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Mitochondrial proteomics as a selective tool for unraveling Parkinson's disease pathogenesis

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Parkinson's disease (PD) is a neurodegenerative disease characterized by the large-scale loss of dopaminergic neurons in the substantia nigra and the formation of protein aggregates that accumulate in the cytoplasm of the remaining dopaminergic neurons. Most cases arise sporadically, while the precise cause remains obscure. This lack of understanding as to the etiology of PD continues to serve as a major barrier for delivering effective therapeutics. Mitochondria are potent integrators and coordinators of apoptosis, necrosis and cell survival. Neurotoxin-based and genetically modified animals, which mimic aspects of the core pathologies seen in human PD, support a role for oxidative stress, production of reactive oxygen species in excess and mitochondrial dysfunction in PD pathogenesis. This and other similar discoveries provide a convergence point for an explosion of morphological, biochemical, molecular, cell and animal model studies for investigating the contribution made by mitochondrial dysfunction to PD pathology. Proteomics screening technologies have proved to be a valuable aid in the investigator's tool bag, by which to confirm a prominent role for mitochondrial proteins in PD pathology. Here, we discuss how improved understanding of the mitochondrial proteome through the application of high-throughput proteomics, combined with genetic studies and pharmacological manipulations to influence mitochondrial dynamics and functions, promises to give insights into PD's underlying disease mechanisms. Ultimately, such insights may pave the way towards designing novel strategies for providing symptomatic, neuroprotective and restorative therapeutic options to PD patients.

KEYWORDS: biomarker • mitochondrial electron transport chain • mitochondrial function • mitochondrial proteomics • Parkinson's disease

Parkinson's disease (PD) is the most frequently occurring movement disorder and the second most common neurodegenerative disease after Alzheimer's disease (AD), affecting approximately 1% of those aged over 65 years and over 6 million people worldwide [1]. The disease is clinically recognized by a triad of motor-related symptoms, namely bradykinesia, rigidity and resting tremor [2]. The growing socio–economic burden associated with PD has spurred attempts at aligning innovative new research methods with the many unanswered questions still surrounding PD pathology, in the hope that their application may eventuate towards a cure. Furthermore, current drug therapies for treating PD confer only limited symptomatic relief and associate with long-term side effects. Hence, it is wise to place emphasis on understanding the causes of the disease in order to prevent or delay disease onset in the first place. Such an approach necessarily involves identifying mechanisms and potential risk factors associated with the disease.

Parkinson's disease has as its most defining pathological feature the large-scale destruction of dopaminergic neurons that arise in the substantia nigra pars compacta (SNpc), located in the mesencephalic brain region. The loss of dopamine (DA)-producing neurons results in DA deficiency in the striatum, where these neurons project towards and terminate at. An additional characteristic, which PD shares with other neurodegenerative diseases such as AD, is the abnormal processing of mutant or damaged

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proteins. This results in their accumulation and aggregation in intracytoplasmic inclusions termed Lewy bodies (LBs) and Lewy neurites, found present within both dopaminergic and non-dopaminergic neurons of selective brain structures that include, but are not limited to, the SNpc [3]. α-synuclein (SNCA) forms the main protein constituent of LBs [4], while also containing many other constituents. The comprehensive list remains under investigation. Several proposals have been made to explain the aggregation of SNCA and the formation of LBs, including reduced proteasomal breakdown, defects in lysosomal degradation and loss-of-function mutations in enzymes that partake in the ubiquitin conjugation/deconjugation pathway. All these pathological mechanisms can potentially impair the cell's natural defences for protecting against the harmful cellular effects that stem from protein aggregation [5]. Experimental studies have provided evidence that in PD patients, the cell's protective mechanisms, such as the mitochondrial antioxidant superoxide dismutase (SOD) and glutathione, for processing free radicals, are overwhelmed by the abundance of nonmetabolized SNCA oligomers. This defensive barrier breakdown may modify the structure of proteins, lipids and mitochondrial DNA (mtDNA), thereby contributing towards the occurrence and progression of PD [6].

Epidemiological evidence strongly suggest a role for environmental factors, including pesticides, such as insecticides, herbicides and fungicides, commonly used in agricultural practice, placing those exposed at risk for developing PD [7]. Although most PD cases arise spontaneously, in a small subset of cases, a clear genetic etiology for PD is discernable. This includes the detected presence of mutations in the genes encoding for the proteins SNCA, parkin, DJ-1 [8], PINK1 and leucine-rich repeat kinase 2, also known as dardarin (see **Table 1**) [9]. Currently, however, increasing recognition is given to the importance of experimental evidence, derived, for instance, from twin studies, which implicates a complex interplay between genetic and environmental factors that confer PD susceptibility (**Figure 1**) [10].

