as a redox-sensitive chaperone as well as a sensor for oxidative stress and apparently protects neurons against oxidative stress and cell death. Mutations in this gene are the cause of autosomal recessive early-onset PD [77]. Hayashi and colleagues suggested that DJ-1 is an integral mitochondrial protein that directly plays a role in maintenance of mitochondrial complex I activity [78]. PINK-1 is a serine/threonine protein kinase that localizes to mitochondria. It is thought to protect cells from stress-induced mitochondrial dysfunction. Mutations in this gene cause one form of autosomal recessive early-onset Parkinson disease [6].

Taken together these findings suggest a pivotal role for mitochondrial-mediated oxidative stress in PD pathogenesis.

2.2. Mitochondrial Metabolism: NAD^+/NADH Ratio. Several studies suggest that changes in energy metabolism in the brain are not merely a consequence of neuronal loss but rather a contributory factor to the progression and development of the disease [79–83]. The decrease in mitochondrial bioenergetics capacity and altered redox status is linked through various mechanisms including interconvertible reducing equivalent pool: NAD^+ and reduced β-nicotinamide adenine dinucleotide (NADH) [84]. NAD^+ and NADH are major factors for numerous energy metabolism-associated redox reactions and mitochondrial functions but also calcium homeostasis, ageing, and cell death [85]. Additionally, NAD^+/NADH ratio is a powerful regulator of glycolysis, TCA cycle, and oxidative phosphorylation. Our group recently observed that NAD^+/NADH ratio is significantly increased in PD cybrids when compared with CT cybrids (Esteves et al., submitted). By using an oxygen electrode, we observed that although whole cell basal oxygen consumption was comparable between the PD and CT cybrids, the proton leak was increased and maximum respiratory capacity was decreased in the PD cybrids [86].

Multiple families of enzymes catalyze various biochemical reactions by consuming NAD^+. Among these are the recent identified sirtuins (SIRTs) that have a catalytic domain characterized by its requirement for NAD^+ as a cofactor. SIRTs are a family of deacetylase enzymes that are able to remove the acetyl group from ε-amine of lysine and have been found in a wide variety of subcellular locations. So far, in humans there are seven identified SIRTs (SIRT1 to SIRT7) that play a role in a wide variety of important biological processes, including transcriptional silencing, DNA recombination and repair, apoptosis, axonal protection, fat mobilization, and ageing [87]. The deacetylase and ADP ribosylase activity of SIRTs suggests that they could act as metabolic or oxidative sensors, regulating cellular machinery according to the information they receive. Resveratrol, an activator of SIRT1 [88], protected SH-SY5Y cells against dopamine-induced cytotoxicity by reducing intracellular oxidative stress [89]. Additionally, it was shown to exert a neuroprotective effect on 6-OHDA rat model of PD [90] and to significantly protect mice from MPTP-induced motor coordination impairment, hydroxyl radical overloading, and neuronal loss [91]. On the other hand, a work from 2009 showed that SIRT1 overexpression did not exert any neuroprotective effect against neuronal damage induced by MPTP

in mice [92]. A dramatic reduction in the expression of SIRT1 occurred when an acute stress was induced by addition of several neurotoxins (MPP^+, rotenone, KA, or 3NPA) to the culture medium. However, in human samples of PD and dementia with LBs, there were no changes in SIRT1 expression [93]. So far, no work has been published regarding SIRT1 involvement in alpha-syn oligomers clearance in PD. In an MPTP-treated primate model of PD, caloric restriction, wich upregulates SIRT1, prevented the function of locomotor function and preserved striatal dopamine [94]. SIRT1 is known to deacetylate approximately a dozen of known substrates, such as peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1α). PGC1α is of major importance for PD etiopathogenesis because it plays a central role in the regulation of cellular energy metabolism and stimulates mitochondrial biogenesis. Work from our group demonstrated that PD cybrids with inherent mitochondrial dysfunction have a decrease in SIRT1 activity that was correlated with a decrease in PGC1α levels indicating that mitochondrial biogenesis is affected in our PD model [86]. However, more studies need to be performed in order to substantiate this data. The role of SIRT2 in neurodegenerative disorders is less established. Nevertheless, North and coworkers provided data revealing that SIRT2 is involved in cell cycle regulation via the deacetylation of α-tubulin [95]. More recently, Outeiro and coworkers reported an intriguing connection between SIRT2 and PD that suggested that inhibition of the human SIRT2 rescues cells from alpha-syn-mediated toxicity [96]. The exact mechanism whereby SIRT2 inhibition affects alpha-syn aggregation remains uncertain. One proposed mechanism is through alpha-syn interaction with alpha-tubulin [97–99]. Moreover, it was shown that PD cybrids have an increased free/polymerized tubulin ratio indicating that microtubule destabilization may mediate alpha-syn oligomers accumulation [98]. Apha-tubulin acetylation is associated with microtubule stabilization, so one possibility is that an increase in SIRT2 activation due to increase in NAD^+ levels increases alpha-tubulin deacetylation leading to microtubules network disruption which potentiates alpha-syn oligomerization (Esteves et al., submitted). So far, little is known about the remaining SIRTs involvement in PD. Overall, SIRTs targeting may be therapeutically beneficial in PD pathogenesis by manipulating the pathways responsible for PD neurodegeneration not only regarding alpha-syn oligomerization but also mitochondrial biogenesis. However, despite exciting data, the feasibility of developing SIRTs-based therapy for PD and other neurodegenerative diseases needs to be demonstrated in animal models, and finally in human trials.

2.3. Calcium Homeostasis Deregulation. Disturbances in calcium homeostasis can affect neuronal functionality since calcium is one of the most important elements in cellular signaling. An increase in cytosolic calcium concentration can arise from two sources: the major intracellular calcium store which is the endoplasmic reticulum and the extracellular space. Moreover, mitochondrial function is based on the ability to uptake calcium and to accumulate it in the matrix, being this process dependent on the mitochondrial membrane potential. The primary role of mitochondrial calcium is the stimulation of OXPHOS [100]; thus, any perturbation in mitochondrial or cytosolic calcium results in profound alteration of cellular function. Rotenone was shown to increase the susceptibility of cells to calcium overload with subsequent cell death in SH-SY5Y neuroblastoma cells [101]. Sheehan and coworkers showed that PD cybrids sequestered less calcium in their mitochondria than CT cybrids, due to their decreased mitochondrial function [102]. Subsequently, our work showed that PD cybrids have a significantly higher concentration of cytosolic calcium as compared to CT cybrids and in addition show less capacity in mitochondrial calcium buffering, indicating that mitochondrial normal function is impaired [103].

In addition, an increased activity of calcium-stimulated phospholipase A_2 in the putamen is reported to be due to either decreased dopaminergic striatal input or degenerative processes in dopamine nerve terminals [104]. Dopaminergic neurons from substantia nigra express calcium buffering proteins which effectively sequester calcium without using ATP. Expression of the calcium buffering protein, calbindin, reduces vulnerability to mitochondrial toxins [105] and seems to confer resistance to MPTP [106]. Interestingly, dopaminergic neurons that do not express the Ca^{2+}-buffering protein, calbindin-D28k, are selectively lost during PD progression [107]. In addition, dopaminergic neurons that express relatively high levels of another calcium-buffering protein, calretinin, appear to be resistant to degeneration in PD [108].

So far, it has been demonstrated that alpha-syn regulates calcium entry pathways and, consequently, that abnormal alpha-syn levels may promote neuronal damage through deregulation of calcium homeostasis [109].

3. Mitochondrial Metabolic Control of Cellular Traffic: Microtubules Involvement

Cytoskeletal injury is likely to be responsible for altered rearrangement and movement of organelles, being a common feature of several neurodegenerative diseases including PD. Microtubules are highly dynamic polar structures that play critical roles in diverse cellular functions. These include cell motility and division, organelle transport, cell morphology, and organization. Intracellular transport of protein and organelle cargo is one of microtubules' most important function, and is an essential requirement for all mammalian cells. In addition, microtubule-mediated transport is crucial to maintain the function and structure of neurons [110]. Microtubules dynamic instability requires an input of energy to undergo polymerization and depolymerization cycles. This dynamic process is extremely regulated and is influenced by many factors, where the concentration of free tubulin is the driving force [111].

Several lines of evidence suggested that microtubules disruption may be involved in PD. In fact, tubulin was shown to colocalize with alpha-syn in LBs. Also, tubulin folding is

dependent on ATP and GTP hydrolysis, and mitochondrial impairment with subsequent energy failure is one of PD hallmarks [112, 113]. Toxins that affect ATP production may affect the tubulin polymerization/depolymerization process leading to an increase in free tubulin. In fact, MPP^+, a known complex I inhibitor that induces PD-like symptoms, depolymerizes microtubules in PC12 cells [114, 115]. Later, Cappelletti and coworkers provided the first evidence that microtubules instability is specifically affected by MPP^+ [116]. Moreover, rotenone, another complex I inhibitor, was also shown to potentiate microtubules depolymerisation *in vivo* and *in vitro* [117] by binding to the colchicine site on tubulin heterodimers [118]. Ren and Feng found that microtubule depolymerization induced by rotenone caused vesicle accumulation in the soma and killed serotonergic neurons through a mechanism dependent on serotonin metabolism in the cytosol [119].

Microtubule depolymerization induces disruption in axonal transport, which leads to an accumulation of damaged organelles, aggregated/misfolded proteins, and vesicles. What happens in dopaminergic neurons is that dopamine leakage from the vesicles to the cytosol promotes an increase in oxidative stress induced by dopamine oxidation which culminates in cell death [120]. Treatment of cotransfected cells or primary mesencephalic neurons with colchicine, vinblastine, or nocodazole reversed alpha-syn-mediated inhibition of DAT activity providing insights for alpha-syn regulation of DAT activity, namely, by tethering the transporter to the microtubular network [121]. Furthermore, treating cells with nocodazole, a microtubule-disrupting agent, resulted in an increase in the number of small aggregates particles. Indeed, perturbation of microtubule system due to inhibition of microtubule assembly or due to deletion of genes involved in microtubule biogenesis stimulates alpha-syn aggregation and toxicity [122]. Also Webb and coworkers found that microtubule disruption with nocodazole inhibits autophagosome-lysosome fusion, what can decrease the degradation of alpha-syn oligomers [123]. Our group demonstrated that taxol, a microtubule stabilizer, was able to reduce alpha-syn oligomers accumulation in PD cybrids [98]. Intriguingly, the process of alpha-syn fibril formation was shown to be stimulated by tubulin and two other microtubule-associated proteins [124–126]. This means that when microtubule network is disrupted, the amount of free tubulin increases triggering alpha-syn fibrillization. Indeed alpha-tubulin directly interacts with alpha-syn [127]. On the other hand, Alim and collaborators found that alpha-syn is a functional microtubule-associated protein, and mutant forms of alpha-syn lose this potential [97]. Alpha-syn overexpression induces not only microtubule disruption but also impairs microtubule-dependent trafficking and induces neuritic degeneration in SH-SY5Y cells [128]. Moreover, Saha and colleagues found that the movement of alpha-syn mutant forms associated with PD through axons of cultured neurons has reduced transport rates. In addition, phosphorylation of alpha-syn in serine-129 also reduces its axonal transport [129].

Mitochondria use cytoskeletal proteins as tracks for their directional movement. Interaction of mitochondria with microtubules and their movement along microtubule tracks depends on MAPs [130]. The cytoskeletal system regulates not only mitochondrial movement but also their morphology and function. Therefore, damage to microtubules interferes with mitochondria movement through axons. On the other hand, mitochondria damage perturbs transport of mitochondria through axons, increasing their retrograde movement. These changes in mitochondria dynamics lead to a decrease of mitochondria numbers in axons and mitochondria accumulation in cell bodies [131, 132]. Studies from 2002 revealed that depletion of mitochondria numbers and function in axons occurs in neurodegenerative disorders [133, 134]. Since mitochondria are ATP suppliers and microtubules need ATP to accomplish their function, damage to mitochondria will have a profound effect on axonal transport and as a consequence axonal maintenance and function [135]. Normal mitochondria are expected to generate sufficient amounts of ATP but also to divide [136]. Indeed, mitochondrial biogenesis occurs by fission of pre-existing mitochondria. Fission allows the dilution of damaged macromolecules and also prevents excessive mitochondria enlargement. In fact, enlarged mitochondria are less likely to be degraded by mitophagy, which potentiates their progressive failure [137]. Initial enlargement may occur because of oxidative damage to proteins responsible for mitochondrial fission or mutations in the corresponding nuclear genes. Moreover, mitochondrial turnover may also decrease due to decreased lysosomal capacity associated with lipofuscin overload. It is also called "ageing pigment" and has been considered a reliable biomarker for the age of neurons. Lipofuscin is an intralysosomal, polymeric substance, primarily composed of cross-linked protein residues and formed due to iron-catalyzed oxidative processes. Because it is undegradable and cannot be removed via exocytosis, lipofuscin accumulation is inevitable during ageing [138, 139]. The accumulation of lipofuscin within postmitotic cells is a recognized hallmark of ageing occurring with a rate related to longevity.

