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**Title Page****Mitochondrial dysfunction in the limelight of Parkinson's disease pathogenesis****Rebecca Banerjee<sup>1</sup>, Anatoly A. Starkov<sup>1</sup>, M. Flint Beal<sup>1</sup> and Bobby Thomas<sup>1,\*</sup>**

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**Abstract**

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder with unknown etiology. It is marked by widespread neurodegeneration in the brain with profound loss of A9 midbrain dopaminergic neurons in substantia nigra pars compacta. Several theories of biochemical abnormalities have been linked to pathogenesis of PD of which mitochondrial dysfunction due to an impairment of mitochondrial complex I and subsequent oxidative stress seems to take the center stage in experimental models of PD and in postmortem tissues of sporadic forms of illness. Recent identification of specific gene mutations and their influence on mitochondrial functions has further reinforced the relevance of mitochondrial abnormalities in disease pathogenesis. In both sporadic and familial forms of PD abnormal mitochondrial paradigms associated with disease include impaired functioning of the mitochondrial electron transport chain, aging associated damage to mitochondrial DNA, impaired calcium buffering, and anomalies in mitochondrial morphology and dynamics. Here we provide an overview of specific mitochondrial functions affected in sporadic and familial PD that play a role in disease pathogenesis. We propose to utilize these gained insights to further streamline and focus the research to better understand mitochondria's role in disease development and exploit potential mitochondrial targets for therapeutic interventions in PD pathogenesis.

**Key words:** mitochondrial dysfunction, mitochondrial DNA, electron transport chain, permeability transition pore,  $\alpha$ -synuclein, parkin, PINK1, DJ-1, LRRK2

## Introduction

Mitochondria, the power house of living cells and regulators in cell survival and death are especially complex and delicate organelles. Mitochondria easily succumb to diverse assaults either generated in situ or those imposed from extracellular environment. Mitochondrial dysfunction results in a dwindling supply of cellular energy, a failure in maintaining cellular homeostasis, and activation of cell death pathways which could underlie selective dopaminergic neurodegeneration in Parkinson's disease (PD) [1]. PD is characterized pathologically by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), reduction of dopamine and its metabolite levels in the basal ganglia and formation of Lewy bodies [2]. Clinically PD patients manifest symptoms of progressive rigidity, bradykinesia, tremor and postural instability [3, 4] and depend solely on symptomatic relief treatments [5]. To date there is no cure for PD, the etiopathology of PD is unresolved, and hence the quest to define disease mechanisms continues. Whether mitochondrial dysfunction is the cause or effect of PD pathogenesis is debatable. In this review we have provided an update of PD research pursued over the last three decades that underpins the participation of mitochondria in the dopaminergic neuronal demise in PD.

### **Mitochondrial dysfunction in the idiopathic form of disease**

The causes to the idiopathic form of PD accounting for almost 95% of cases are unknown, and several theories are suggested in the etiology of the disease. In this section we review the most relevant features involved in mitochondrial dysfunction that are associated with the disease.

#### *Mitochondrial electron transport chain complexes in PD*

The seminal discovery that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) causes PD-like symptoms in humans [6] and the ensuing rapid unraveling of its molecular mechanism of toxicity strongly stimulated the interest of PD research community to the role of mitochondria in PD pathology.

In non-human primates, MPTP intoxication recapitulates most of the clinical and pathological hallmarks of PD except for the presence of Lewy bodies [7-9]. The revelation of MPTP mechanisms has resulted in a gold mine of information regarding the involvement of mitochondria and led to subsequent development of other toxin-induced animal models of PD. MPTP is metabolized to its toxic form MPP<sup>+</sup> (1-methyl-4-phenylpyridinium ion) by mitochondrial monoamine oxidase (MAO) [10], specifically by MAO B [11] and is rapidly concentrated in the mitochondria by an energy-dependent process [12]. Once accumulated, it specifically inhibits the oxidation of NAD (nicotinamide adenine dinucleotide) -linked substrates [13] by blocking the electron transfer through the complex I of the electron transport chain (ETC) somewhere in the proximity of its quinone binding site [14]. It also inhibits the activity of a key TCA (tricarboxylic acid) enzyme KGDHC ( $\alpha$ -ketoglutarate dehydrogenase complex), thereby impairing the ATP synthesis and inducing “energy crisis” *in vitro* [15] and *in vivo* [16]. The rather selective toxicity of MPP<sup>+</sup> to dopaminergic neurons in SN (substantia nigra) was explained by the fact that MPP<sup>+</sup> is selectively accumulated by the dopamine uptake system involving the dopamine transporters (DAT) [17]. Human platelets which also express DAT [18] have been shown to accumulate MPP<sup>+</sup> with high efficiency and suffer from mitochondrial failure as a result of MPP<sup>+</sup> exposure [19]. Human platelets also contain high levels of MAO B and share a number of properties with aminergic neurons including receptors, uptake sites and storage granules for amine neurotransmitters. As platelets are more available for research than PD brain samples or muscle biopsy, they were a natural choice of PD tissue samples for earlier studies on the status of complex I in PD. Several laboratories reported low complex I activities in either PD platelet homogenates or mitochondria isolated from PD platelets, although the degree of its inhibition varied within a wide range. The activities of other respiratory chain complexes in PD platelets were even more variable, that is some laboratories found impaired complex II [20], complex II + complex III [21], and complex IV activities [22], whereas others reported a selective complex I inhibition [21, 23] (See Table 1 for details). It is noteworthy that no distinction was made between familial PD patients and sporadic PD patients in these earlier studies, and their treatment status

had not always been reported. The latter is of importance because some neuroleptics are known to inhibit complex I *in vitro* [24], and L-dopa (levo-dopa) treated rats also exhibit lowered complex I activity in brain mitochondria [25]. However, these factors were accounted for in the study by Haas and colleagues, who found a decreased activity of complex I and other ETC complexes in platelets from untreated PD patients [21]. Additionally about an equal number of studies and in particular more recent ones did not find any inhibition in complex I or other ETC complexes in platelets isolated from either sporadic or familial PD patients which were either receiving treatment or untreated at the time of analysis [26-31]. There is no consensus in most of the reported data on ETC activities in other PD tissues (Table 1), with earlier reports finding ETC deficiencies whereas later reports contradicting these findings. Earlier reports [29, 32-34] point to a specific deficiency in complex I activity in SN but not in other brain sub-regions. However, more recently lowered complex I activity was shown in purified mitochondria isolated from PD frontal cortex [35, 36]. A selective decrease in a number of subunits in complex I was also demonstrated in PD brain [37]. Others could not confirm this finding [38], but a recent study also reported some abnormalities in assembly and oxidation of complex I subunits in PD cortex mitochondria [36]. Several studies showed impaired complex I, II+III, and IV in PD muscle [39-41], whereas others reported no differences [29, 42, 43], and findings on the ETC deficiency in PD lymphocytes [20, 44] are counter-balanced by the evidence on the absence of such deficiencies [45]. Such inconsistency in the experimental data could likely be explained by significant methodological differences in all these studies, e.g. difficulties in obtaining the relevant tissue samples, individual variability of PD patients and many other factors, as was recently suggested [35]. Most of these problems can surely be overcome in the future studies, however it is felt that a definite and reliable answer to whether there is ETC dysfunction in PD mitochondria can not be obtained by an individual laboratory. It likely will require a coordinated effort of an international consortium, well standardized assay procedures, and a statistically meaningful number of thoroughly characterized tissue samples. As it stands now, the deficiency in ETC complexes appears to be at least an idiopathic molecular feature of PD, but the question whether it

firmly belongs to other molecular landmarks of PD neuropathology can not be answered with certainty. One could argue whether ETC deficiency is etiological in PD. However the presence of reduced complex I function in less affected regions in the CNS and in platelets indicates that it could be an early pathogenic event in PD. Ample data strongly suggest that toxin-induced ETC disruption per se is sufficient to trigger the death of nigrostriatal neurons and to cause PD-like symptoms in animals and humans. Thus, despite discrepancies in ETC functions reported from various laboratories, a systemic complex I deficiency might be a predominant feature in PD pathogenesis.