Mitochondrial dysfunction as a central pathological mechanism in PD

Mitochondria fulfill a wide range of cellular roles, including regulating oxidative phosphorylation and generating cellular energy. These subcellular organelles furthermore regulate aspects as diverse as lipid metabolism, cell signaling, Fe-S cluster formation, cell growth, maintaining a constant intracellular calcium milieu as well as programmed cell death through the release of pro-apoptotic factors, such as cytochrome c [11]. Since these are critical for maintaining normal neurophysiological functions, such as neurotransmission, synaptic maintenance and neuronal survival in eukaryotic cells, it is not surprising that their dysfunction has been implicated in PD pathogenesis. Such an association places focus on the mitochondrial proteome as a point of convergence between genetic and environmental-based hypotheses concerning PD pathogenesis. For instance, although the functions of the proteins associated with PD pathology remain to be precisely defined, an extraordinary number of proteins implicated in PD's disease processes are localized in or interact with mitochondria [12]. Additionally, although rare, a number of studies provide evidence that abnormalities in mtDNA may contribute to PD pathogenesis [9].

Following a brief overview of experimental evidence derived from the use of animal models and human studies, which indicate a major role for mitochondrial pathology as an underlying cause of PD, we review here the contributions made by proteomic technologies for better understanding the role of mitochondrial proteins in PD's disease processes. It is anticipated that proteomic applications may result in more effective therapies that target the defective mitochondrial mechanism seen in PD.

Experimental toxins support a role for mitochondrial pathology in PD

Mitochondrial dysfunction leads to increased oxidative stress through the formation of reactive oxygen species (ROS). In turn, the damage incurred to proteins induced by exposure to excessive levels of ROS leave proteins incapable of performing their normal physiological functions. Animal models, based on the toxic damage induced by the neurotoxin 6-hydroxydopamine (6-OHDA) are used extensively for accurately mimicking aspects of human PD in rats [13]. Although the precise mechanism underlying 6-OHDA-induced area-specific neuronal damage occurs remains to be ascertained, studies making use of the 6-OHDA rat model have shown repeatedly that this involves mitochondrial functional impairment [14]. For instance, 6-OHDA has been shown to generate iron-dependent oxidative stress and deplete antioxidant (e.g., glutathione) levels, while it was also reported that 6-OHDA inhibits complexes I and IV of the mitochondrial electron transport chain (METC) [15,16]. Furthermore, the presence of 6-OHDA was seen to associate with increased activity of monoamine oxidase (MAO)-B, the enzyme bound to the outer mitochondrial membrane for catalyzing the oxidation of monoamines. It was shown that 6-OHDA is readily auto-oxidized and oxidatively deaminated by MAO, yielding hydrogen peroxide (H_2O_2), which, in turn, generates hydroxyl radicals (-OH), the most reactive of these being ROS [17,18], as well as producing corresponding *p*-quinone. 6-OHDA quinine triggers a cascade of oxidative reactions, resulting in the formation of an insoluble polymeric pigment that is related to neuromelanin [19].

In addition, using traditional protein detection assays, including immunoassays and western blot analyses, for analyzing the dopaminergic rat cell-line PC12, Gorman and colleagues provided evidence that exposure to 6-OHDA induced apoptosis through the mitochondrial release of cytochrome c and Smac/Diablo, caspase-3 activation, cleavage of PARP and nuclear condensation [20]. 6-OHDA was also seen to stimulate cell survival, by increasing heat-shock protein (Hsp) 25 levels, which protected cells against 6-OHDA-induced apoptosis and Hsp70. Taken together, these findings indicate that 6-OHDA's mechanism of dopaminergic toxicity may, at least in part, derive from its ability to induce oxidative stress [21].

Table 1. Pathogenic mutations in the Parkinson's disease-associated genes encoding for mitochondrial proteins and their effects on mitochondrial morphology and function.