An increasing body of data emphasizes the crucial role of mitochondrial dynamics in mitochondria function. Mitochondria are not a static and autonomous organelle but, instead, a highly dynamic one. This organelle undergoes continual cycles of fusion (the combination of two mitochondria into a single organelle) and fission (the separation of long, tubular mitochondrion into two or more smaller parts) [140, 141]. These dynamic processes regulate mitochondrial function by enabling mitochondrial recruitment to critical subcellular compartments, content exchange between mitochondria, mitochondrial shape control, mitochondrial communication with the cytosol, and mitochondrial quality control. In fact, this highly dynamic balance between mitochondrial fission and fusion controls mitochondrial morphology, length, size, and number and also regulates mitochondrial function and distribution. Increasing evidence suggests that abnormal mitochondrial dynamics is involved in mitochondrial dysfunction which may mediate neuronal death in PD models. Interestingly, MPP^+ and rotenone were shown to induce mitochondrial fragmentation dependent on the fission protein GTPase

Dynamin-related protein 1 (Drp1) [142–144]. Similarly, 6-OHDA-induced mitochondrial fragmentation in SH-SY5Y cells suggests that excessive mitochondrial fission might be mediating neurotoxicity induced by complex I inhibition [145]. In human fibroblasts, acute exposure to high-dose rotenone resulted in decreased mitochondrial membrane potential that caused mitochondrial fragmented morphology [146]. Also chronic exposure to rotenone was reported to reduce mitochondrial movement in differentiated dopaminergic SH-SY5Y cells [147]. These results suggest that PD toxins hamper fission/fusion machinery. Work obtained with two PD-related genes also highlighted microtubules involvement in PD as well as mitochondria dynamics impairment. For instance, parkin was shown to strongly bind to microtubules and to ubiquinate tubulin [120]. In addition, PINK-1 was found to have a role in the trafficking of mitochondria along microtubules suggesting that loss of PINK-1 function besides altering mitochondrial morphology and dynamics can also alter microtubule function [148]. It was also suggested that the PINK-1/Parkin pathway promotes mitochondrial fission and/or inhibits fusion by negatively regulating fusion proteins function, such as Mitofusin-1 and -2 (Mfn1 and Mfn2) and Optic atrophy 1 (Opa1), and/or positively regulating Drp1 [149]. Moreover, the loss of mitochondrial integrity in PINK-1 and parkin mutants can be due to reduced mitochondrial fission [150]. In Drosophila, PINK-1 and parkin mutant phenotypes were markedly suppressed by overexpression of Drp1 or downregulation of Opa1 indicating that the PINK-1/Parkin pathway regulates mitochondrial remodeling process by promoting mitochondrial fission [151]. Additionally, PINK-1 deficiency is also linked to mitochondrial pathology in human cells and Drosophila, which can be rescued by Exner et al. [152]. Lutz and colleagues demonstrated that an acute downregulation of parkin in human SH-SY5Y cells severely affects mitochondrial morphology and function, a phenotype comparable with that induced by PINK-1 deficiency. Furthermore, they showed that loss of parkin or PINK-1 function increases Drp1-dependent mitochondrial fragmentation [153]. Narendra and colleagues demonstrated that dysfunctional mitochondria, with a low membrane potential, recruit parkin to the mitochondria and induce the mitochondria to undergo fusion and mitophagy [154]. Furthermore, the loss of PINK-1 function elicited oxidative stress and mitochondrial turnover that was coordinated by the autophagic and fission/fusion machineries [155, 156].

Overall, mitochondrial-mediated microtubule disruption leads to a rapid accumulation of protein aggregates and/or damaged organelles such as mitochondria contributing to neurodegeneration as seen in PD.

4. Protein Aggregation: Alpha-Synuclein Oligomerization

When misfolded proteins cannot be refolded or degraded (by the proteasome or by the lysosome), the cell activates an alternative way of defence, sequestering abnormal and/or toxic proteins into aggregates. There is convincing data pointing to protein aggregation role in both familial and sporadic PD pathogenic process. Indeed, the presence of spherical protein inclusions is found in the cytoplasm of surviving nigral neurons, named LBs. It is thought that fibrillar alpha-syn is the building block of LBs. Indeed, alpha-syn is also the most sensitive marker for LBs, implying that it is necessary for LBs formation [157]. Nevertheless, LBs contain many proteins other than alpha-syn, including neurofilaments and other cytoskeletal proteins, suggesting that they might be important in aggresome formation. Alpha-syn is a small, highly charged 140-amino acid residue protein predominantly expressed in CNS, whose function is still poorly understood. However, several functions have been pointed out. It is thought that alpha-syn plays a role in the regulation of dopamine biosynthesis via activation of protein phosphatase 2A that reduces tyrosine hydroxylase phosphorylation, the rate-limiting enzyme in dopamine production [158, 159], and reducing the amount of available active mitogen-activated protein kinases [160] and phospholipase D [161]. Furthermore, several studies suggest that alpha-syn is involved in modulating synaptic transmission, the density of synaptic vesicles, and neuronal plasticity [162–165]. In addition, alpha-syn can act as molecular chaperone preventing aggregation of other proteins *in vitro*. In fact, alpha-syn can activate Hsp70, and more interestingly its structure is very similar to heat shock proteins [166, 167]. It also provides a supportive role in the folding/refolding of SNARE proteins critical for neurotransmitter release, vesicle recycling, and synaptic integrity [168]. Interestingly, alpha-syn knockout mouse showed no signs of neurodegeneration suggesting that alpha-syn is not required for neuronal development [165, 169]. SNCA gene mutations are very rare (three missense point mutations A30P, A53T, and E46K) however, they have helped to elucidate molecular mechanism of intracellular accumulation of alpha-syn. Transgenic mice expressing human wild-type alpha-syn gene develop several of clinical and pathological features of PD, such as accumulation of LBs, the loss of dopaminergic terminals in the basal ganglia, and associated motor impairments [170]. Overexpression of alpha-syn in substantia nigra results in loss of dopaminergic neurons, phosphorylation of alpha-syn at Ser129, and activation of caspase-9 [171]. Moreover, A53T and A30P alpha-syn transgenic mice develop neuronal mitochondrial degeneration and cell death [172]. Interestingly, A30P and A53T mutant alpha-syn are known to self-associate more rapidly than the wild-type forms facilitating aggregation into fibrils [173–176]. However, transgenic mice with A30P alpha-syn mutation do not exhibit alpha-syn inclusions [177]. In contrast, A53T alpha-syn transgenic mice develop neuronal synucleinopathy [178].

Alpha-syn can interact with many proteins, like calpain 1 that can cleave alpha-syn in the C-terminal region further enhancing its fibrillization process [179]. Likewise, alpha-tubulin induces alpha-syn fibrillization in yeast, rat brain, and human brain [122, 125]. Moreover, work from our lab demonstrated that in PD cybrids with a complex I defect and ATP depletion both calpain 1 over-activation and free alpha-tubulin increase trigger alpha-syn oligomerization [98, 103]. Numerous studies now support the hypothesis that

alpha-syn oligomerization is the key step driving neuronal damage in PD. Indeed, the idea that oligomers, rather than the fibrils, are the pathogenic aggregate species that are responsible for neuronal cell death in PD is emerging. In fact, based on several observations, it has been suggested that LB formation does not cause but, instead, protects against neurodegeneration [180, 181]. Interestingly, neuropathological analysis of PD patients' brains has shown that neurons containing LBs seem healthier than neighbouring neurons [182, 183]. Although the mechanism underlying the cytotoxicity of the oligomers is not clear [184, 185], earlier studies showed that alpha-syn protofibrils disrupt synthetic vesicles *in vitro* [186, 187] causing imbalance of cellular ions and, thus, cell death. Several alpha-syn posttranslational modifications lead to the formation of stable oligomers. These include nitration, oxidation, phosphorylation, and interaction with iron. Oxidative modification of alpha-syn via dopamine adducts may facilitate aggregation [175]. Similarly, recent data demonstrated that stable overexpression of alpha-syn in SH-SY5Y cells or exposure of cells to dopamine facilitated alpha-syn oligomerization [188]. Dopamine was shown to modulate differently the stability of protofibrils and fibrils composed of wild-type or mutant forms of alpha-syn [189]. Wakamatsu and coworkers showed that truncated human alpha-syn is deleterious to the development and survival of nigral dopaminergic neurons [190]. Likewise, a work from 2007 demonstrated that aggregated alpha-syn mediates dopaminergic neurons toxicity *in vivo* [191].

Other mechanisms showed that alpha-syn aggregation might be closely related to oxidative reactions which may play a critical role in PD neurodegeneration [192]. For instance, abnormalities in copper and H_2O_2 homeostasis were shown to play critical roles in the metal-catalyzed oxidative oligomerization of alpha-syn [193]. Additionally, by using antibodies to specific nitrated tyrosine residues in alpha-syn, extensive and widespread accumulation of nitrated alpha-syn protein inclusions was demonstrated in protein inclusions of PD brains [72]. Subsequently, it was found that in HEK 293 cells stably transfected with wild-type and mutant alpha-syn, nitrative and oxidative insults induced the formation of alpha-syn aggregates [194]. Our results in PD cybrids indicate that mitochondrial-driven ROS production induces alpha-syn oxidation and oligomerization. Alpha-syn aggregation was significantly decreased upon antioxidant treatment (CoenzymeQ or GSH) [30].

Other important covalent promoter of aggregation is phosphorylation. Alpha-syn carries a number of potential sites for phosphorylation. Indeed, Ser129 phosphorylation of alpha-syn strongly modulates interactions between alpha-syn and synphilin-1 and the formation of inclusions. The levels of soluble alpha-syn oligomeric species are increased by phosphorylation at Ser129 [195]. It was shown that alpha-syn is extensively phosphorylated in aggregates from synucleinopathies patients [196]. In addition, polyunsaturated fatty acids were reported to promote oligomerization, suggesting that alpha-syn may aggregate via interaction with cell membranes [197]. One proposed mechanism for oligomers toxicity, as previously mentioned, is the formation of pores by ring-like intermediates. Moreover, iron was shown to specifically induce alpha-syn aggregation suggesting its role in aggregate formation [198].

Evidences suggest that mitochondrial dysfunction could induce alpha-syn misfolding. In fact, treatment with rotenone resulted in an increase of detergent-resistant alpha-syn aggregates in COS-7 cells expressing wild-type alpha-syn [199]. Moreover, mutant and wild-type alpha-syn interacts with mitochondrial cytochrome c oxidase, a key enzyme of the mitochondrial respiratory system [200]. Li and coworkers demonstrated that a portion of alpha-syn is present in the membrane of mitochondria in normal dopaminergic neurons [201]. Under overexpression conditions, alpha-syn may translocate to the mitochondria and cause enhanced toxicity in response to subtoxic concentrations of mitochondrial toxins [202]. Using SHSY cells overexpressing alpha-syn A53T mutant or wild-type, as well as isolated rat brain mitochondria, it was shown that alpha-syn localizes at the mitochondrial membrane causing oxidative stress [76]. More recently, Devi and colleagues showed that mitochondria of PD-vulnerable substantia nigra and striatum had significant accumulation of alpha-syn and decreased complex I activity [203]. Another interesting study demonstrated that during the ageing process in yeast, functional mitochondria are required for alpha-syn toxicity [204]. Furthermore, our work provided data showing that PD cybrids harbouring inherent mitochondrial impairment developed alpha-syn oligomers accumulation. Alpha-syn oligomerization in our studies was triggered by several different pathways all dependent on mitochondria malfunction (reviewed by [205]).

Although more studies need to be performed in order to elucidate alpha-syn function, it is quite undeniable that its oligomerization seems to be the key step that drives both pathology and cellular damage in PD.