*Pesticidal exposure and its link to mitochondrial dysfunction.*

Epidemiological studies have clearly established the role of environmental factors posing an increased risk of developing PD [46]. Earlier epidemiological evidence from twin studies and more recently published genome-wide single nucleotide polymorphism analyses of a population cohort of parkinsonian patients strongly suggest that idiopathic PD does not develop due to genetic heritability [47, 48]. This, along with geographic variations in PD incidence, suggests that sporadic PD is most likely because of environmental factors, perhaps in combination with genetic susceptibilities. Exposure to agricultural chemicals such as pesticides has been postulated as a potential environmental risk factor for the disease [49]. This relationship of pesticide exposure and PD appears strongest for exposure to herbicides and insecticides, especially following long durations of exposure. The most concrete evidence in humans comes from a Taiwanese study which showed that exposure to paraquat for more than 20 years was associated with PD [50].

Paraquat (1,1'-dimethyl-4,4'-bipyridinium, CAS# 4685-14-7) belongs to a class of quaternary ammonium herbicides. Chemically, paraquat closely resembles MPTP and it was tested as a PD model soon after the discovery of MPTP [51]. This extensively used herbicide has been linked to PD in epidemiological surveys [52], and a case study reported parkinsonism in a farmer acutely exposed to the related herbicide diquat dibromide [53]. In rodents, paraquat caused selective degeneration of



dopaminergic neurons [54], dopamine depletion,  $\alpha$ -synuclein protein aggregation and increased oxidative stress [55]. Paraquat also induced an increase in the  $\alpha$ -synuclein level in SN and frontal cortex and caused intraneuronal aggregation of  $\alpha$ -synuclein in the SNpc [56].

Although both MPP<sup>+</sup> and paraquat inhibit mitochondrial complex I, there are substantial differences in the action of these toxins [57]. Paraquat entry into neurons is not mediated by the dopamine transporter [58], and it does not bind to rat and mouse striatal dopamine D<sub>1</sub> and D<sub>2</sub> receptors. Therefore, there is no molecular basis for this compound to accumulate selectively in the SN dopaminergic neurons, as is the case with MPP<sup>+</sup>. Paraquat also has a much lower binding affinity toward complex I. *In vivo* exposure to MPTP and rotenone, but not paraquat, inhibited binding of <sup>3</sup>H-dihydrorotenone to complex I in brain mitochondria [57]. Similar to MPP<sup>+</sup>, paraquat induces significant ROS production through redox cycling [59]. To note, we have compared *in vitro* ROS-inducing efficiency of complex I –targeting pesticides MPP<sup>+</sup> and pyridaben with that of paraquat, and found that the latter is a more potent (~10 folds) ROS inducer. Moreover, the onset of ROS production by this compound in isolated mouse brain mitochondria actually preceded the inhibition of complex I, which was not the case with pyridaben and MPP<sup>+</sup> (our unpublished results). Taking into account that paraquat can generate ROS in non-mitochondrial enzymatic reactions [60], we think it is quite possible that the mechanism of paraquat toxicity to SN neurons is entirely different from that of MPP<sup>+</sup>. This suggests that paraquat kill cells through failure in antioxidant defenses and oxidative damage to cytosolic proteins [61], whereas MPP<sup>+</sup> causes toxicity through bioenergetics failure. However, in both scenarios mitochondria are primary mediators of cell death as they are the major source of paraquat-induced ROS production in the brain [62].

It was noted that geographic overlap in use of paraquat and another ETC toxin, maneb could indicate a multiple-hit environmental model in which both compounds act synergistically on the dopaminergic system [54, 63, 64]. Maneb ([2-[(dithiocarboxy) amino] ethyl] carbamodithioato)](2-)-κS,κS']manganese, CAS# 12427-38-2) belong to a class of manganese-containing dithiocarbamate

fungicides. Exposure to maneb has been associated with parkinsonism [65]. Laboratory studies suggest that dithiocarbamates can cause dopamine depletion and induce degeneration of dopaminergic neurons [55]. There have been case reports of parkinsonism in agricultural workers exposed to maneb [65, 66]. In mitochondria, maneb binds to complex III, thereby blocking the oxidative phosphorylation, inhibiting respiration and inducing ROS generation [67]. Maneb also inhibits the ubiquitin proteasome pathway [68]. Thus, maneb-induced mitochondrial impairment could be augmented by a derangement in protein degradation machinery.

In rodents, simultaneous injections of both paraquat and maneb exhibited higher toxicity than injections of each chemical alone [54, 63, 64], with nigrostriatal degeneration and other pathological features that closely resemble sporadic PD. Recent studies hints that the mechanism of synergistic action of maneb and paraquat is modulated by the expression of major mitochondrial pro-apoptotic proteins. Individually, paraquat and maneb induce Bak-dependent cell death, but together they trigger Bax-dependent cell death. They do so by increasing the expression of three strong Bak inhibitors, Bfl-1, Bcl-xL and Mcl-1, and by inducing Bax activators that include Bak and Bim [69, 70].

Recently, rotenone rat models of PD have been developed that reproduce essential biochemical and behavioral human PD features such as irreversible cell death of dopamine neurons and formation of Lewy body-like cytoplasmic inclusions [71-73]. Rotenone is a classical and reasonably selective inhibitor of mitochondrial complex I. *In vitro*, rotenone completely prevents the oxidation of pyruvate and many other physiological substrates, thereby inhibiting ATP synthesis in mitochondria. Moreover, rotenone and other complex I inhibitors stimulate ROS production by mitochondria [74]. To note, mammalian mitochondrial complex I is a very complex enzyme, composed of at least 49 individual polypeptides. This enzyme is the proton pump capable of converting the chemical energy of NADH into the electrochemical proton gradient across the inner membrane of mitochondria. Mitochondria utilize the proton gradient to synthesize ATP. The NADH is derived from multiple reactions of oxidation of various nutrients in mitochondria; in order to maintain their normal rates, NADH has to be re-oxidized

to NAD<sup>+</sup>, similar to as it happens in glycolysis. This reoxidation is catalyzed by complex I, which is therefore important for both an efficient ATP synthesis and for the normal functioning of the overall catabolic machinery of mitochondria. Moreover, several of the individual polypeptides comprising complex I have been shown to interact with non-ETC proteins participating in other cellular processes. Some of these proteins are “suspiciously” associated with cell death pathways believed to be important in PD. For example, a recently discovered component of complex I called GRIM-19 (gene associated with retinoid-IFN-induced mortality) physically interacts with a serine protease *HtrA2/Omi* (High temperature requirement protein A2) and enhances its pro-apoptotic activity [75]. In humans, point mutations in *HtrA2* are a susceptibility factor for PD (*PARK13 locus*). Moreover, *HtrA2* interacts with a putative mitochondrial protein kinase *PINK1*; mutations in the latter are associated with the *PARK6* autosomal recessive locus for susceptibility to early-onset PD [76]. Upon activation of the p38 stress-sensing pathway, *HtrA2* is phosphorylated in a *PINK1*-dependent manner at a residue adjacent to a position found mutated in patients with PD. *HtrA2* phosphorylation is decreased in brains of PD patients carrying mutations in *PINK1*. We mentioned these facts here to illustrate that a simple and common perception that “complex I inhibition causes bioenergetics impairment causes ATP depletion causes cell death” might be too simplistic as complex I dysfunction may cause cell death by another mechanism which is yet to be discovered. In fact, ATP depletion had been ruled out as a cause of selective neurotoxicity of rotenone in rodents [77]. Furthermore, a recent study provides some interesting insights on the role of complex I in mediating cell death. 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 [78]. However, deletion of *Ndufs4* did not affect the survival of dopaminergic neurons in these midbrain cultures. Interestingly dopaminergic neurons were more sensitive than other neurons in these cultures to cell death induced by rotenone, MPP<sup>+</sup>, or paraquat treatment. Contrary to the expectation, absence of complex I activity did not rescue dopaminergic neurons, from these mitochondrial toxins through