Gene	Locus, chromosome location	Subcellular localization	Mitochondrial pathology	
			Knock-out animal models	*Human mutations*
PINK1	PARK6; 1p35	• The complete protein is transcribed in the nucleus, translated in the cytoplasm, and imported intact into the mitochondria • Subsequent processing and intramitochondrial sorting • May localize to the inner mitochondrial membrane • The C-terminus may be exposed to the intermembrance space [207]	**Flies** • Male sterility. Impaired sperm contained swelled nebenkern, a specialized mitochondrial derivative • Loss of the outer mitochondrial membrane • Increased apoptosis • Reduced levels of mtDNA and mitochondrial protein • Reduced ATP level • Expression of the antiapoptotic *drosophila* homolog of human Bcl-2 recovered mtDNA, ATP, mitochondrial protein levels and suppressed mitochondrial dysfunction • Muscle cell apoptosis • Expression of the *Parkin* transgene showed that Parkin acts downstream of PINK1 to maintain mitochondrial integrity and function [208] • Male sterility • Apoptotic muscle degeneration • Defects in mitochondrial morphology • Increased sensitivity to oxidative stress • Fragmented mitochondrial cristae [69]	• Reduced mitochondrial membrane poteintial [209]
Parkin	PARK2; 6q25	• Imported into the mitochondrion • Localized to mitochondria in dividing cells [186]	**Mice** • Decreased METC activity in striatum • Decreased antioxidant protein levels • Increased sensitivity to rotenone-induced apoptosis [210] **Flies** • Mitochondrial abnormalities • Apoptosis [68]	• Reduced Co-1 and Co-IV activities in peripheral blood [207]
DJ-1	PARK7; 1p36	• Present in mitochondrial intermembrane space and matrix [72] • May translocate to the outer mitochondrial membrane during oxidative stress [211]	**Mice** • Increased sensitivity to MPTP and oxidative stress [212]	

The inheritance mode for all genes is autosomal recessive.
METC: Mitochondrial electron transport chain; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces parkinsonism in mice by recapitulating the hallmark cellular pathology of human PD, including death of dopaminergic neurons in the substantia nigra, thereby decreasing dopaminergic input to the striatum [22,23]. These characteristics have made MPTP one of the most extensively used experimental tools used for studying aspects of PD in animals. Strong interest in a possible mitochondrial dysfunction that underpins PD pathology was generated when the primary molecular mechanism of toxicity for MPTP was discovered [24]. It is widely believed that the MPTP-induced selective dopaminergic neuron loss involves a series of enzyme-regulated steps involving MAO [25], particularly MAO-B [26] in glial cells, which converts MPTP to its toxic metabolite, MPP^+ (1-methyl-4-phenylpyridinium ion). MPP^+ is then selectively taken up into dopaminergic neurons via the DA re-uptake transporter. Here, it accumulates rapidly in mitochondria to inhibit the oxidation of NAD (nicotinamide adenine dinucleotide)-linked substrates [27]. Mitochondrial inhibition is brought about by MPP^+ blocking electron transfer at Complex I (Co-I) of the METC, within proximity of its quinone binding-site [28]. Additionally, MPTP shows the ability to induce energy failure, by inhibiting the