5. Intracellular Mechanisms of Degradation

5.1. Calpain-Mediated Proteolysis. Sustained mitochondrial dysfunction in PD can release calcium, contributing to aberrant calcium homeostasis in the cytosol leading to activation of calpains. Indeed, mtDNA-depleted cells exhibit an increase in calpain activation [206]. Calpains are calcium-activated neuronal proteases that belong to a highly conserved family of cysteine proteases, thus, they are widely expressed in the CNS and are preferentially involved in the degradation of short-lived proteins [207]. Its structure consists of a cysteine-proteinase domain combined with a calmodulin-like Ca^{2+}-binding domain. In fact, the activities of the representative classical, ubiquitous mammalian calpains, μ-calpain (calpain 1), and m-calpain (calpain 2) are regulated by Ca^{2+} concentration. They exist as a proenzyme heterodimer (80 kDa and 29 kDa) in resting cells but are activated by calcium through autolytic processing (to produce a heterodimer of 78 kDa and 18 kDa). Furthermore, *in vitro* studies showed that calpain 1 and 2 differ in the calcium concentration required to become activated; calpain 1 requires a calcium concentration in the μmolar range, and calpain 2 requires mmolar range [208]. It is widely known that calpastatin, a ubiquitously expressed 110 kDa protein,

is the sole endogenous inhibitor of calpains [209, 210]. Overall, calpains are intracellular nonlysosomal calcium-regulated cysteine proteases that mediate regulatory cleavages of specific substrates and are involved in a number of processes during differentiation, life, and death of the cell. These proteases cleave diverse proteins species, including receptors, ion channels, cytoskeletal components, proteases, oncogenic proteins, and cell signalling proteins. Although calpain activation was initially implicated in the necrotic process, several studies have indicated that these proteases play a prominent role in apoptotic processes. In fact, calpains are able to be activated via caspase-mediated cleavage of calpastatin during initiation of apoptotic execution [211]. Active caspase-3 can cleave calpastatin, thereby promoting uncontrolled calpain activation. Although calpains may enhance caspase activity, they can also function to block the activation of caspases. For example, calpains can cleave caspase-9 rendering it incapable of activating caspase-3 and preventing the subsequent apoptotic cascade [212]. Moreover, procaspase-3 is a calpain substrate. In PC12 cells, MPP^+ induced calpain activation and AIF release which was prevented by calpain inhibitors [213].

Cdk5 belongs to a group of serine/threonine kinases best characterized for their role in cell cycle progression. This kinase was shown to have a role in the pathogenesis of PD. Cdk5 has been colocalized with LBs in PD patients' brains. Conversion of the cdk5 activator p35 to the more stable p25 form may lead to increased cdk5 activity. Consistent with this possibility, an increase in p25 levels was shown after MPTP treatment. Several reports suggest that the p35-to-p25 conversion is mediated through calpain activation [214–216]. Increased expression of calpain in the mesencephalon of PD patients and primate PD models has been demonstrated, suggesting a calpain involvement in PD pathogenesis [217]. Additionally, enhanced calpain expression and activity is observed in the substantia nigra and locus coeruleus of PD patients. Increased calpain expression and cell death in spinal cord in C57BL/6N mice were also reported following MPTP treatment [218]. The presence of calpains in morphologically abnormal dopaminergic fibers as well as in LBs further supports calpains involvement in PD. Most interestingly, inhibition of calpain blocks neuronal degeneration and restores behavioural function in experimental models of PD [219]. Likewise, the inhibition of mitochondrial complex I by MPP^+ and rotenone activated calpain and seems to constitute an early event in the neurodegenerative process prior to caspase-3 activation [220, 221]. Moreover, rotenone-induced degeneration of spinal cord motor-neuron in male Lewis rats progressed with upregulation of calpain and caspase-3 [222].

It is now well accepted that alpha-syn oligomerization is pivotal in progression of PD pathology and cellular damage. Subsequently, it has been described in other diseases that involve protein aggregation, the protective effect of calpain inhibition. Haacke and coworkers showed that coexpression of the highly specific cellular calpain inhibitor calpastatin abrogated fragmentation and the formation of inclusions in cells expressing pathological ataxin-3 suggesting a critical role of calpain in the pathogenesis of Spinocerebellar ataxia type 3 [223]. Moreover, Goñi-Oliver and colleagues provided data that established the first direct evidence that calpain promotes GSK-3 truncation in a way that has implications in signal transduction, and probably in pathological disorders such as AD [224]. In 2003, it was shown that parkin accelerates the degradation of alpha-syn via the activation of calpain leading to the prevention of alpha-syn-induced cell death [225]. Also in 2003, it was demonstrated that alpha-syn was a substrate of calpain 1 [179]. Latterly, the same group showed data revealing that calpain-mediated cleavage near and within the middle region of soluble alpha-syn with/without tyrosine nitration and oxidation generates fragments that are unable to self-fibrillized [226]. As opposed, Dufty and colleagues in 2007 showed that cleavage of alpha-syn by calpain occurs in PD brain and calpain-cleaved fragments of alpha-syn colocalized with activated calpain [227]. These results suggest that calpain may link alpha-syn to its disease-linked oligomerization, aggregation, and subsequent formation of LBs. Our group showed that PD cybrids have increased cytosolic calcium levels correlated with an increase in calpain activation and expression which subsequently potentiates the formation of toxic alpha-syn oligomers. In addition, our results showed that calpains inhibition decreased the toxic alpha-syn oligomers and increased the nontoxic insoluble alpha-syn aggregates, preventing caspase-3 activation [103].

Demarchi and collaborators showed that calpain modulates macroautophagy in mammalian cells [228]. Indeed, in calpain-deficient cells, autophagy is impaired, thus contributing to the apoptotic switch. Recent data showed that in AD the lysosomal autophagic system may not degrade tau in the normal adult rat brain. However, inhibition of autophagy may induce tau proteolysis through calpain activation [229]. So far, no data was shown confirming the correlation between autophagy and calpains in PD.

Overall, a role for calpains-mediated proteolysis is strongly postulated in the pathogenesis of PD. More studies need to be performed to address if calpains inhibition offer a potential therapeutic target in the neurodegenerative process of PD.

5.2. Autophagy: A Quality Control System. Age causes a progressive accumulation of wornout organelles and subcellular structures that reduces the cellular and molecular efficiency of various biological processes that are required for maintaining homeostasis and survival. This accumulation of extracellular/intracellular damaged proteins has been frequently used as a biomarker of ageing [230]. Moreover, it seems to reflect an unbalance between the capability/activity of the quality control systems and the amount of altered proteins in the cells. Autophagy is ubiquitous in eukaryotic cells and is the major mechanism involved in the clearance of oxidatively or otherwise damaged/worn-out macromolecules and organelles, controlling thus cell death. Autophagy is a process traditionally regarded as a cellular response to stress typically related to nutrient deprivation, toxin exposure, infection, or oxidative stress. In contrast to the ubiquitin-proteasome pathway which degrades mostly short-lived proteins, autophagy is primarily involved in breaking down proteins with long half-lives and damaged

organelles [231]. Moreover, is a highly organized and probably quite specific intralysosomal degradation pathway regulated by a large family of genes, the ATG family [232, 233]. Over 30 atg genes have been identified in yeast and at least 11 have orthologs in humans. For instance, atg6 is also known as beclin and atg8 is commonly called LC3 in mammals [234, 235]. There are three main types of autophagy in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [236]. The differences among the various autophagic mechanisms depend of the type of cargo, route, and mechanism for its delivery to lysosomes. Macroautophagy involves any type of cellular material, including large organelles, such as mitochondria, being sequestered within a double-membrane-bounded vacuole called autophagosome. Autophagosomes receive acid hydrolases by fusion with late endosomes or lysosomes. Subsequently, the degradation of its content progresses and matures to a secondary lysosome or residual body depending on whether degradation is complete or partial, respectively [237–239]. Microautophagy involves macromolecules and small organelles that enter lysosomes through invagination of its membrane [240, 241]. CMA is a mechanism for selective digestion of a particular group of soluble cytosolic proteins with a KFERQ motif [233, 242]. Once this sequence is recognized, these proteins are directly translocated to the lysosomal membrane.

Alterations in the autophagic machinery have been implicated in the pathogenesis of PD. Indeed, an increased number of autophagic vacuoles and related structures of autophagy have been found in PD patients [243]. In addition, autophagic vacuoles are also described within mesencephalic dopaminergic neurons in several experimental models of PD [244, 245]. LC3-II and Beclin-1 expressions were significantly increased in brains from humans with Dementia with LBs and in transgenic mice overexpressing A53T alpha-syn, as compared with respective controls [246]. However, the lysosome-associated membrane protein 1 (LAMP1), cathepsin D (CatD), and Heat Shock Protein 73 (HSP73) immunoreactivity were significantly decreased within PD nigral neurons when compared to age-matched controls [247]. There is controversy over whether this increase in autophagic markers is protective or instead causes neuronal death. It has been suggested that this increase is responsible for neuronal death. However, enhanced degradation of deleterious aggregates or disrupted organelles can be protective [248].

Autophagy is indeed an important process in a variety of human diseases caused by toxic, aggregate-prone, intracytosolic proteins which became inaccessible to the proteasome when they form oligomers [249, 250]. Depending on its conformational state and cellular conditions, alpha-syn can be degraded by autophagy [251]. CMA and macroautophagy were shown to be important pathways for wild-type alpha-syn degradation in neurons [252]. Being a soluble protein, alpha-syn can be degraded by CMA because it contains the CMA recognition targeting motif [253]. None of the 3 mutations in familial forms of PD affect the CMA targeting motif. Nevertheless, the wild-type protein, contrary to the mutant forms, is readily translocated into the lysosomal lumen whereas the mutant forms remain bound to the complex not only blocking its degradation but also the degradation of other substrates. Moreover, posttranslational modifications of alpha-syn that promote its oligomerization have a similar effect, suggesting that alpha-syn oligomers may block lysosomal translocation and CMA [248]. For example, in mouse ventral medial neuron cultures, SH-SY5Y cells, and isolated mouse lysosomes, dopamine-modified alpha-syn is not only poorly degraded by CMA but also blocks degradation of other substrates by this pathway [254]. While the narrow proteasome barrel excludes the entry of oligomers/aggregates of alpha-syn, such substrates can be degraded efficiently by macroautophagy. The ability of alpha-syn to inhibit proteasome activity is related to its propensity to assemble into filaments [255]. Indeed, fibrillar forms of the protein usually get jammed inside proteasome blocking its activity [256]. Interestingly, disruption of autophagy by RNA interference significantly increased alpha-syn oligomer accumulation *in vitro*, confirming the significance of macroautophagy in alpha-syn clearance. In addition, rotenone-induced alpha-syn aggregates were cleared following rapamycin stimulation of autophagy [246]. Rapamycin inhibits the mammalian target of rapamycin (mTOR), a negative regulator of autophagy [257]. Rapamycin has been shown to degrade all forms of alpha-syn in a stable inducible PC12 cell model [251]. These findings support the concept that neuronal autophagy is essential for protein homeostasis as well as for the reduction of the potentially pathogenic alpha-syn oligomers. Coexpression of Beclin-1, a regulator of the autophagic pathway, was found to activate autophagy, reduce accumulation of alpha-syn, and ameliorate associated neuritic alterations in a neuronal cell line that overexpressed alpha-syn. This neuronal cell line showed lysosomal accumulation and alterations in autophagy [258].

Ageing seems to affect mitochondria particularly. Mitochondria undergo the most dramatic age-related changes, such as structural deterioration like swelling and loss of cristae, sometimes complete destruction of the inner membrane, decreased respiration, and low ATP production [137]. Often they are extremely enlarged and are called "giant" mitochondria [259, 260]. Nonfunctional mitochondria are important sources of ROS which contribute to the accumulation of damaged and nonfunctional components in the cells which is characteristic of ageing. In addition, because of mitochondrial ROS generation, protein damage occurs and mtDNA mutations accumulate at an accelerating rate. Subsequently, this leads to the synthesis of abnormal mitochondrial proteins exacerbating mitochondrial dysfunction.

During recent years, accumulating evidence has been gathered showing that mitochondria also may be subject to selective degradation through mitophagy. Mitophagy is important to the elimination of dysfunctional mitochondria and mutated mtDNA since DNA repair in mitochondria is much less robust than in the nucleus [261]. There are many reports showing mitochondria within autophagosomes [262–264]. It was then proposed that damaged mitochondria are captured and digested by the lysosome.