inhibition of complex I. This study casts doubt upon the generally accepted notion that complex I is the primary mechanism of toxicity of PD-linked neurotoxins. These results have implications on how experimental results using such mitochondrial toxins are interpreted in the context of cell death in PD. In this regard, a recent study provides new insights into the mechanism of rotenone-induced neurotoxicity and the role of complex I *in vivo* [79]. This study used the rotenone toxicity rat model combined with a stereotactic viral delivery of complex I “substitute”, enzyme called “alternative NADH dehydrogenase” (Ndi1). The latter is a single-polypeptide NADH dehydrogenase found in yeast mitochondria that does not generate proton gradient but catalyzes normal NADH oxidation. An expression of the Ndi1 protein in the rat SN strongly protected neuronal cells from rotenone-induced loss of tyrosine hydroxylase positive dopamine neurons and decreased oxidative damage to DNA in these neurons [79]. These findings have two very significant ramifications. First, it demonstrates that neuronal cell survival depends on the ability of complex I to re-oxidize mitochondrial NADH rather than on its ability to generate proton gradient or its interaction with other proteins. This greatly contributes to our understanding of the mechanistic aspects of complex I and ETC impairments in cell death. Second, this study brightly illustrates that current knowledge of these mechanistic aspects is, if incomplete, already sufficient enough to design a mitochondria-targeted *in vivo* treatment ameliorating a toxin-induced neuronal death. This development brings new hope that therapeutically relevant interventions for human PD would be possible to design in near future.

#### Role of mitochondrial DNA in PD

The fact that cellular organelles such as mitochondria can house their own DNA has further added to the functional complexity. It is strongly believed that proximity of mitochondrial DNA (mtDNA) to ROS generated as a consequence of respiratory chain function could render mtDNA vulnerable to mutations [80, 81]. A dysfunction in complex I could lead to a defective ROS producing system that in turn may affect the mtDNA which encodes 7 of the 49 protein subunits of the complex I

enzyme. Strong support for mtDNA encoded defect comes from studies which showed that complex I defects from PD platelets are transferable into mitochondrial deficient cybrid cell lines [82, 83]. These defects are associated with increased free radical production, increased susceptibility to MPP+, and impaired mitochondrial calcium buffering [84]. This suggests that the complex I deficits in PD are inherited either from the mitochondrial genome or from alteration in somatic mtDNA. In PD, the maternal inheritance pattern of mtDNA mutation is rare and may be a mere coincidence [85-87]. However, a number of studies provide genetic evidence that mtDNA abnormality may contribute to PD pathogenesis. A novel mitochondrial 12SrRNA point mutation was found in a pedigree with Parkinsonism, deafness and neuropathy [87]. There is also evidence for occurrence of Parkinsonism in association with the Leber's optic atrophy mitochondrial mutation G11778A [88]. Analysis of mtDNA found no homoplasmic mtDNA point mutations in coding regions in PD subjects in either DNA isolated from white blood cells or from the SN [89, 90]. Whereas low level heteroplasmic mutations in the ND5 subunit of complex I were reported to be associated with PD, [91] the level of heteroplasmy was much less than seen in diseases caused by mtDNA mutations. Contrary to these our recent study found higher burden of homoplasmic mtDNA mutations in PD subjects from SN of younger patients who have not lost a significant number of dopaminergic neurons (M Flint Beal unpublished observations). Human mtDNA exhibits region specific variation in indigenous populations [92] that can be organized into phylogenetic trees. The haplotype J and K are associated with mild uncoupling of mitochondria, allowing adaptation to colder climates, which would lead to reduced mitochondrial ROS production. Haplotype J, determined by a single nucleotide polymorphism (SNP) at 10398G and haplotype K in the control region of mtDNA, reduced the incidence of PD by 50% in patients of European ancestry [93]. Furthermore in the same study, a SNP at 9055A of ATP6 reduced the risk in women, and SNP 13708A within the NADH dehydrogenase 5 gene was protective in individuals older than 70 years of age (73% reduction). Consistent with these findings the haplotype cluster UKJT produced a 22% decrease in the risk of getting PD [94]. In a Finnish population the super cluster JTIWX increased the risk of both PD

and PD with dementia. The JTIWX cluster was associated with a 2 fold increase in non-synonymous substitutions in the mtDNA genes encoding complex I subunits [95].

Additional evidence to the role of mtDNA in PD was found by an increased proportion of mtDNA deletion observed in PD patients as compared to age-matched control subjects associated with a decrease in cytochrome oxidase activity [96, 97]. These mutations were considered somatic as each neuron harbored its own unique set of mtDNA deletions [97]. On the contrary, such mtDNA deletions in the nigral neurons have also been reported to be increased in the elderly human population [98]. Mitochondrial genome sequence from SN of PD patients and control subjects has not shown any distinct pathogenic mutation responsible for PD [90]. However, polymorphisms in human mtDNA conferring certain mtDNA haplotypes have been indicated to influence the risk for developing PD [93-95, 99, 100] and a polymorphism noted in a nuclear-encoded mitochondrial complex I subunit could relate to the susceptibility to PD [99]. Recently, mutations/polymorphisms in mitochondrial transcription factor A (TFAM) gene that regulates transcription of mtDNA and copy number has been suspected for its association in PD [101, 102]. Interestingly ablation of TFAM in nigrostriatal dopaminergic neurons of mice is known to cause progressive loss of SNpc dopaminergic neurons and L-DOPA responsive motor deficits due to reduced mtDNA expression and respiratory chain deficiency [103]. Another enzyme regulating mtDNA replication is the mtDNA polymerase gamma 1 (POLG1). POLG1 is a nuclear encoded protein and imported into the mitochondria to localize in the inner mitochondrial membrane. Mitochondrial DNA synthesis, replication and repair are controlled by POLG1 [104] and mutations might lead to mitochondrial pathology and DNA deletions. It has been shown that mutations in POLG1 results in a wide range of complex syndromes including autosomal dominant or recessive progressive ophthalmoplegia and parkinsonism [105-108]. PD associated with POLG1 mutations are L-DOPA responsive, characterized by loss of SNpc dopaminergic neurons lacking Lewy bodies, and by the presence of multiple mtDNA deletions and mtDNA contents in muscle [105, 109]. Muscle biopsies are also characterized by reductions in the activities of mitochondrial complex I, III, and IV [105, 109].

However, it seems that POLG1 mutations do not always necessarily result in PD. A study conducted on a large number of sporadic PD patients from UK and Italy does not conform to the possibility of linking common POLG1 polymorphisms in PD [110]. Overall in the present scenario the factors responsible for causing abnormalities in mtDNA are unclear and also whether alterations in mtDNA are a primary or secondary event in PD pathogenesis. Despite these confounding and scanty evidences, the role of mtDNA cannot be overruled and further investigations are warranted to better understand its role in PD.

#### Mitochondrial permeability transition and PD

The mitochondrial permeability transition pore (PTP) phenomenon had been known and studied over more than 40 years but still remains somewhat mysterious and enigmatic. In the recent decade, it has drawn a renewed interest chiefly because of its perceived role in cell death by either apoptosis or necrosis, which is extensively reviewed elsewhere [111]. There are also numerous and recent reviews detailing available evidences for the role of PTP in neural cell death induced by ischemia, trauma, and various neurodegenerative diseases [111-114]. However, we should emphasize here that despite all this wealth of information, there is actually no strong and unambiguous evidence supporting the role of PTP in any of neurodegenerative disease at the level of organism.

The “classical” permeability transition pore (PTP) is a large channel in the inner mitochondrial membrane which is normally closed and can be opened by  $\text{Ca}^{2+}$  accumulation in mitochondria and many other factors. Opening of this channel renders mitochondria incapable of ATP production and frequently results in severe damage to mitochondrial ultrastructure. The PTP is not merely a stochastic “damage” of mitochondrial ultrastructure but a specific mechanism controlling the permeability of the inner mitochondrial membrane. Several signature features define this mechanism. In mammalian mitochondria, PTP has a defined size; it is permeable to solutes with molecular mass less than ~1,500 D [115, 116]. The opening of PTP is fully reversible *in vitro* [116, 117], whereas it can be opened and closed repeatedly without extensive damage to mitochondria if the experimental conditions are properly

selected. *In vitro*, opening of the “classical” PTP results in a large-amplitude mitochondrial swelling, an obligatory decrease in the membrane potential [118, 119] and, if allowed to stay open for some time, results in eventual loss of matrix solutes such as NAD [120], which inhibits the respiration of mitochondria, and GSH, which stimulates ROS production [121]. The role of ROS in PTP activation is somewhat controversial. Although many reports suggest that ROS can induce PTP opening [118], almost all of these studies employed exogenous oxidants added to mitochondria or cells. The role of endogenously produced ROS in PTP opening remains to be elucidated [122]. On the other hand, PTP opening stimulates ROS production by isolated mitochondria [123, 124] and in cells [125].

The molecular identity of the protein(s) that actually form the PTP channel remains a mystery. Past studies identified several proteins involved in the PTP formation or modulation, such as voltage dependent anion channel (VDAC), adenine nucleotide transporter (ANT), and more recently, mitochondrial phosphate transporter (PIC) [126], though none of these proteins are currently thought to directly form the PTP-channel [126-128]. Until recently, mitochondrial (ANT) was viewed as the most likely candidate on the role of PTP-forming protein [126, 128]. ANT may also interact with another matrix protein, cyclophilin D (CYPD) [129], which is a target of cyclosporine A. Although the role of CYPD in modulating the  $\text{Ca}^{2+}$  threshold for PTP activation had recently been strongly confirmed [130-133], the role of ANT in the PTP formation was strongly challenged [134]. To note, one of the earliest observations implicating  $\text{Ca}^{2+}$ -induced mitochondrial dysfunction in PD-related cell death was that MPP<sup>+</sup> with 6-hydroxydopamine or dopamine strongly stimulated  $\text{Ca}^{2+}$  release from mitochondria and hydrolysis of intramitochondrial pyridine nucleotides [135]. This was followed by the finding that MPP<sup>+</sup> caused mitochondrial swelling and the release of cytochrome c [136], calcium efflux and membrane depolarization [137], all of which were inhibited by cyclosporine A thereby implying a classical PTP. A monoamine oxidase metabolite of dopamine, 3,4-dihydroxyphenylacetaldehyde, induced PTP in mitochondria isolated from neural cells [138]. Also, promethazine which is a weak PTP inhibitor [139], prevented the MPP<sup>+</sup>-induced decrease in the mitochondrial membrane potential,



inhibited  $\text{Ca}^{2+}$ -induced PTP in isolated brain mitochondria and strongly reduced the loss of SN neurons [140]. Recently, we have found that genetical ablation of PTP modulator cyclophilin D, which significantly increases the resistance of mitochondria to  $\text{Ca}^{2+}$ -induced PTP opening, also strongly protects mice from acute MPTP-neurotoxicity (Thomas et al., manuscript in preparation). Interestingly, neuroprotective effect in the cyclophilin D null mice were only limited to acute MPTP neurotoxic paradigm and not observed in sub-acute MPTP neurotoxicity [141]. These findings suggest that cyclophilin D mediates only acute-toxicity paradigms and may not play a role in apoptosis seen in sub-acute regimen.

Thus, sufficient evidence supports the induction of PTP by MPP+, at least at the level of isolated brain mitochondria. As discussed earlier, this PD toxin is an inhibitor of complex I, and in this regard it is very interesting that complex I had long been implicated in the modulation of PTP opening and it was suggested that complex I may be a part of the PTP assembly [142]. However, given the complexity and insufficient knowledge of PTP composition and regulation, it is clear that further research should answer some basic questions first, such as “what protein(s) form the PTP channel?”, “what proteins modulate the PTP opening directly, by protein-protein interactions?”, “how to assess and modulate the PTP status *in vivo*, in brain cells?”. Answering these questions will immensely help to establish whether PTP plays a role in the etiology of PD and develop therapeutic compounds targeting PTP.

### **Mitochondrial dysfunction and the genetic causes of disease**

In the past decade, a rapidly expanding list of Mendelian-inherited gene mutations has provided tremendous insight to the mechanisms leading to loss of the nigrostriatal dopaminergic neurons in familial PD which constitutes about less than 10% of the total PD cases. In recent years, a plethora of studies channeled towards exploring the multifunctional aspects of these Mendelian gene mutations point towards their potential deleterious role in the disruption of mitochondrial harmony and subsequent mitochondrial failure culminating in neuronal dysfunction and death. We have briefly highlighted below

the clinical association of five genes (*α-synuclein*, *parkin*, *PINK1*, *DJ-1* and *LRRK2*) in PD that have been suggested to have a direct or indirect role in mitochondrial dysfunction in disease pathogenesis.

### *α-synuclein*

Missense mutations in *α-synuclein* gene (PARK1 & 4 locus) and in addition to genomic triplications of a region of *α-synuclein* gene are associated with autosomal dominant PD [143-146]. *α-synuclein* is a fibrillar aggregation prone protein which attains an increased propensity to aggregate due to the presence of its hydrophobic non-amyloid beta component domain. Fibrillar form of *α-synuclein* forms a major structural component of Lewy bodies and is believed to contribute in PD pathogenesis due to a toxic gain of function. Although both mitochondrial dysfunction and protein aggregation are implicated in PD pathogenesis it was not clear until recently that these two processes are interrelated and complement each other in disease pathogenesis. New emerging evidences from both *in vitro* and *in vivo* studies suggest a strong pathogenic role of *α-synuclein* in causing mitochondrial dysfunction. Studies suggest that neurodegeneration observed in mice harboring human A53T *α-synuclein* mutant is linked to mitochondrial damage. These mice show mitochondrial accumulation of human *α-synuclein* and exhibit mitochondrial degeneration associated with increased mtDNA damage and impaired activity of the electron transport chain complex IV cytochrome oxidase [147]. Overexpression of human *α-synuclein* in mice also increases susceptibility to neurodegeneration following administration of mitochondrial toxins like MPTP and paraquat [148, 149], whereas loss-of-function of *α-synuclein* is known to promote resistance to mitochondrial toxins like MPTP, 3-nitropropionic acid, and malonate [150]. These findings suggest a complementary relationship between expression of *α-synuclein* to inhibition of mitochondrial functions considering that administration of mitochondrial electron transport chain inhibitors in rodents and in cell culture systems are known to cause aggregation and formation of *α-synuclein* inclusions [71, 151-153]. There is also data suggesting that inhibition of the ubiquitin proteasomal pathway *in vitro*