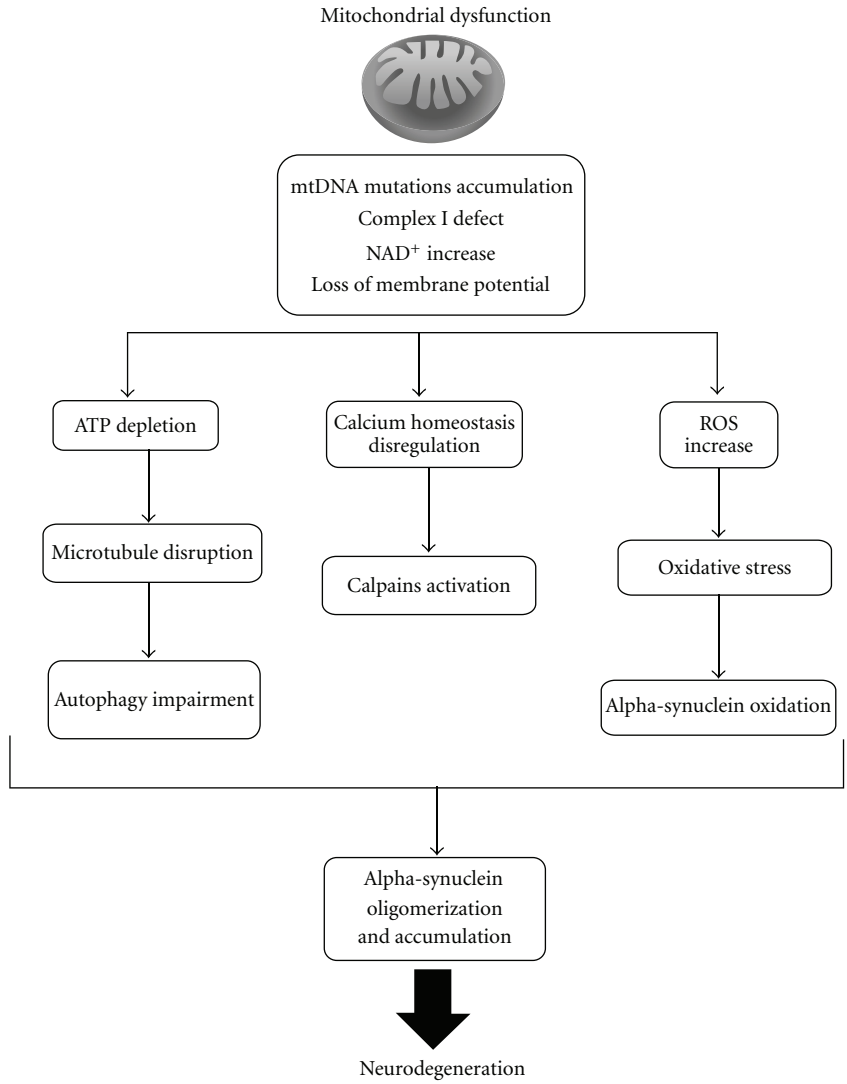

Figure 1: The mitochondrial cascade hypothesis for PD states that the inherited electron transport chain gene combinations determine basal ETC efficiency and ROS production. This defines the rate at which acquired mtDNA alterations occur determining when mitochondrial impairment reaches a threshold that activates the pathologic characteristics of PD. Mitochondrial impairment in PD is characterized by a complex I defect, which leads to ATP depletion and NAD^+/NADH ratio imbalance promoting microtubule disruption. Moreover, mitochondrial membrane potential is lost and calcium homeostasis is deregulated, which leads to calpains activation. In addition, the levels of mitochondrial ROS are significantly increased triggering oxidative stress. These all prompt alpha-syn oligomerization either by interaction with free tubulin, calpains or by oxidation. The end result is alpha-syn oligomerization and accumulation of alpha-syn oligomers as well as of disrupted organelles culminating in neurodegeneration.

The evidence for mitophagy comes from a work in yeast that identifies a specific protein in the mitochondrial outer membrane Uth1p, whose presence is necessary for mitochondrial macroautophagy. Degradation of mitochondrial proteins did not occur in mutant strains carrying null mutations of Uth1p [265]. Additionally, autophagic sequestration of mitochondria is further supported by the observation that protein markers of mtDNA are lost together with protein markers of mitochondrial inner membrane such as cytochrome oxidase subunit IV and subunit I. Parkin and PINK-1 PD-related proteins were recently associated to mitophagy. Chu and coworkers showed that MPP^+ induced autophagy and mitochondrial degradation that was inhibited by siRNA knockdown of autophagy proteins Atg5, Atg7, and Atg8 [266]. Moreover, it was shown that Parkin promotes autophagy of damaged mitochondria and further supports that PD pathogenesis implicates a failure in the elimination of dysfunctional mitochondria [154]. Furthermore, stable knockdown of pink-1 induced oxidative stress and mitochondrial fragmentation in SH-SY5Y cells through autophagic and fission/fusion machineries [156]. A report from 2010 demonstrated a new interaction between PINK-1 and Beclin1, a key proautophagic protein. PINK-1 significantly enhanced basal and starvation-induced autophagy,

which was reduced by knocking down beclin1 expression [267]. Another report provided data linking PINK-1 and parkin to the selective autophagy of mitochondria [268].

In PD, autophagy is of extreme importance once it allows cells to rid themselves of damaged and superfluous mitochondria, as well as other organelles and most interestingly of protein aggregates accumulation.

6. Concluding Remarks

PD affects 1% of the population at age of 65 and up to 5% of the population by age 85 [269] and is the second most common neurodegenerative disorder after AD. Efforts have been made in order to elucidate PD trigger mechanisms within the cell and further consequences. Compelling data suggests that mtDNA alterations may lead to a massive mitochondrial impairment and thus triggering the death of dopaminergic neurons. When mitochondrial dysfunction reaches a critical threshold inside the cell it rouses ATP depletion, oxidative stress, and calcium homeostasis deregulation that in turn induces microtubule disruption, alpha-syn oxidation, and calpains activation. In addition, the fusion and fission machinery is impaired as well as autophagy leading to the accumulation of alpha-syn oligomers and disrupted organelles (Figure 1). This accumulation creates a positive feedback loop causing the pathology and ultimately the death of dopaminergic neurons. Further studies need to be performed to understand how and when mitochondrial dysfunction starts and why it affects a specific group of neurons. The therapeutic challenge might start acting at an mtDNA level or at a microtubule level improving the degradation of alpha-syn oligomers and disrupted organelles.

Abbreviations

PD: Parkinson's disease
alpha-syn: Alpha-synuclein
LBs: Lewy Bodies
MPTP: 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine
mtDNA: Mitochondrial DNA
OXPHOS: Oxidative phosphorylation
ROS: Reactive oxygen species
MPP^+: 1-methyl-4-phenylpyridinium
AD: Alzheimer's disease
6-OHDA: 6-hydroxydopamine
POLG1: mtDNA polymerase gamma 1
NAD^+: β-Nicotinamide adenine dinucleotide
NADH: Reduced β-NAD^+
SIRTs: Sirtuins
PGC1α: Peroxisome proliferators activated receptor gamma coactivator-1 alpha
CMA: Chaperone-mediated autophagy
CNS: Central Nervous System
Mfn1 and Mfn2: Mitofusin-1 and -2
Opa1: Optic atrophy 1
Drp1: GTPase Dynamin-related protein 1
LRRK2: Leucine-rich repeat kinase 2
NAM: Nicotinamide
CNS: Central Nervous System
BDNF: Brain-derived neurotrophic factor
GDNF: Glial cell line-derived neurotrophic factor.

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Review Article

Mitochondrial Dysfunction in Parkinson's Disease: Pathogenesis and Neuroprotection

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Mitochondria are vitally important organelles involved in an array of functions. The most notable is their prominent role in energy metabolism, where they generate over 90% of our cellular energy in the form of ATP through oxidative phosphorylation. Mitochondria are involved in various other processes including the regulation of calcium homeostasis and stress response. Mitochondrial complex I impairment and subsequent oxidative stress have been identified as modulators of cell death in experimental models of Parkinson's disease (PD). Identification of specific genes which are involved in the rare familial forms of PD has further augmented the understanding and elevated the role mitochondrial dysfunction is thought to have in disease pathogenesis. This paper provides a review of the role mitochondria may play in idiopathic PD through the study of experimental models and how genetic mutations influence mitochondrial activity. Recent attempts at providing neuroprotection by targeting mitochondria are described and their progress assessed.

1. Introduction

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder, the second most common age-related neurodegenerative disease after Alzheimer's disease [1]. It can be characterised clinically by rigidity, resting tremor, and postural instability [2]. These symptoms result from the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and a subsequent depletion of dopamine in the striatum [2].

The etiopathogenesis of PD is still not fully understood. 95% of cases are sporadic: a multifactorial, idiopathic disorder resulting from contributions of environmental and genetic susceptibility. The remaining 5% is the result of genetic mutations, of which there are several types, many only recently identified. However, old age remains the greatest risk factor, with 0.3% of the entire population affected, rising to more than 1% in the over 60 s and 4% in those over the age of 80 [3]. The two forms of PD share pathological, biochemical, and clinical features, with dysfunction of mitochondria and associated molecular pathways representing a bridge between the two forms of PD as well as the natural ageing process.

Most mitochondrial dysfunction results from damage to complex I—or NADH (nicotinamide adenine dinucleotide phosphate):ubiquinone oxidoreductase—the first and most complex protein in the electron transport chain [4]. It is a large protein, consisting of 42 or 43 subunits [5] on the inner mitochondrial membrane, which forms part of the oxidative phosphorylation system [6]. Defects to complex I are thought to be central to the pathogenesis of PD, and many other known cell death pathways play a role in complex I-mediated dopaminergic cell death, such as Bax transcription activation [7]. Moreover, the presence of intramitochondrial enzymes such as cytochrome *c* and their role in apoptosis [8] may make mitochondria particularly vulnerable to pathogenic events when physiological mechanisms become disrupted. Oxidative stress can increase the releasable pool of cytosolic cytochrome *c* [9]—a crucial initiator of the caspase apoptotic signalling cascade [10]—through peroxidation of cardiolipin [7], a mitochondrial-specific lipid. Defects in complex I lower the threshold for Bax-mediated

mitochondrial-dependent apoptosis [9], a crucial event in the degeneration of dopaminergic neurons in the SNpc [11]. This advances the suggestion that mitochondrial dysfunction is a common link and a point of convergence to different pathogenic pathways.

Mitochondrial dysfunction is implicated further in sporadic PD by the finding that complex I inhibition can decrease proteosomal activity, which in turn renders dopaminergic neurons more susceptible to damage by some neurotoxins [12]. This suggests that a decline in proteasomal activity could be the mechanism by which the ubiquitin-proteasomal system is impaired, leading to the development of pathological protein aggregates.

The implication of mitochondrial dysfunction in PD began with the accidental exposure of 4 Californian drug users to 1-methyl-4-phenyl-1,2,3,6-tetrahydopyridine (MPTP) (see [13] for a summary of the discovery). Their intravenous administration of the meperidine analogue resulted in selective death of cells in the substantia nigra [14]. The toxin has now been extensively used in mice and nonhuman primates as an experimental model for PD [1], which has allowed the molecular basis of neuronal death to be followed. The effect is systemic, with human platelets also vulnerable to MPP^+-induced toxicity [15]. The unravelling of the action of MPTP has led to a great deal of information regarding the involvement of mitochondrial dysfunction. Armed with further information regarding the molecular pathogenesis of PD, investigators have identified further toxins with the capacity to be used for PD experimental models [16]. All have mitochondrial dysfunction and the related activation of cellular pathways at the heart of their effects.

2. Mitochondrial Dysfunction in PD and Oxidative Stress

Oxidants, including hydrogen peroxide and superoxide radicals, are produced as byproducts of oxidative phosphorylation, making mitochondria the main site of ROS generation within the cell. This is a normal situation and basal levels of ROS can be limited by a range of antioxidants. However, in pathological situations, where mitochondrial respiratory defects occur, the amount of ROS produced by the electron transport chain increases dramatically, swamping the antioxidant protection mechanisms. PD has been shown to produce these conditions. A postmortem description of complex I deficiency in the substantia nigra of PD patients led to the direct link between mitochondrial dysfunction and the disease [17], which has since been repeatedly observed [18]. Respiratory chain defects can also be found in platelets [19] and other highly oxidative tissues, such as skeletal muscle [20], in idiopathic PD.

Perier et al. [9] has linked increased ROS levels to complex I inhibition with the use of neurotoxin models. Complex I defects can lower the threshold of apoptosis mediated by mitochondria through depletion in ATP production and the generation of free radicals [9]. Elevated ROS levels can also result in damage to phospholipids and polyunsaturated free fatty acids (PUFA), which are both highly prevalent in the brain and very susceptible to oxidative damage. In PD, lipid peroxidation is increased in the SN up to the time of death compared to age-matched controls, as shown by lower PUFA content—an index of the amount of available substrate available for lipid peroxidation—in post-mortem tissue [21]. Levels of markers of oxidative damage to proteins are also increased in PD post-mortem tissues, with a twofold increase in the SN [22, 23]. Two reasons are given for this region-specific protein carbonyl rise: the symptomatic PD treatment levodopa contributes to this oxidation [22], and oxidative damage is higher in dopaminergic neurons [23]. Studies have sought to find if this oxidation, and subsequent disease progress, is related to excess production of ROS or inadequate and impaired detoxification by the endogenous antioxidants. Analysis of superoxide dismutase (SOD) levels show no change in SOD1 (cytosolic isoform) levels, but there is an increase in activity of the inducible mitochondrial isoform SOD2 [24]. This suggests that mitochondria are the location of increased ROS production and are the site of the first line of defence against oxidative stress.