leads to accumulation of unfolded proteins causing mitochondrial dysfunction and mitochondrial dependent cell death suggesting that both mitochondrial dysfunction and protein aggregation may have convergent mechanisms leading to neurodegeneration [154]. A possible connection between mitochondrial dysfunction and protein aggregation as in the case of *α-synuclein* could be explained by the fact that *α-synuclein* is a modulator of oxidative stress. Consistent with this notion more than twofold increases in specific carbonyl levels of three metabolic proteins such as carbonic anhydrase 2, alpha-enolase, and lactate dehydrogenase 2 in brains of human A30P *α-synuclein* transgenic mice were reported. These findings imply that proteins associated with impaired energy metabolism and mitochondria are particularly prone to oxidative stress associated with mutant *α-synuclein* [155]. Additionally, presence of *α-synuclein* mutations may significantly modify proteomic organization *in vivo* that may in turn affect multiple cell survival pathways to impact disease development. A recent study in presymptomatic A53T *α-synuclein* fly model of PD using global isotopic labeling strategy combined with multidimensional liquid chromatography and tandem mass spectrometry revealed significant changes in protein interaction networks [156]. These changes in normal proteomic organization resulted in improper functioning of multiple pathways in addition to mitochondrial defects to induce PD. Together, these data suggests that on one hand formation of *α-synuclein* inclusions could be initiated by an impaired mitochondrial function whereas on the other hand impaired mitochondrial function may result in abnormal protein aggregation. However, precisely how these two processes are linked to each other and is associated with the pathogenesis of disease process warrants a more detailed investigation.

There is also a great deal of interest amongst researchers to identify whether *α-synuclein* associates with mitochondria during pathologic conditions to disrupt mitochondrial integrity. A recent study showed that the N-terminal 32 amino acids of human *α-synuclein* contain a cryptic mitochondrial targeting signal, which is crucial for mitochondrial targeting of *α-synuclein* [157]. Mitochondrial

targeted *α-synuclein* was shown to predominantly associate with the inner mitochondrial membrane whereas *α-synuclein* lacking the mitochondrial targeting signal failed to associate with the mitochondria. Interestingly accumulation of *α-synuclein* in the mitochondria of human dopaminergic neurons caused reduced mitochondrial complex I activity and increased production of ROS which was not observed in *α-synuclein* lacking the mitochondrial targeting signal. Investigation of mitochondria from SN, striatum, and cerebellum of postmortem PD patients and controls showed the constitutive presence of *α-synuclein* in the mitochondria of all three brain regions from normal subjects. Interestingly, mitochondria isolated from SN and striatum but not cerebellum from PD subjects showed significant accumulation of *α-synuclein* and decreased complex I activity. Blue native gel electrophoresis and immunocapture analysis further revealed the association of *α-synuclein* with mitochondrial complex I suggesting that pathological consequences such as complex I inhibition might be associated due to mitochondrial accumulation of *α-synuclein* [157]. It will be of great importance to understand what triggers the mitochondrial translocation of *α-synuclein* in addition to its mitochondrial targeting by the cryptic mitochondrial targeting signal. A possible mechanism is suggested to be improper cytosolic environment during cellular abnormalities with oxidative stress being a major culprit. A recent study demonstrated that cytosolic acidification induces translocation of *α-synuclein* from the cytosol to mitochondria. This translocation of *α-synuclein* was shown to occur rapidly under artificially-induced low pH conditions and especially as a result of pH changes during oxidative or metabolic stress. Interestingly *α-synuclein* binding was shown to be facilitated by low pH-induced exposure of the mitochondria-specific lipid cardiolipin. These results imply a direct role for *α-synuclein* in mitochondrial physiology, especially under pathological conditions [158].

Thus both genetic and biochemical abnormalities in *α-synuclein* lead to its physical association with mitochondria. This in turn impacts mitochondrial physiology to affect normal functioning of

dopaminergic neurons. The focus now should be on how these factors are interrelated to provide a cause and effect relationship to development of PD due to abnormalities in *α-synuclein*.

### Parkin

Mutations in the *parkin* gene (PARK2) were reported to cause early onset juvenile form of autosomal recessive parkinsonism [159]. *Parkin* mutations serve as the most common cause of young onset PD, although mutations in patients with delayed-onset cases have also been reported [160, 161]. *Parkin* is a RING finger containing protein and has been shown to function as an ubiquitin E3 ligase *in vitro* [162]. It is known to mediate polyubiquitination reaction to clear aggregation prone substrates for proteasomal degradation, and the loss of its E3 ligase activity has been suggested to result in accumulation of toxic substrates leading to autosomal recessive form of PD [163]. In addition to its role in the proteasomal machinery more recent studies have suggested a role of *parkin* in mediating proteasomal independent polyubiquitination of lysine 63-linked process or even monoubiquitination [164]. Such modifications are known to mediate functions involved in transcriptional regulation, protein trafficking and neuroprotective signaling [165, 166]. Numerous studies indicate that a potential function of *parkin* might be in the mitochondria. Although *parkin* protein is ubiquitously expressed, subcellular fractionation studies demonstrate that *parkin* is associated with the outer mitochondrial membrane [167, 168] suggesting a potential role in modulating mitochondrial functions. In dividing SH-SY5Y neuroblastoma cells *parkin* is localized to the mitochondria whereas upon differentiation it translocates to Golgi apparatus and also nucleus. This phenomenon of *parkin* departing the mitochondria and locating itself to other intracellular structures is also observed when cells are treated with inhibitors of electron transport chain such as rotenone, mitochondrial uncouplers and cell cycle blockers [169]. It was further demonstrated that this mitochondrial export of *parkin* is mediated by opening of the mitochondrial permeability transition pore. Interestingly upon transient transfection, wild type *parkin* seems to be imported into the mitochondria whereas the amount of protein import was much less when

cells were transfected with *parkin* mutants. Kuroda and colleagues also demonstrated a novel function of *parkin* whereby overexpression of *parkin* resulted in transcription and replication of mtDNA in proliferating cells, a phenomenon blocked by knockdown of *parkin* by siRNA. Transcription of mtDNA in proliferating cells was a result of direct functional association of *parkin* with TFAM. These data suggests *parkin* is involved in mitochondrial transcription and replication. *In vivo* studies also support a potential role of *parkin* in the mitochondria. Gene knockouts of *parkin* mouse and flies are known to exhibit dramatic mitochondrial defects [68, 170]. These include swollen mitochondria, severely fragmented cristae, decreased abundance of several proteins subunits of the mitochondrial electron transport chain complex I and IV, and decrements in mitochondrial respiratory capacity [68, 170]. In contrast to *parkin* knockout mouse models the mitochondrial abnormalities in drosophila models representing loss of *parkin* function show dopaminergic neurodegeneration, reduced lifespan, and increased apoptosis in flight muscles [171, 172].

One possible function of *parkin* in the mitochondria might be to maintain normal antioxidant status. Downregulation of *parkin* in PC12 cells resulted in a significant decrease in reduced glutathione levels and superoxide dismutase activity [173]. In *parkin* mutant flies dopaminergic neurodegeneration is enhanced by loss-of-function mutations of the glutathione S-transferase S1 (GstS1), a rate limiting enzyme in glutathione synthesis [174]. Dopaminergic neurodegeneration observed in *parkin* mutant flies were significantly suppressed by overexpression of GstS1 suggesting that *parkin* might be involved in regulation pathways responsible for glutathione metabolism. This is also consistent with the ability of allyl disulfide and sulphoraphane, activators of phase II detoxifying genes to rescue dopaminergic neurodegeneration in *parkin* mutant flies by directly affecting abundance of reduced glutathione levels [175]. In SH-SY5Y cells transcriptional induction of tyrosinase causes overproduction of intracellular dopamine and its oxidative metabolites leads to increased generation of both cytosolic and mitochondrial reactive oxygen species to cause apoptosis. Stable expression of wild type, but not mutant *parkin* in these cells resulted in blockade of tyrosinase-induced apoptosis. Wild type *parkin* significantly

attenuated the tyrosinase-induced activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase signaling whereas PD associated *parkin* mutants failed to attenuate tyrosinase induced activation of stress activated protein kinase signaling and subsequent apoptosis [176]. These *in vitro* findings further gains support from *in vivo* fly models where the JNK is strongly activated in the dopaminergic neurons of *parkin* mutant flies and the activation of the JNK signaling pathway results in shrinkage of dopaminergic neurons with decreased tyrosine hydroxylase level and impaired locomotion. Consistent with this, both epistatic analysis and mammalian cell studies showed that *parkin* inhibits the JNK signaling pathway in an E3 ligase activity-dependent manner [177]. This suggests that *parkin* may play crucial role in mitochondria dependent stress signaling pathway in impacting apoptosis. Further evidence to its role in apoptosis is strengthened from studies where inducible overexpression of *parkin* has been shown to delay ceramide-induced mitochondrial swelling leading to blockade of cytochrome c release and subsequent caspase 3 activation in differentiated PC12 cells [167]. Interestingly, apoptosis was abrogated in these cells by disease-causing mutants and treatment with proteasome inhibitor suggesting a role of *parkin* in mitochondria dependent cell death. The mitochondrial dependence of *parkin* in impacting cell death is also consistent with findings where treatment of midbrain dopaminergic neuronal cultures showed increased susceptibility to mitochondrial complex I inhibitor rotenone-induced death [178]. Similar effects have also been observed in primary cultures from skeletal muscle derived from *parkin* knock-out mice, where absence of *parkin* resulted in greater sensitivity to cytotoxicity following treatment with mitochondrial complex I inhibitor rotenone and a mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone [179]. Interestingly, severe mitochondrial respiratory chain deficits have also been detected in peripheral leukocytes taken from human PD patients with *parkin* mutations [180]. Taken together these observations provide convincing evidence for a major role of *parkin* in directly regulating mitochondrial functions that probably is evolutionarily conserved.

*Parkin* has also been shown to be instrumental in rescuing mitochondrial dysfunction caused due to pathogenic mutations associated with familial PD. This includes mitochondrial dysfunction due to

*α-synuclein* overexpression and *PINK1* loss-of-function. Loss of *parkin* function has been shown to exacerbate mitochondrial damage in *α-synuclein* transgenic mice [181]. The mitochondrial damage in these double mutant mice with *α-synuclein* overexpression and *parkin* deficiency includes mitochondrial morphological alterations and reduced complex I mediated mitochondrial respiration in substantia nigra. This suggests that *parkin* deficiency may provide increased vulnerability to alterations in mitochondrial functions due to *α-synuclein* overexpression. Although it is yet to be determined how *parkin* is involved in these mitochondrial functions. *Drosophila PINK1* mutants exhibit increased sensitivity to mitochondrial stress with reduced ATP levels and mtDNA content. Similar to *parkin* mutant flies mitochondria in *PINK1* mutant flight muscles are swollen with fragmented cristae [182-184]. Furthermore, *parkin* overexpression can rescue mitochondrial alterations induced due to loss of human *PINK1* [185]. These studies further demonstrated that mutant *PINK1* associated mitochondrial dysfunctional phenotype was remarkably reversed by overexpression of *parkin*. It is yet unclear how *parkin* rescues these mitochondrial anomalies. *PINK1* and *parkin* has been shown to physically interact in neuroblastoma cell lines [164] although the functional implications of such interactions are unclear. It might be possible that *PINK1* being a kinase might regulate *parkin* function posttranslationally either by direct phosphorylation or by other signaling mechanisms. Additionally, a recent study suggests that *parkin* plays a concerted role with *PINK1* in regulating mitochondrial dynamics through the mitochondrial fission process [186]. The fact that *parkin* can rescue *PINK1* loss-of-function associated mitochondrial abnormalities unequivocally suggests an inherent role of *parkin* in the maintenance of mitochondrial functions.

### *PINK1*

Mutations in phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (*PINK1*) cause a rare form of autosomal recessive PD [187]. Postmortem brains of PD patients with *PINK1* heterozygous mutations display nigrostriatal neuronal loss with Lewy bodies [188]. Nuclear encoded



*PINK1* gene is translated in the cytoplasm and imported into the mitochondria through an N-terminal mitochondrial targeting sequence, and bears a kinase domain to function as a serine/threonine kinase [187, 189]. *PINK1* mutations are believed to result in impaired phosphorylation of its substrates probably in the mitochondria to cause PD. The sub-mitochondrial localization of *PINK1* has been intensely debated which is crucial for identifying the physiological substrates of *PINK1* and its mode of action in the context of PD pathogenesis. The majority of studies demonstrate that *PINK1* localizes in the inner mitochondrial membrane [188-190], whereas others suggest that *PINK1* associates with the intermembrane space [76, 189, 190] or even the outer mitochondrial membrane [188] with the kinase domain facing the cytoplasm [191].

Accumulating evidence suggests that *PINK1* mutations may result in the loss of *PINK1* function resulting in PD [192-194]. There are numerous *in vivo* studies which support this notion. In *Drosophila*, loss of *PINK1* function produced mitochondrial morphological defects such as fragmented cristae, increased sensitivity to oxidative stress, loss of nigrostriatal dopaminergic neurons and apoptotic flight muscle degeneration [182-184]. The pathological phenotype observed in *PINK1* mutant flies resembled those of *parkin* mutant flies. Overexpression of *parkin* has been shown to block mitochondrial dysfunction and associated pathology due to *PINK1* loss-of-function. This suggests that both *parkin* and *PINK1* share common mechanistic pathways in impacting mitochondrial dysfunction *in vivo* with *parkin* acting downstream of *PINK1* [182-184]. Recently, mice deficient in *PINK1* demonstrated significant mitochondrial defects such as impaired mitochondrial respiration, and significant defects in complex I and also complex II-IV activity in the striatum of younger animals. Interestingly these mitochondrial functions were normal in the cerebral cortex of younger animals but they were significantly impaired in the older animals suggesting that aging in addition to *PINK1* loss-of-function might influence mitochondrial respiration. These changes were also accompanied by significantly reduced activity of the Krebs cycle associated enzyme aconitase. Loss of *PINK1* also showed increased exacerbation to mitochondrial respiration upon exposure to oxidative stressors such as H<sub>2</sub>O<sub>2</sub> and mild heat shock. This

suggests that mitochondrial dysfunction together with increased oxidative stress may result in dysfunction of nigrostriatal dopaminergic neurons [195]. These mice also result in impaired dopamine release and synaptic plasticity in the striatum which is crucial for normal dopamine neuronal function especially during disease condition [196]. Interestingly, mitochondrial pathology and respiratory chain defects were detected in the peripheral tissues in PD patients with *PINK1* mutation [197]. Recently, a PD patient with *PINK1* mutation demonstrated defects in mitochondrial respiratory and ATP synthesis in fibroblasts [198] suggesting an unequivocal role of *PINK1* in mitochondrial function. *In vitro* studies, using various cell lines, demonstrate that an overexpression of wild type *PINK1*, but not PD-linked *PINK1* mutants inhibited mitochondrial cytochrome c release and blocked neuronal apoptosis [199, 200]. Downregulation of *PINK1* in human cell lines by RNA interference results in abnormal mitochondrial morphology and reduced mitochondrial membrane potential which were rescued by wild type *PINK1* but not PD associated mutants [185]. Interestingly, aberrant mitochondrial morphologies such as swollen, truncated and fragmented mitochondria were also observed in fibroblasts cells from patients carrying the Q126P and G309D *PINK1* mutations suggesting a role of *PINK1* in maintenance of mitochondrial integrity [185].