3. Experimental Models and Mitochondrial Dysfunction

The discovery by Langston and colleagues in 1983 that the neurotoxin MPTP causes specific and irreversible damage to dopaminergic neurons and generates Parkinsonian symptoms [14] caused a surge in activity in PD research. The use of a reproducible model allowed for the pathogenic events to be studied, which has led to the gradual unravelling of many of the biochemical disturbances (although some steps remain unknown). Several other compounds induce dopaminergic cell death, with all affecting mitochondrial physiology.

3.1. MPTP. The metabolism of MPTP occurs in a complex and stepwise fashion [25]. As it is highly lipophilic, the compound can rapidly (within seconds) cross the blood-brain barrier (BBB) after systemic administration. The protoxin MPTP is metabolised within the brain to the unstable molecule 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by monoamine oxidase (MAO), specifically the MAO-B subtype enzyme within nondopaminergic neurons due to the cellular localisation of the enzyme [26]. Then, probably due to spontaneous oxidation, it forms the active toxin 1-methyl-4-phenylpyridinium (MPP^+) [27]. MPP^+ is then released via an unknown mechanism into the extracellular space. It is the next step which accounts for the selectivity of the toxin: as MPP^+ is a polar molecule it cannot freely enter cells, unlike its precursor, MPTP. The toxin does however have a high affinity for plasma membrane dopamine transporter (DAT), as the use of DAT inhibitors [28, 29] or the absence of DATs [30] prevent its uptake. Once inside dopaminergic neurons, there are at least three routes which MPP^+ can take [31]: it can take the vesicular pathway, bind to vesicular monoamine transporters and translocate MPP^+ into synaptosomal vesicles [32]; it can interact with various cytosolic enzymes by remaining in the cytosol [33], or can be concentrated within the mitochondria [34]. The effect of the latter of these three routes will now be discussed. However,

it should be noted that a cascade of deleterious effects [31] contribute to a greater or lesser extent to dopaminergic cell death, and no factor alone is the sole cause of the degeneration.

MPP^+ enters the mitochondria passively due to the large transmembrane potential of mitochondrial membranes and accumulates at the matrix. The levels of MPP^+ in the mitochondria can reach saturation within a few minutes [34]. The rate of the toxin's uptake depends on its intramitochondrial concentration, which inversely affects the transmembrane potential [34]. This equilibrium is disrupted when mitochondria become anaerobic or the transmembrane potential gradient is disturbed by the presence of an uncoupling agent [34].

Once MPP^+ has entered the mitochondria in this potent and rapid manner, the toxin can affect various functional elements of the organelles. For example, the tricarboxylic acid cycle enzyme α-ketoglutarate dehydrogenase is inhibited by MPP^+ [35]. But the main cause of mitochondrial dysfunction involves the compound's action on complex I of the respiratory chain. MPP^+ binds to complex I soon after its uptake by dopaminergic neurons. Rotenone (discussed below) is a classic complex I inhibitor. Its binding is blocked by the presence of MPP^+ [36], confirming the site of the toxin's action. It has been found that the binding of MPP^+ is required at two distinct sites (one hydrophilic and one hydrophobic) for complete NADH inhibition, but it provides a much weaker inhibition of complex I than rotenone [4, 37]. The binding to the first of these sites affects the coupling between the PSST and NADH dehydrogenase (ND) 1 subunits (20 and 36 kD, resp.) of complex I, while the location of the second site, although apparently causing a more potent a more potent inhibition of complex I enzymatic activity [4], is not precisely known. The flow of electrons is interrupted in a dose- and time-dependent manner, leading to an acute deficit in ATP production, particularly in the striatum and ventral midbrain [38, 39]. However, due to the low levels (around 20%) of ATP depletion which actually occurs *in vivo* in whole mice brain tissues [38] and the evidence that there is little or no neuronal loss when residual ATP levels are maintained above 20% [11], there is thought to be more prominent factors causing MPTP-induced dopaminergic cell death than a MPP^+-related reduction in ATP levels. In addition to this, complex I activity is required to be reduced by more than 50% to cause significant ATP depletion [40]. The role of complex I inhibition in MPP^+ toxicity is further questioned by Choi et al. [41], who found the toxin caused dopaminergic cell death occurred despite a lack of the *Ndufs4* gene, essential for complex I assembly and function. The importance of MPP^+ binding can be supported, however, by improvements in ATP production and alleviation of degeneration after efforts to stimulate mitochondrial respiration by way of a bypassing of the complex I blockade [42].

Mitochondria are a major source of ROS, with up to 2% of all oxygen consumed by mitochondria being converted to superoxide [43]. The production of ROS is increased after MPP^+ inhibition of complex I [32] by more than 40% over control levels [11], which works in parallel with other reactive molecules including nitric oxide, a molecule produced by the nitric oxide synthase enzyme in the brain [44]. Transgenic mice with increased brain activity of the scavenging enzyme SOD showed resistance to MPP^+ toxicity [45]. This protective effect did not affect the block on complex I activity, showing the importance and potency of free radical damage in the MPTP model. This enzyme has been localised in the intramembrane space where it acts as a physiological protective mechanism against superoxide toxicity [46]. The deleterious impact of ROS in the MPTP model mirrors the results of studies conducted on human post-mortem samples [47]. It is likely that a depletion of ATP and increased ROS production, following complex I impairment, trigger molecular cell death pathways such the activation of procell death protein Bax [7], and it is these ROS-initiated pathways, rather than an acute energy shortage, which result in the greatest proportion of MPTP-induced degeneration.

It has been proposed that MPTP may also cause dopaminergic cell death through a reduction in the number of lysosomes [48], cytoplasmic organelles containing hydrolytic enzymes important in the degradation of cytoplasmic proteins, and other organelles through the process of macrophagy [49] (also see mitophagy, which is discussed in Section 5.3). ROS, levels of which are elevated due to mitochondrial dysfunction, cause permeabilisation of lysosomes leading to defective clearance and accumulation of the double-bonded sequesters of the cytosolic material to be degraded, autophagosomes (APs). Lysosomal depletion and neurodegeneration subsequently occur after ectopic release of lysosomal proteases. Induction of lysosomal biogenesis attenuated the MPTP-induced cell death [48], while rapamycin, an inhibitor of the cellular kinase mammalian target of rapamycin (mTOR) known to induce autophagy, can restore lysosomal levels and reduce AP accumulation [48, 50]. This represents a further pathway by which ROS released in energy-deprived cells cause cell death in this model.

3.2. Rotenone. Rotenone is used commonly as a pesticide in the UK and United States. Its use in South America dates back to the 17th century as an agent to paralyse and surface fish. Epidemiological studies have established a link between contact with pesticides and an increased risk of Parkinson's [51], although the short half-life of the compound, particularly in sunlight (1–3 days), and the fact that it does not leach from soils mean the chance of PD being caused directly from environmental exposure to this pesticide is very low, with others leading to the aforementioned epidemiological trend [52]. Rotenone is a specific inhibitor of complex I and causes its deleterious effects through oxidative damage, with only mild depletion of ATP levels [53]. This toxicity can be prevented with the administration of antioxidants [53]. Rotenone rapidly crosses the BBB thanks to its lipophilic structure, with maximal CNS concentrations reached within just 15 minutes [54]. Within cells, rotenone freely crosses into subcellular compartments including mitochondria, in which the toxin impairs oxidative phosphorylation by binding to the PSST subunit of complex I [4]. Interestingly,

due to this promiscuous manner of cell entry, rotenone does not concentrate within dopaminergic cells selectively, unlike MPTP, although cell death is specific to these cells. Therefore, dopamine cells appear to be particularly sensitive to the resultant complex I dysfunction [55]. Again unlike MPTP, rotenone leads to the presence of Lewy bodies [56], the proteinaceous pathologic biomarker of sporadic PD. These inclusions are immunoreactive to α-synuclein and ubiquitin [56]. The evidence that rotenone, a classic complex I inhibitor, causes aggregation of Lewy bodies may mean that mitochondrial dysfunction has a role in the development of these pathologic protein inclusions in PD [57]. Furthermore, recent evidence shows that rapamycin protects against rotenone-induced apoptosis by the induction of autophagy in SH-SY5Y human neuroblastoma cells [58]. This provides a further link between mitochondrial dysfunction and the toxic effect of rotenone, with the clearance of the impaired organelles induced by rapamycin preventing increases in cytochrome *c* levels and apoptotic pathways [58].

3.3. 6-OHDA. 6-hydroxydopamine (6-OHDA) is a hydroxylated analogue of dopamine, first isolated in 1959 [59]. It has been used most extensively in rodents. By virtue of its structure, 6-OHDA possesses a high affinity for many catecholamine membrane transporters including DAT and norepinephrine transporters, allowing the compound to freely enter both dopaminergic and noradrenergic neurons [16]. This leads to a lack of specificity, as damage can be caused centrally and peripherally to these signalling pathways. Therefore, administration needs to be precise: stereotaxic injection is usually the route used to create a Parkinsonian lesion (6-OHDA does not cross the BBB well spontaneously, thus systemic administration does not result in the accumulation of neurotoxic concentrations), which represents a technical challenge. Once in catecholamine cells, 6-OHDA causes damage through reactive oxygen species and quinones [60]. The fine molecular details of neuronal death are more difficult to understand with this model, however, as the ways by which cytotoxic events occur may differ depending upon the distance between them and the site of injection [16]. Despite this, some of these details have begun to be uncovered, including the induction of autophagy through activation of extracellular signal-regulated kinase (ERK) 1/2 [61, 62]. Inhibition of the ERK pathway confers neuroprotection in 6-OHDA-treated cells [62]. The mitochondrial localisation of ERK2 in particular causes enhanced autophagy to levels which cause a pathologic reduction in ATP production due to the degradation of healthy mitochondria [61], providing a molecular component in 6-OHDA cytotoxicity.

4. Mitochondrial DNA

An extra level of complexity is added to mitochondria as they can house their own mitochondrial DNA (mtDNA), possibly due to an ancestry to free-living bacteria that became trapped to form eukaryotic cells [63]. This confers an extent of genomic autonomy to these organelles, but given their precarious yet vital position and specific vulnerability to toxins, it may increase the likelihood of pathogenic alterations occurring. Mitochondrial DNA is prone to point mutations at a higher frequency than DNA in other parts of the genome. This is due to a lack of repair mechanism, small mitochondrial genome size (around 16 kb in length), and the close proximity of the genome to the site of ROS production at the inner mitochondrial membrane. Consequently, an accumulation of mtDNA mutations occurs throughout life and results in precipitation of pathological disorders due to a gradual degradation of mitochondrial efficiency [64–66]. As PD development is intrinsically linked with age, this is an attractive hypothesis to couple with what is known about the disorder's pathogenesis. There have been various reports to back this up [67, 68], although no single gene defect appears likely to cause more damage than others, with gene mutations combining in a complex manner [69]. However, the role of mitochondrial point mutations in ageing has been questioned by Vermulst et al. [70], who found that mutation frequency was 11-times lower than previously reported [71] and did not have an effect on ageing in wild-type mice. The group also debated the validity of previous data on the basis of the PCR and cloning strategies used, techniques that are limited by polymerase infidelity and cloning artefacts. In another contradiction to the link between mtDNA defects and age, a higher rate of homoplasmic mtDNA mutations has been found in PD patients from younger subjects, who have had less significant SN dopaminergic loss (M.F. Beal, unpublished findings, cited in Banerjee et al. [72]). Thus, the correlation between mtDNA damage, age, and PD incidence is by no means indivisible.

Most neuropathological studies have been limited to skeletal muscle, which provides an accessible tissue, important in the diagnosis of mtDNA disorders and often involves functional muscle impairment. Muscle from these patients has been found to feature respiratory chain-deficient muscle fibres. These particular fibres showed higher levels of mtDNA deletion than normal fibres. Muscle specific mtDNA mutations were found in this postmitotic tissue, demonstrating the propensity mtDNA has to mutate and for these point mutations to accumulate [73]. Mitochondrial proliferation is linked to apoptosis and a high amount of mutations [74].

It has been found that complex I defects (a 25% deficiency was shown) from PD patients can be transferred into mitochondrial deficient platelet cybrids [75]. These defects can affect calcium homeostasis by altering mitochondrial membrane potential, an elevation of ROS production and reduced ATP production [76]. Since mtDNA encodes 7 of the 49 protein subunits of the complex I enzyme, it is fair to surmise that any defects to this genomic structure would have palpable functional disturbances.