The cytoprotective role of *PINK1* has been demonstrated by studies using cellular and *in vivo* models of PD. Loss of *PINK1* function by siRNA resulted in increased sensitivity to MPP<sup>+</sup>-toxicity in primary cortical neurons which were abrogated by overexpression of wild type *PINK1* [201]. This protective effect of *PINK1* overexpression was mediated by its kinase domain which was abolished by PD-associated *PINK1* mutants. These were also consistent with *in vivo* studies where adenoviral overexpression of *PINK1* in midbrain dopaminergic neurons rescued toxicity against the parkinsonian neurotoxin MPTP [201]. Interestingly, the cytoprotective phenotype of *PINK1* was achieved merely by the presence of cytosolic *PINK1* lacking a mitochondrial targeting sequence. These results are indicative of the crucial role of cytoplasmic *PINK1* rather a mitochondrial kinase in impacting its cytoprotective function. To further emphasize this phenomenon a recent study showed that *PINK1* localization in the

mitochondria spans the outer mitochondrial membrane, with the N-terminus being inside the mitochondria, whereas the C-terminal kinase domain faces the cytosol [191]. This pattern of *PINK1* assembly may provide clues to its interaction with other cytosolic proteins such as *parkin* and also other substrates of *PINK1*.

Recently, two potential substrates known to mediate *PINK1* anti-apoptotic function were identified. These include a) TNF receptor-associated protein 1 (TRAP1), a mitochondrial molecular chaperone also known as heat shock protein 75 [190] and b) *HtrA2*, a mitochondrial serine protease, also known as *Omi* which is also a candidate gene for PARK13 [76]. *In vitro* studies by Pridgeon et al., suggests that *PINK1* binds and associates with TRAP1 in the mitochondrial intermembrane space and the innermembrane. Additionally, using a kinase assay *PINK1* was shown to phosphorylate TRAP1 in test tubes and also in cell models. In cells, TRAP1 phosphorylation by *PINK1* was shown to prevent cytochrome c release and apoptotic cell death induced by H<sub>2</sub>O<sub>2</sub>. Furthermore disease-linked and kinase mutants of *PINK1* were unable to phosphorylate TRAP1 to promote cell survival, while absence of TRAP1 prevented the ability of wild type *PINK1* to protect cells against apoptosis. The TRAP1 mode of action in inhibiting mitochondrial cytochrome c release is unknown but it seems that TRAP1 acts downstream of *PINK1* to block apoptosis. Plun-Favreau and colleagues identified the mitochondrial serine protease *HtrA2*, also known as *Omi*, as a putative binding partner of *PINK1*. The functional interaction between *Omi* and *PINK1* was brought about by phosphorylation of *Omi* by the p38-kinase in the presence of a functional *PINK1*. Interestingly the phosphorylation of *Omi* was completely absent in postmortem brain tissue from patients who developed PD due to *PINK1* mutations. The *Omi* phosphorylation was able to rescue mouse embryonic fibroblasts against PD-causing toxins such as 6-hydroxydopamine, rotenone and other stressors causing apoptosis. These cytoprotective effects of *Omi* was abolished by PD causing *PINK1* mutations suggesting that *Omi* functions downstream of *PINK1* to rescue against mitochondrial cell death. This is in agreement with *Omi* in rescuing against mitochondrial dysfunctions leading to PD [202] since during apoptosis *Omi* is released from the mitochondrial

intermembrane space to the cytosol to act on the IAPs (inhibitor of apoptosis) to activate the caspases downstream. Additionally Omi is known to degrade unfolded mitochondrial proteins to serve as a protein-quality control agent. The identification of both TRAP1 and Omi as potential substrates for *PINK1* and that these substrates cooperate with *PINK1* to rescue mitochondrial apoptosis against stressors further reinforce the role of mitochondrial dysfunction in neurodegeneration associated with PD. However, it is yet to be determined whether TRAP1 and *Omi* are authentic substrates *in vivo* and if yes, how they influence loss of *PINK1* in PD.

More recently *PINK1* has also been shown to play a role in the process of mitochondrial dynamics by interacting with proteins involved in the fission and fusion machinery. The mitochondrial fission is a process controlled by drp1 (dynamin related protein 1) and FIS1 (mitochondrial fission protein) involving separation of mitochondrion into two or more smaller parts where as mitochondrial fusion controlled by Mitofusin (mfn) and Optic atrophy 1 (OPA1) involves combination of two mitochondria into a single organelles. A balance between fission and fusion machinery is crucial for the normal mitochondrial function, *PINK1* has been suggested to promote mitochondrial fission and to regulate mitochondrial morphology in *Drosophila* and several mammalian models [186, 203, 204]. Genetic manipulations in *Drosophila* by overexpression of drp1 protein known to promote mitochondrial fission and knockdown to mfn and OPA1 that inhibit mitochondrial fusion has been shown to suppress *PINK1* mutant phenotype of dopaminergic neurodegeneration and loss of flight muscles [186, 203]. On the other hand some studies also point toward a significant role of mitochondrial fusion proteins in rendering neuroprotective phenotype in models of oxidative stress and mitochondrial stressors [205, 206]. These suggest that it is very premature to determine if mitochondrial fission and/or fusion may play a definitive role in PD pathogenesis and requires further exploration.

#### *DJ-1 and LRRK2*

*DJ-1* mutations associated with PD are rare and account for 1-2% of early onset forms of the disease [207]. Several *in vitro* and *in vivo* studies demonstrate that silencing *DJ-1* increases susceptibility to cell death whereas overexpressing *DJ-1* provides cytoprotective effects against cell death. The cytoprotective effect of *DJ-1* seems to be specific for death due to oxidative stress but not to non-oxidative stresses, since *DJ-1* knockout flies show increased sensitivity to H<sub>2</sub>O<sub>2</sub> and the redox cycler paraquat [208, 209]. The modification of cysteine residues on *DJ-1* to cysteine-sulfinic and cysteine-sulfonic acids under oxidative conditions is suggested to be required for its cytoprotective action during oxidative stress-induced cell death [208, 210, 211]. It is thus suggested that *DJ-1* functions as a redox sensor to exert protective effects by undergoing cysteine modifications on *DJ-1*. Recent studies also suggest that *DJ-1* may exert neuroprotective effects through several different mechanisms especially by acting as a transcriptional coactivator, a protease or a molecular chaperone [148]. At this juncture, unlike *parkin* and *PINK1*, evidence for the role of *DJ-1* in mitochondrial function is minimal. Endogenous *DJ-1* is localized to mitochondrial matrix and the intermembrane space although a predominant portion of *DJ-1* localizes to the cytosol and a small fraction in the nucleus [212, 213]. It is believed that the mitochondrial localization of *DJ-1* might suggest a potential role in mitochondrial functions. There is evidence that *DJ-1* functions as an atypical peroxiredoxin-like-peroxidase and that loss of this function results in an impaired mitochondrial ROS scavenging [214]. In conjunction with this, mice lacking *DJ-1* are more susceptible to dopaminergic neurotoxicity and oxidative stress induced by MPTP [215]. Also nigrostriatal dopaminergic neurons were highly vulnerable to paraquat in *DJ-1* deficient mice, the toxicity being associated with lowered ATP levels and inhibited proteasomal function [216]. However the exact role of *DJ-1* in mitochondria and the importance of mitochondria in developing such phenotypes remain to be established. It has been shown that during oxidative stress *DJ-1* is upregulated and translocates to the mitochondria [217], where its functional form resides as a dimer [213]. Mitochondrial targeted *DJ-1* showed increased protection against oxidant induced cell death by functioning as an antioxidant. However, it is yet to be determined if *DJ-1* undergoes such

dynamic regulation *in vivo* to render its antioxidant action in the mitochondria. It was recently demonstrated that *DJ-1* functions to rescue against oxidative stress by its RNA binding ability in an oxidation dependent manner to modulate functioning of mitochondrial genes and genes involved in glutathione metabolism and other generic cytoprotective signaling [218]. This suggests a possible universal mechanism by which *DJ-1* may defend against cytotoxicity during oxidative stress and mitochondria associated cell death. There is also evidence from *in vitro* studies that *DJ-1* physically interacts with familial PD linked genes such as *PINK1* [219] and with *parkin* during oxidative stress [220] but such phenomenon and their implications to disease pathogenesis is yet to be proven *in vivo*. A possible function of the association of *DJ-1* with *parkin* and *PINK1* might be to alleviate oxidative stress during mitochondrial dysfunction and cell death. This notion gains support from *in vivo* studies where loss-of-function of *PINK1* and *parkin* mediated neurodegeneration in flies can be alleviated by overexpression of antioxidant enzymes [174, 221]. Since defective functioning of *parkin* and *PINK1* lead to mitochondrial dysfunction and increased ROS production a plausible function of *DJ-1* is suggested to act as a sensor for oxidative stress associated with mitochondrial respiration [222].

Mutations in leucine-rich repeat kinase 2 (*LRRK2*) gene cause autosomal dominant PD [223, 224]. This gene has obtained considerable attention because of the presence of *LRRK2* mutations beyond familial cases of disease with evidence of partial penetrance which is age dependent. *LRRK2* encodes a 2527 amino acid multidomain, 280 kDa protein belonging to ROCO protein family that includes a Rho/Ras-like GTPase domain, a protein kinase domain of the MAPKKK family, as well as a WD40-repeat and a leucine-rich repeat domain. An additional domain C-terminal to the GTPase domain, termed COR (for carboxy-terminal of Ras), is of unknown function. Point mutations have been found in almost all of the identified domains. The presence of mutations in several different domains, as well as the lack of deletions or truncations, along with dominant inheritance, is consistent with a gain-of-function mechanism. The precise physiological role of this protein is unknown but the presence of multiple functional domains suggests involvement in a wide variety of functions. A possible mechanism

of *LRRK2* action is at mitochondria. There is evidence from subcellular fractionation studies that about 10% of *LRRK2* is associated with the mitochondrial fraction [225]. Another study showed an association of *LRRK2* with TOM20, a marker of the outer mitochondrial membrane [226]. The strongest evidence for localization of *LRRK2* to mitochondria comes from a recent immunohistochemical study [227]. The authors generated polyclonal antibodies to the amino or carboxy termini of *LRRK2* to examine the subcellular and immunohistochemical distribution of *LRRK2*. The subcellular fractionation studies showed the presence of *LRRK2* in microsomal, synaptic vesicle-enriched and synaptosomal fractions from rat brain including mitochondria. Since, as discussed above, mitochondria are implicated in the pathogenesis of PD, the localization of *LRRK2* to mitochondria could play a critical role in PD pathogenesis.

Overall, familial gene products which reside in the mitochondria or elsewhere in the cell could be crucial players in the maintenance of mitochondrial homeostasis. Deleterious mutations affecting the vital functions of these proteins therefore could dysregulate mitochondrial functions by interaction through a maze of signaling pathways. Based on the evidences of intensive studies conducted *in vivo* and *in vitro* to resolve the impact of gene or environment or a complex interaction of both in PD might eventually expose mitochondria as the responsible personnel in the neuronal death in PD.

### **Concluding remarks and future perspectives**

Ample empirical evidence as detailed in this review suggests a major role of mitochondrial dysfunction in the pathogenesis of PD. Deficits in mitochondrial complex I activity in combination with increased oxidative stress, and aging associated damage to mitochondrial DNA are currently known to dominate as key biochemical abnormalities associated with pathogenesis of sporadic PD. Factors that potentially trigger these mitochondrial abnormalities which ultimately lead to PD are still elusive. It is suggested that mitochondrial anomalies could result from exposure to environmental factors such as pesticides and other daily use chemicals that have been shown to cause PD both in humans and animal

models. In addition familial mutations result in PD due to mitochondrial dysfunction that includes complex I deficits, increased ROS production, and impaired mitochondria-dependent cytoprotective signaling cascades. Together these further reinforce and validate the importance of mitochondrial functions not only in sporadic PD but also in familial forms of the disease. Although much is known about the familial gene mutations and their impact on mitochondrial functions, a definitive mitochondrial pathway in disease pathogenesis due to these mutations is yet to emerge. Based on the current knowledge of mitochondrial alterations in sporadic and familial PD it could be speculated that strategies aimed at correcting the above mentioned biochemical abnormalities might be useful to halt or slowdown the progression of PD. In this regard some of the candidate drugs which showed great efficacy in experimental models of PD have already made it to clinical trials. Preliminary clinical trial data on Coenzyme Q10 and creatine known to act by improving mitochondrial electron transport chain functions have shown some promise. There is also a great deal of enthusiasm due to recent identification of novel mitochondrial targets such as PGC-1 $\alpha$  (peroxisome proliferator-activated receptor gamma co-activator) and the sirtuin family of enzymes that are known to modulate aging, mitochondrial biogenesis, metabolic homeostasis and mitochondria-dependent cell death. These observations hold promise for future development of neuroprotective strategies in PD by targeting mitochondrial dysfunction. It is important to remember that PD is a multi-factorial disease and mitochondrial dysfunction may only be a part of this complex process. Future research should thus focus on developing neuroprotective strategies by targeting multiple pathways involved in disease process including those targeted to alleviate mitochondrial dysfunction in PD.

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**Table 1. Impaired activity of ETC complexes in PD.**

Merged cells indicate that the reported activity was measured over a span of two or more ETC complexes, e.g. complex II+ complex III or complex I + III. Changes in activities are presented in % from control; numbers indicate the number of individual samples assayed; “m” or “c” indicates whether isolated mitochondria (m) or crude homogenates (c) were used for assessing the enzyme activities; n.d. stands for “no difference found”; “n.a.” stands for “not assessed”.

Sample	Complex I	Complex II	Complex III	Complex IV	Reference
Platelets, m	- 54%, 10	n.d., 10		n.d., 10	Parker et al., 1989 [23]
Platelets, m	- 16%, 25	n.a.	n.a.	n.a.	Krige et al., 1992 [228]
Platelets, m	- 52%, 27	n.a.	n.a.	-30%, 27	Benecke et al., 1993 [22]
Platelets, m	-25%, 13	-20%, 13		n.d., 13	Haas et al., 1995 [21]
Platelets, c	-26%, 20	-20%, 20	n.a.	n.a.	Yoshino et al., 1992 [20]
SN, c	-38%, 9			n.a.	Schapira et al., 1990 [38]
SN, c	-30%, 9	n.d., 9		n.a.	Schapira et al., 1990 [38]
SN, c	-42%, 7				Schapira et al., [32]
SN, c		n.d., 7		n.d., 7	Schapira et al., [32]
SN, c		-33%, 7		n.a.	Janetzky et al., [34]
Frontal crtx, m	-50%, 5	n.d., 5	n.d., 5	n.d., 5	Parker et al., [35]
Cortex, m	-30%,	n.a	n.a.	n.a.	Keeney et al., 2006 [36]
muscle, m	-40%, 5	-48%, 5	-40%, 5	n.a.	Bindoff et al., 1991 [41]
muscle, m	-71%, 21	n.d., 21	-34%, 21	n.d., 21	Blin et al., 1994 [40]
muscle, m	-26%, 8	n.d., 8	-68%, 8	n.d., 8	Cardellach et al., 1994 [39]

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