The SN may be particularly susceptible to the accumulation with age of somatic mtDNA mutations due to their age-related loss, high oxidative capacity, and sensitivity to mitochondrial dysfunction [77]. Indeed, this group show high levels of mtDNA deletions associated with decreased cytochrome oxidase activity in SN neurons of both aged and PD patients and demonstrated mitochondrial respiratory chain impairment. Mitochondrial DNA deletions of over 43% and 52% of total mtDNA in individuals over the age

of 70 and in PD patients, respectively, were found [77]. This data, combined with that of the independent study carried out by Kraytsberg et al. [71] show that parkinsonism enhances the impairments already suffered by aged SN neurons due to mtDNA damage. However, despite this correlation, it is not clear whether mtDNA mutations are the primary cause of mitochondrial dysfunction or exist secondary to the organelle's functional impairment. Reeve et al. [78] outlines the hypothesis that somatic mtDNA mutations occur as a result of oxidative stress and clonally expand. Cells affected by PD (i.e., neurons in the SN) are more likely to contain mtDNA mutations due to the stresses that these cells are subject to. This provides a composite conclusion of both schools of thought, whereby mtDNA mutations are originally secondary to the primary cause of disease and/or cellular stress, but once clonally expanded can contribute to the pathogenesis of the disease by participating in cell death pathways.

Outside of a role in conferring functional deficits, human mtDNA exhibits region-specific variation in indigenous populations, allowing these haplogroups to be phylogenetically organised. Geographical-dependent replacement mutations and amino acid conservation substitutions have allowed our ancestors to survive more northern climates and higher altitudes [79]. This has consequences in health today, with some mtDNA haplotypes influencing PD expression. Van der Walt et al. [80] found a single-nucleotide polymorphism at 10398G in individuals classed as haplogroup J, which confers neuroprotection, particularly in women. The haplotype causes an amino acid change from threonine to alanine in the ND3 subunit of complex I [80]. A disease-specific link with the mtDNA cluster UKJT has also been found and is reported to cause a 22% reduction in population-attributable risk for PD [81]. In a study of a Finnish population, an excess of nonsynonomous substitutions to complex I genes in mtDNA in the JTIWX supercluster increased the risk of PD and PD with dementia by 2.5-fold. This supports the theory that the total number of substitutions in mtDNA conveys the risk of PD rather than individual mutations [82].

5. Genetic Causes of PD and Mitochondria

Heritable cases of PD make up a very small proportion of PD cases, but a heritable factor may predispose an individual to the development of the disease [83] and are involved in early onset forms of the disorder [84–86]. As discussed so far, mitochondrial dysfunction is central to the pathogenesis of PD, thus it follows that the gene defects outlined here also directly or indirectly affect mitochondrial function.

5.1. α-Synuclein. This soluble, acidic, presynaptic nerve terminal protein has an increased tendency to aggregate due to its hydrophobic non-β-amyloid domain, and forms the major structural component of Lewy bodies, as well as acting as the precursor protein for amyloid plaques in Alzheimer's disease [87]. Physiologically in the substantia nigra, it may have a role in the filling and refilling of synaptic vesicles [88]. Three missense mutations, A53T [89], A30P [90] and E46K [91], and one triplication [92] of this 140-amino acid protein have so far been found to cause an autosomal dominant inherited form of PD. It has been discovered in yeast that functional mitochondrial DNA is essential for α-synuclein toxicity [93]. Evidence also suggests a role for α-synuclein in causing mitochondrial dysfunction. A study of the A53T transgenic mouse, in which the polymerisation process which ultimately converts monomers into amyloid fibrils is accelerated, established that these mice develop dysmorphic mitochondria in brain stem and spinal cord cells [94]. It has also been found *in vitro* that overexpression of α-synuclein impairs mitochondria structure and function [95]. In addition to this, cytochrome *c* interacts with mitochondria in Lewy bodies to promote their aggregation [96], as well as activating cell death pathways. Mitochondrial DNA damage was also found in A53T mice, while nuclear DNA remained intact [94], demonstrating the increased vulnerability of mtDNA. Wild-type α-synuclein is protective and lowers staurosporine-induced caspase-3 activity and p53 expression [97], but these effects can be reversed by the addition of 6-OHDA, thus shifting this antiapoptotic protective function to one which contributes to the aggregation of α-synuclein [98], suggesting an indirect mechanism by which mitochondria interact with cell death pathways. Mutant α-synuclein expression *in vitro* show reduced proteasomal activity without direct toxicity, although they were more sensitive to apoptotic-mediated cell death along with mitochondrial depolarisation and caspase-3 and -9 elevation when treated with subtoxic concentrations of a proteasomal inhibitor [99], linking the paths of mitochondrial dysfunction and protein aggregation. But it remains unclear whether mitochondrial dysfunction precedes α-synuclein inclusion formation or vice versa. They are, however, intrinsically linked. α-synuclein associates with mitochondria during pathologic conditions: a sequence in the N-terminal of human protein contains a mitochondrial-targeting signal that leads to α-synuclein associating with the inner membrane of the mitochondria, resulting in complex I impairment and increased ROS production [100], increased protein tyrosine nitration and a decreased mitochondrial transmembrane potential [101]. The cell death pathway mediator, cytochrome *c*, is also released upon this aggregation [102]. The association of α-synuclein and mitochondria was especially significant in PD-vulnerable brain regions, that is, SNpc and striatum [100]. Rotenone-induced aggregation of α-synuclein, and the subsequent decrease in ATP levels, was reversed by the removal of the complex I inhibitor [103], highlighting the importance of mitochondrial impairment to aggregate formation. The possibility that the combination of α-synuclein and mitochondria is a protective measure can be countered by evidence that α-synuclein-null mice are resistant to mitochondrial toxins, including MPTP [104, 105]. It has been suggested that abnormal cytosolic conditions are required for this signalling to become active and translocation to take place: it occurs rapidly in low intracellular pH [106], a condition which may be caused by ROS overproduction and metabolic stress. Thus, biochemical abnormalities interact with genetic modifications to the α-synuclein gene resulting in the impairment of mitochondrial function and

subsequent neuronal degeneration. These interactions are demonstrated in Figure 1.

5.2. Parkin. Mutations to the parkin gene (PARK2) are linked to an autosomal recessive juvenile form of the disease [107], constituting the most common cause of young onset PD. Hundreds of such cases have been found in patients from almost all ethnic backgrounds [108]. The parkin gene encodes a 465 amino acid RING finger-containing protein. Like other RING finger proteins, parkin acts as a ubiquitin E3 ligase. It has an important role in the function of ubiquitin, which is primarily involved in the targeting of aggregation-prone substrates for degradation by proteosomes, by conferring substrate specificity (parkin's function is reviewed [109]). This function has been shown *in vitro* [110]. Thus, mutations to parkin may cause loss of this ligase activity resulting in accumulation of toxic substrates and degeneration, with many potential substrates for parkin [109]. The fact that these substrates do not fit into a common pathway may be due to the broad range of regulatory functions, alongside protein aggregate degradation, which ubiquitin fulfils. These include signal transduction, postreplicative DNA repair, endocytosis protein trafficking, and regulation of transcription and translation [111].

But parkin's most reproducible, and possibly most relevant, activity is its neuroprotective activity against a variety of pathogenic factors. At least, some of this neuroprotection is afforded by delaying mitochondrial swelling and rupture and the subsequent cytochrome *c* release and caspase-3 activation, as demonstrated in cells overproducing parkin [112]. The importance of parkin in the normal function of mitochondria as a controller of cytochrome *c* release, and therefore apoptosis, is underlined by the inverse relationship expression levels of parkin share with cytochrome *c* release [113]. Parkin can become associated with the mitochondria, particularly the outer membrane, suggesting a direct and local protective effect [114]. The protein is involved with the regulation of transcription and replication of mitochondrial DNA in proliferating cells, stimulating the organelle's biogenesis—an effect blocked by parkin short-interfering RNA (siRNA) knockdown [114]. This has been backed-up *in vivo*: parkin-null mice show reduced mitochondrial respiratory capacity as well as increased protein and lipid peroxidation [115] leading to nigrostriatal defects but not loss of dopaminergic neurons [116]. Mitochondrial morphological changes have been observed, but this led only to disruption of complex I function in nigral mitochondria and did not result in cell death [117]. Abnormal morphological branching of mitochondria and lowered complex I-mediated ATP production have been described in parkin-mutant fibroblasts, but these functional impairments did not occur when parkin was knocked down by 50% [118]. An increased susceptibility to oxygen radical damage has been observed in parkin-null *Drosophila* [119]. A comprehensive study has confirmed the association between parkin and mtDNA, leading to protection from oxidative stress and stimulation of mitochondrial genome damage [120]. These functions were impaired in parkin-deleted human fibroblasts [120]. Thus, functional parkin is important in the maintenance of mitochondrial antioxidant defences and protection of mtDNA.

5.3. PINK-1. PINK1 (PTEN-induced putative kinase 1) gene mutations are the second most common cause of autosomal recessive, early-onset parkinsonism [86]. This 581 amino acid protein features a serine/threonine kinase domain, which is the site of most missense mutations, leading to impairment of kinase activity [86]. Like parkin, PINK-1 has been shown to be protective in a number of stress paradigms [121–123]. Nigrostriatal neuronal loss has been observed in post-mortem brains of PD patients with PINK1 mutations [124]. This study also showed the localisation of PINK1 to mitochondria. It is here that PINK1 mutations are thought to cause PD by impaired phosphorylation of its substrates, although the precise mitochondrial location of PINK1 association is unclear [125].

Mutations of PINK1 result in a loss of the protein's function, leading to PD. It is in mitochondria that the impaired phosphorylation is most deleterious: mitochondrial cristae are fragmented in PINK1 mutants, and there is increased susceptibility to oxidative stress [126]. PINK1 mutants share many phenotypic characteristics with parkin mutants [127, 128]. It could then be delineated that the two proteins act in a common pathway, with parkin functioning downstream of PINK1, as transgenic expression of parkin ameliorated all PINK1 impairments, but not vice versa [127–129]. Further interplay between these proteins has recently been uncovered (see Figure 2). Recent evidence has suggested that PINK1 along with parkin has a role in the mitochondrial targeting of autophagy, a process by which cells degrade intracellular material via the employment of lysosomes [130]. Mitophagy, the mitochondrial-specific form of the process, is important in the disposal of damaged or stressed organelles. The process is upregulated after disturbances in mitochondrial membrane permeability (reviewed in [131]). Accumulation of mutated α-synuclein protein also initiates this compensatory mechanism [132] (although parts of the autophagic-lysomal system may be impaired in these cases, as levels of the lysosomal protease cathepsin D are reduced [133, 134]). Parkin is selectively recruited from the cytosol to mitochondria with low membrane potential, where the protein mediates the degradation of the dysfunctional organelle (induced in this study using the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP)) by lysosomes [135]. PINK1 acts as the biochemical signal allowing parkin to identify dysfunctional mitochondria, the levels of which are regulated by voltage-dependent proteolysis [136]. The E3 ligase function of parkin, which is constitutively repressed, becomes activated upon PINK1-dependent mitochondrial localisation [137]. Thus, defective mitophagy due to a lack of parkin recruitment to dysfunctional mitochondria by PINK1 causes the accumulation of impaired mitochondria, and a subsequent buildup of ROS and proapoptotic proteins. The importance of this system is furthered by the evidence that VPS41, a protein known to traffic proteins to lysosomes in yeast, is neuroprotective in 6-OHDA and rotenone cellular models by blocking the apoptotic cascade and enhancing protein clearance, although the toxin-induced mitochondrial

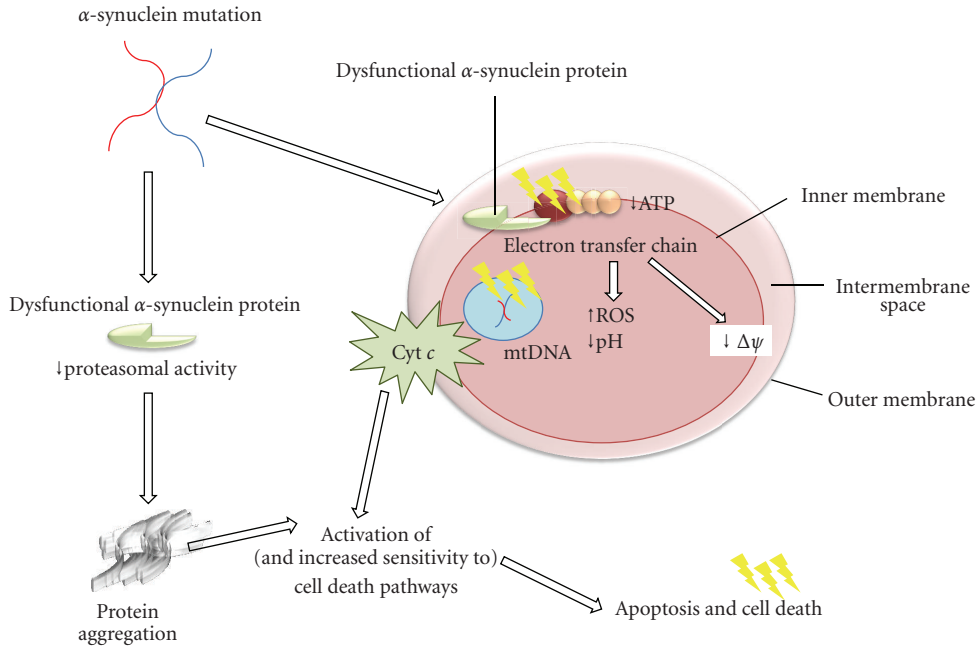

Figure 1: Genetic mutation of α-synuclein and subsequent protein and biochemical alterations. Modifications to the α-synuclein gene cause dysfunction of its protein product. Proteasomal activity becomes impaired, leading to an increased tendency for the protein to aggregate. Mutated α-synuclein protein also localises at the inner mitochondrial membrane, causing complex I dysfunction. ATP production is subsequently reduced, as is the transmembrane potential of the organelle. Increased levels of ROS result, and with mtDNA being particularly susceptible to ROS damage, further cell stress occurs. The acidic cytosolic environment created by ROS and metabolic impairment results in the activation of cell death mediators, such as cytochrome *c*. Apoptotic pathways are initiated and cell death subsequently occurs.

membrane potential disruption was not affected [138]. Vives-Bauza et al. [139] expands upon the functions of parkin and PINK1 by describing a role in mitochondrial trafficking to the perinuclear region—a subcellular area associated with autophagy, again suggesting the proteins function in mitochondrial turnover. These functions are repressed in knockout models of the two proteins involved, implying that a lack of these events play a role in pathogenesis. Moreover, overexpression of parkin enhanced the protective effects of mitophagy, with autophagy proteins such as Atg7 and LC3/Atg8 involved in mitochondria remodelling [140]. This provides a link between morphology and some active processes of mitochondria which impact upon cell survival.

Morphological changes of the tubular networks which mitochondria form impact cell survival, with a balance between the processes of fission and fusion required to maintain morphological integrity. Mitochondria are highly dynamic organelles, and these processes are ongoing. It has been found that overexpression of PINK1 promotes mitochondrial fission while inhibition of the protein causes excessive fusion [141, 142]. The PINK1/parkin pathway thus achieves its effect of mitochondrial integrity by promoting fission [142, 143]. Ageing affects mitochondrial activity and how it reacts to a lack of PINK1: at 3-4 months, impaired mitochondrial function after PINK1 knockout was observed in the striatum but not in the cerebral cortex; however, at 2 years of age, this impairment also occurred in the cortex [144]. Impairments to mitochondrial respiration in this study were induced by cellular stress-mediators, as well as a reduction in the activity of the Kreb's cycle enzyme aconitase, underlining the protein's protective role in mitochondria to intrinsic and extrinsic factors. Examination of fibroblasts from a patient affected by a PINK1 mutation has revealed low mitochondrial respiratory activity and enhanced oxygen radical production due to complex I inhibition [145], demonstrating clinically the effects found experimentally.

5.4. DJ-1. A rarer cause of early-onset forms of PD is mutations to the gene encoding the 189-amino acid protein, DJ-1. Affecting the PARK7 gene, they account for 1-2% of these cases [85]. As with the other proteins outlined, mutant studies demonstrate an increased susceptibility to cell death. Knockdown of DJ-1 by siRNA led to populations of the human neuroblastoma cell line SH-SY5Y becoming susceptible to several oxidative insults including hydrogen peroxide, MPP^+ and 6-OHDA [146]. Conversely, DJ-1 overexpression in this cell line leads to increased resistance to these insults and reduced intracellular ROS levels [147]. This protection is apparently selective against environmental oxidative stress *in vivo*, as shown in the paraquat-treated DJ-1-null *Drosophila* model [148]. Furthermore, the levels of DJ-1 modification increase with age, leading to significant increases in oxidative stress and inactivation of the protein's function [149]. This shows that DJ-1 modifications may have a role in sporadic PD in aged animals, in addition to familial early-onset cases. DJ-1-deficient mice show hypersensitivity to MPTP—manifested by increased dopaminergic neuronal loss and striatal denervation [150]. Embryonic cortical

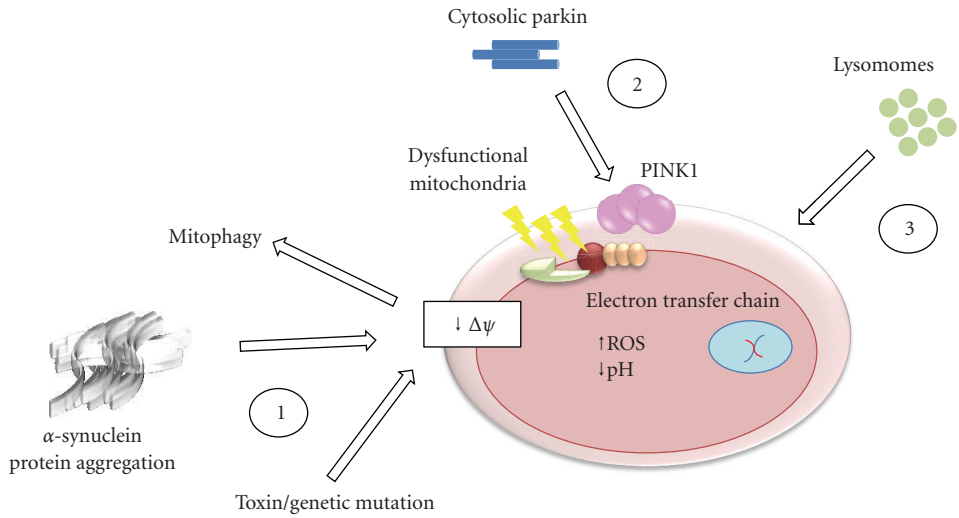

Figure 2: The physiological association of parkin and PINK1 proteins in mitophagy. (1) Genetic mutations or the introduction of toxins lead to various impairments, including depletion of ATP production at the electron transfer chain. A buildup of ROS leads to a more acidic environment within the mitochondrion, as well as inducing a decrease in the mitochondrial transmembrane potential after the opening of permeability transition pores. This is the signal for mitophagy to occur. (2) The process is induced through the interaction of cytosolic parkin with mitochondria-associated PINK1. PINK1 acts as the biochemical signal for parkin to identify damaged mitochondria. (3) Parkin then mediates the lysosomal degradation of the dysfunctional organelle.

neurons have shown increased sensitivity to oxidative stress [150] and proteasomal inhibition leading to apoptosis [151]. All impairments were reversed by restoration of DJ-1 expression [150]. DJ-1 is activated by an oxidative cytoplasmic environment [152], thus its protective mechanism works in an on-demand manner. This group describe a role for DJ-1 as a redox-sensitive molecular chaperone, which inhibits α-synuclein aggregate formation. The complex I inhibitor paraquat decreased proteasome activity along with ATP and regulatory subunit levels in DJ-1 deficient mice, but not wild-type littermates [153]. In addition to this, a transcription factor, expression nuclear factor erythroid 2-related factor 2 (Nrf2), which induces cytoprotective genes, was reduced. This provides evidence that DJ-1 acts as a regulator of transcription. The mechanism by which DJ-1 protects against oxidative stress has been investigated: the protein has been shown to regulate the MAP3 kinase apoptosis signalling-regulating kinase 1 (ASK1)/thioredoxin 1 (Trx1) complex [154]. ASK1 is a major effector of oxidative cell death and is physiologically inhibited by Trx1. The association is disrupted by oxidative stimuli, and DJ-1-null cells are more susceptible to this dissociation, leading to increased activation of downstream cell death mediators. It has been shown that DJ-1 does not take part in the PINK1/parkin pathway, as overexpression of DJ-1 could not reverse the PINK1 inactivation phenotype [128].

A direct mitochondrial-DJ-1 association has been uncovered. Lev et al. [147] described a cellular redistribution of DJ-1 when cells were exposed to neurotoxins. This is expanded upon by Hayashi et al. [155] who show DJ-1 binding to NADH dehydrogenase (ubiquinone) 1 α-subcomplex 4 (NDUFA4) and ND1, nuclear and mitochondrial DNA-encoding subunits of complex I, demonstrating the importance of DJ-1 in mitochondrial activity. Mitochondrial DJ-1 acts as an atypical peroxiredoxin-like peroxidise, as indicated by the twofold increase in mitochondrial hydrogen peroxide levels in DJ-1 knock-out mice. Cysteine-106 forms the site of the oxidation required for scavenging hydrogen peroxide [156], and potentially other ROS.

5.5. LRRK2. Mutations to the leucine-rich repeat kinase 2 (LRRK2) gene cause a form of autosomal dominant PD. The mutations are associated with familial late-onset PD as well as some sporadic cases of the disorder, which has increased the interest in this gene [157]. The LRRK2 gene encodes a large 2527 amino acid multidomain protein, the physiological function of which is currently unknown. But there are numerous functional domains associated with the protein, including GTPase [158] and kinase domains [157], implying an array of roles. LRRK2 is present largely in the cytoplasm but also associates with the outer membrane of mitochondria [159, 160], suggesting a role in this organelle. However, the role of LRRK2 in PD pathogenesis is questioned by Andres-Mateos et al. [161], who find a sensitivity to MPTP insult of LRRK2 knock-out mice in line with wild-type mice (in contrast to other established gene defects), as well as normal dopaminergic signalling and cell survival levels. Most of the accumulated evidence suggests that a gain-of-function defect causes PD pathology, with an increase in kinase activity prominent in disease development [160, 162]. It has been found by Lin et al. [163] that LRRK2 mutations accelerate the progression of neuropathological abnormalities in α-synuclein transgenic A53T mice by promoting the aggregation of the α-synuclein. These effects are thought to be due to induction of the LRRK2 protein's kinase domain, causing activation of the ERK module [164]. Genetic ablation of LRRK2 in mice had converse effects, suppressing aggregation and delaying

the development of PD-related pathology [163] as did pharmacological inhibition of ERK *in vitro* [164]. However, LRRK2 ablation does not appear to be a viable clinical treatment option. The dopaminergic system of LRRK-null mice appears normal, but it is in the kidneys where the loss of the gene has greatest impact, with a 60-fold increase in the accumulation of α-synuclein and ubiquitinated proteins in aged animals [165]. LRRK2 loss leads to inflammatory responses, oxidative damage and apoptotic cell death [165], meaning mitochondria may be affected. However, this toxicity induced by oxidative stress has been mapped to be caused by the kinase domain, and ERK inhibition restored cell survival to near-control levels [166]. Therefore, it appears that mitochondria play a relatively minor role in LRRK2-related pathology compared to in other gene defects—as demonstrated convincingly by the lack of hypersensitivity to MPTP [161]—with the activity of kinase pathways more involved.

6. Neuroprotective Methods Targeting Mitochondrial Dysfunction

Given their position at the centre of a web of cellular functions and pathways and their role in both idiopathic and familial PD, mitochondria constitute a popular target in efforts to protect dopaminergic neurons, thereby arresting degeneration and maintaining the functional capacity of patients.

6.1. Coenzyme Q10. The most well-known mitochondrial-targeting neuroprotective agent is coenzyme Q10 (also known as ubiquinone). The structure functions primarily as an electron acceptor in the electron transport chain—shuttling electrons between complexes I and II/III—and a potent antioxidant. It can prevent apoptosis by blocking both the binding of Bax to mitochondrial membranes and cytochrome *c* release [167] and inhibiting the opening of the Ca^{2+}-gated mitochondrial permeability transition pore (PTP) and the subsequent apoptotic pathway activation [168]. Coenzyme Q10 can directly scavenge free radicals in the inner mitochondrial membrane through interactions with α-tocopherol [169]. Reduced levels of coenzyme Q10 in mitochondria isolated from PD patients were found [170], as well as a lower serum level of the enzyme in patients with Parkinsonism compared to stroke patients of similar age [171], which showed the potential supplementation of this agent could have. Treatment with differentiated human neuroblastoma cells showed inhibited ROS formation and cell death induced by the herbicide paraquat in repose to coenzyme Q10 [172], while pretreated rat mesenphalic cells were protected against rotenone-induced apoptosis and mitochondrial depolarisation [173]. Preclinical *in vivo* studies augmented this theory with evidence that oral supplementation with coenzyme Q10 reduced striatal dopaminergic neuronal loss in MPTP-treated mice [174] and protected against 3-nitroproprionic acid striatal lesions in rats [175]. A clinical trial used a double-blind, placebo-controlled design, and randomly assigned placebo or coenzyme Q10 at doses of 200, 600 or 1200 mg per day to a total of 80 patients with early PD [176]. Patients were rated on the Unified Parkinson's Disease Rating Scale (UPDRS) over a period of 16 months. Coenzyme Q10 was safe and well tolerated at all dose levels, with the group receiving the highest dose developing PD symptoms at the slowest rate—a slowing a 44%. However, in a study of 130 patients receiving a daily dosage of 300 mg, which led to coenzyme Q10 plasma levels of 1200 mg, the agent had no symptomatic effect [177]. Therefore, despite mixed results from clinical trials, a strong body of evidence exists to merit further investigation into the therapeutic effects of coenzyme Q10 in PD. A large phase III clinical trial following patients for 16 months is due to be completed in 2011. This study will compare the effects of doses of 1200 and 2400 mg per day and placebo in 600 early stage PD patients [178].

6.2. Creatine. Creatine, a nitrogenous guanisine compound, is another potential mitochondria-targeting treatment that has been investigated to clinical trial level. It occurs naturally in vertebrates (produced endogenously by the liver, kidneys, and pancreas) and helps supply energy to muscle and neurons via its conversion to phosphocreatine. This structure can transfer its phosphoryl group to ADP, thereby creating ATP. This reaction is important in maintaining a ready subcellular supply of ATP. Additional supplies of this compound may be especially valuable during times of cellular stress and dysfunction when energy production is compromised. The addition of creatine has been shown to be useful in a number of models of neurodegenerative diseases, including amytrophic lateral sclerosis [179], Alzheimer's disease [180], and Huntington's disease [181]. *In vitro* results show significant neuroprotection of dopaminergic cell numbers and morphology against MPP^+ and 6-OHDA exposure [182]. Oral administration of creatine can protect against MPTP-induced dopaminergic cell loss in mice in a dose-dependent manner [183]; this neuroprotection can be augmented with the addition of the cyclooxygenase-2 inhibitor rofecoxib [184]. The long-term (i.e., 2 years) safety of creatine supplementation has been tested in 60 aged PD patients and approved as safe, but the potential risk to patients with underlying renal problems has been identified [185]. However, a blinded, placebo-controlled clinical trial showed no significant difference in the UPDRS scores of 31 patients with PD compared to the 17 placebo-treated volunteers after 18 months of treatment [186]. But the test did show a lower requirement for symptomatic treatment in the patients who received creatine, which may represent a degree of dopaminergic signalling protection. This success, however limited, has led to a phase III clinical trial being announced [187]. In addition to monotherapy, a combination of creatine and coenzyme Q10 was found to exert synergistic protective benefits against dopamine depletion and cell loss in the MPTP model [188]. The use of creatine to increase levels of readily available ATP in neurodegenerative diseases is promising. The results of a large-scale clinical trial will further elucidate the potential of its therapeutic efficacy.

6.3. SS Peptides. These small, synthetic peptide molecules (named after their designers, Hazel Szeto and Peter Schiller) have been utilised thanks to their ability to penetrate numerous types of cells, including dopaminergic neurons, despite carrying a +3 net charge [189]. Relevant to this paper, is the ability of these peptides to enter mitochondria. Evidence for the peptides' capacity to do this began with fluorescent labelling of a SS-02 analogue, which showed colocalisation with a mitochondrial tracker as they concentrated at the inner mitochondrial membrane (IMM) within isolated neurons [190]. This uptake occurs independently of changes in mitochondrial transmembrane potential [191]. The mechanism behind this targeting is not known, but it may be due to an electrostatic attraction between the cationic peptides and the anionic cardiolipin molecules which form in uniquely high density at the IMM [191]. As mitochondrial potential can be diminished in disease states, this membrane potential-independent uptake property may be advantageous.

These mitochondria-targeting peptides have further neuroprotection weapons, including intrinsic dose-dependent antioxidant properties. Peptides such as SS-02 and SS-31 can scavenge hydrogen peroxide, hydroxyl radical, and peroxynitrite by virtue of their tyrosine residue [190, 192]. This activity also inhibits lipid peroxidation. The localisation of SS-02 and SS-31 to the IMM allows the peptides to prevent mitochondrial swelling and depolarisation [190, 192], as shown in N_2A neuroblastoma cells when depolarisation was induced by *tert*-butylhydroperoxide [193]. Specifically relevant to PD, SS-20 and SS-31 have been shown to be protective in the MPTP mouse model [194]. The latter peptide produced dose-dependent (0.1 to 10 mg/kg) protection against SN dopaminergic neuronal loss and maintained striatal levels of dopamine and its metabolites. SS-20 also protected dopaminergic neurons against MPTP insult, despite its lack of intrinsic ROS scavenging ability. Both peptides were very potent in protecting cultured neurons against MPP^+-induced cell death, with effectiveness at nanomolar concentrations [194]. Impairment of oxygen consumption, ATP depletion, and mitochondrial swelling were all prevented by the peptides in isolated mitochondria treated with MPP^+ [194]. Brain uptake of MPP^+ was not altered in the *in vivo* study, showing that uptake mechanisms were not altered by the peptides. Thus, the properties outlined above such as ROS scavenging and prevention of apoptosis may be utilised to prevent MPTP toxicity.

These peptides undoubtedly offer a promising mitochondria-targeting treatment for PD. However, data so far has come from one group, so although hitherto positive, results should be treated with caution.

6.4. Natural Antioxidants. A number of naturally occurring antioxidants has been shown to have protective effects against the degeneration induced by elevated ROS levels in cases of mitochondrial dysfunction. There is great depth to the number of potentially beneficial compounds which can be derived naturally; for example, there has been over 4000 species of flavanoids, a group of established plant-derived antioxidants, identified [195]. Green tea polyphenols (GTP) have proven to be protective in SH-SY5Y cells against 6-OHDA toxicity [196]. This group find an array of protective effects initiated by GTP including prevention of the decrease in mitochondrial membrane potential, suppressed accumulation of ROS and a scavenging of free radicals in a dose- and time-dependent manner. The efficacy of the green tea component (−)-epigallocatechin 3-gallate (EGCG) has been demonstrated in the MPTP mouse model of PD, with both loss of dopaminergic neurons and striatal dopamine levels attenuated [197, 198] with the authors suggesting that this protection is mediated by the inhibition of NOS expression. But the established antioxidant properties of EGCG also supplement the neuroprotective mechanism, as shown in a yeast-based α-synuclein model in which EGCG, along with a further flavonoid, quercetin, increased growth of α-synuclein cells though potent anti-ROS and metal chelating mechanisms [199]. The route of administration of these products is a great advantage of their use, with the drinking of tea having beneficial effects in this study. Epidemiological evidence shows drinking more than two cups a day of green tea has a protective effect against developing PD [200]. The abundance, accessibility, and safety of this beneficial polyphenol make it attractive to study further as a potential clinical preventative agent.

Oxyresveratrol (OXY), a polyphenol found in high amounts in mulberry wood, has displayed potent scavenging activity against reactive oxygen and nitrogen species in glial cells after hydrogen peroxide exposure [201]. The compound is also less cytotoxic to microglial cells than the antioxidant resveratrol [201]. A study in 6-OHDA-treated neuroblastoma SH-SY5Y cells found OXY significantly reduced the generation of ROS and attenuated apoptotic activities of caspase-3 caused by impaired mitochondria [202]. Adding to the previously realised protective effect OXY has in cerebral ischemia [203] and a profile of OXY as an effective inhibitor of apoptosis and oxidative stress in energy-deprived tissues is becoming established.

Uric acid acts as an antioxidant, scavenging both reactive oxygen and nitrogen species [204]. Epidemiological evidence demonstrates a trend with higher uric acid serum levels correlating with a lower incidence of PD [205–208]. It can also protect against damage to mtDNA by intercepting radicals [209], therefore helping to maintain mitochondrial genomic integrity and prevent the induction of mutations. Uric acid has been found to prevent dopaminergic cell death in rotenone- and homocysteine-treated cells; treatments which cause increased ROS production and exacerbated mitochondrial membrane depolarisation [210]. Guerreiro et al. [211] proposes that uric acid neutralises oxygen species via a Fenton-type chemical reaction, providing *in vitro* dopaminergic neuroprotection. There is much positive data regarding the antioxidative properties of uric acid, and the ease that urate could be administered (i.e., by diet supplementation) is a major advantage of this treatment. However, the potential benefits of increasing urate levels have to be balanced against the risk of developing gout and cardiovascular problems [212]. Currently, participants are being recruited to study the clinical safety of urate elevation

in PD (SURE-PD) by dietary supplementation with inosine [213].

Traditional herbal medicines may be effective in attenuating the accumulation of ROS in the event of mitochondrial dysfunction. Cyperi rhizome (CR), the rhizome of *Cyperus rotundus*, is a traditional herbal medicine used in Korea for stomach disorders. A water extract of CR provided protection *in vitro* against 6-OHDA-induced toxicity through a number of mechanisms including inhibition of both ROS formation and mitochondrial membrane reduction in the first study of the compound in a neurodegenerative disease model [214]. *Uncaria rhynchophylla* is a herb that has been used in Oriental medicine to treat hypertension, convulsions and tremor. An extract has proven to be an effective agent against excitotoxicity in hippocampal slices [215]. However, a study examining the antioxidant properties of a range of extracts against lipopolysaccharide-induced NO release found all but one of the 8 compounds tested displayed weak NO inhibitory effects only [216]. A form of this herb, the hook of *uncaria rhynchophylla* (URH), has proven to be protective in 6-OHDA-treated cells and lesioned rats [217]. *In vitro*, cell apoptosis and ROS levels were significantly attenuated, while the rats showed reduced dopaminergic cell loss. Further tests in other models may help further establish this extract as an effective antioxidant in the treatment of mitochondrial disorders.

It may be necessary and desirable to look further at this branch of antioxidant agents after the failure to slow disease progression in clinical trials examining agents derived from "modern medicine" [218, 219]. At present, however, a lack of data in PD models (particularly when compared to other neuroprotective agents discussed in the paper) prevents the true potential and possible clinical value of these antioxidants being comprehensively evaluated. Research of these substances may become more widespread if putative studies continue to deliver promising results.

7. Conclusion

The positioning and function of mitochondria make these complex subcellular organelles uniquely susceptible to oxidative damage, and impairment to their vital roles exacerbates damage. Their placement at the centre of a plethora of molecular pathways leads to activation of cell death and apoptotic signal cascades, resulting in degeneration. Further adding to the susceptibility of mitochondria is the presence of their own DNA. The nature of this special genome means that mutations are common, particularly in a structure which routinely produces a baseline level of ROS with an inadequate repair mechanism. These mutations accumulate with age, which adds to the PD pathophysiology, since this is a heavily age-related disorder. The use of several neurotoxins as experimental models to reproduce many of the clinical hallmarks of PD both *in vitro* and *in vivo* has allowed the molecular mechanisms of the disease to be investigated further. Common in all types of model is the dysfunction which occurs in mitochondria, often through a direct block of complex I in the electron transport chain, leading to a cascade of further damage. Another milestone discovery in PD research was the delineation of particular protein-encoding genes which, when mutated, lead to the generation of the Parkinsonian phenotype. These mutations are familial in nature, lending subpopulations a degree of susceptibility. The genes allowed further analysis of PD pathogenesis, with mitochondrial dysfunction again proving a deleterious factor. Thus, impairment of these energy-producing organelles provides a link between the common idiopathic form of PD and the rare inherited form of the disease. This makes mitochondria an attractive target for neuroprotective strategies to halt neurodegeneration. Supplementation of Coenzyme Q10 and creatine are methods which have proved successful at pre-clinical level and have been tested in clinical trials, with mixed results. Larger-scale clinical trials, using the information found from previous studies in humans, could help get the best out of these promising strategies. The generation of small peptides, which possess many properties desired in neuroprotective agents, have also shown potential. Various natural antioxidants, including green tea polyphenols, uric acid and extracts from Oriental herbs, have shown potent antioxidant effects, with some demonstrating neuroprotective benefits in a bioenergetically challenged environment, although relatively few studies of these substances in PD models have been carried out. The centring of mitochondria in the pathogenesis of both idiopathic and familial forms of PD mean further neuroprotective strategies should target mitochondria, directly or indirectly, to counter the deleterious and degenerating effects that occur as a consequence of their dysfunction. The ability of present models to recreate mitochondrial impairment to such a degree should allow this process to be tested vigorously and with great accuracy in the laboratory before being presented at the clinical stage.

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