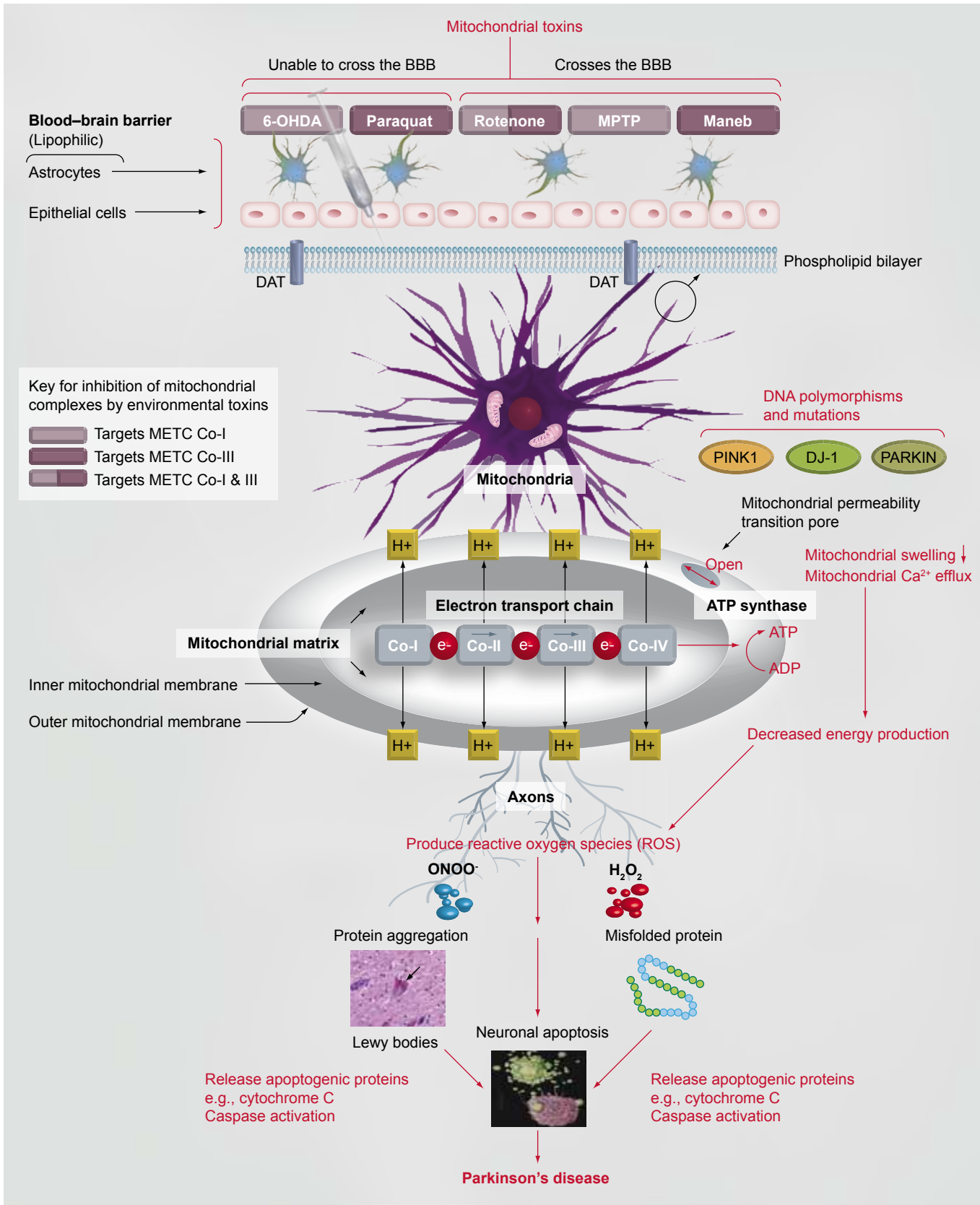

Figure 1. Representation of the mitochondrial electron transport chain. The mitochondrial electron transport chain serves as the main provider of cellular energy and is generated by the flow of electrons down the electron transport gradient. Arrows and text given in red indicate how mitochondrial toxins and mitochondrial-associated genes interfere with normal mitochondrial function, thereby leading to Parkinson's disease.

activity of ketoglutarate dehydrogenase (α-ketoglutarate dehydrogenase complex), an enzyme that is intimately involved in the tricarboxylic acid pathway, involved in ATP synthesis. This effect by MPTP was shown convincingly in both *in vitro* [29] and *in vivo* [30] animal models of PD.

Epidemiological surveys have also identified chronic exposure to the herbicide paraquat as a significant risk factor for developing PD [31]. Whereas paraquat is similar to MPTP in terms of structure and effect (both are capable of inhibiting the METC Co-I), the two toxins' mechanisms of action differ [32]. Paraquat induces toxicity to SN neurons by evoking a failure in antioxidant defences and by incurring oxidative damage to cytosolic proteins [33], as opposed to MPP^+, which induces a failure of the cell's bioenergetic systems, including the mitochondrial energy generating system [34].

Another pesticide, rotenone, was added to the experimenter's toolbag more recently, due to the observation that rotenone rat models of PD reproduce certain key pathological signs, including irreversible death of dopaminergic neurons, the formation of LB-like cytoplasmic inclusions, as well as motor impairments, all of which are reminiscent of human PD [35–37]. A mitochondrial-based mechanism has been proposed for explaining rotenone's neurotoxic effects. Specifically, it has been classified as a selective METC Co-I inhibitor, while rotenone administration also promotes the production of ROS [38].

Taken together, although their mode of action is still a matter of intense discussion, long-used neurotoxins that target dopaminergic neurons and recapitulate features of human PD in animals appear to share mechanisms of toxicity that place mitochondria and the Co-1 of the METC as a central subcellular component of neurodegeneration in PD.

Mitochondrial mutations as a molecular cause of human PD

Several groups have reported reduced activity in METC Co-I activity in both the striatum and SN of PD patients [39–42]. Interestingly, similar observations have been made in peripheral cells, such as in muscle cells and blood platelets taken from sporadic PD sufferers [43]. However, this issue remains controversial, with other groups that had failed to report Co-I inhibition in platelets isolated from either sporadic or genetically induced PD patients [44,45]. The same is true for METC deficiencies detected in the lymphocytes of PD patients [46,47]. However, some investigators have contradicted these results in their work [48]. The finding that SNCA is imported into the mitochondria, where it may impair METC Co-I activity in PD-affected neurons [49], highlights studying mitochondrial proteins as a point of convergence between hereditary and environmental influences for PD development.

The underlying involvement of mitochondria in PD pathology could stem from impaired genes, whose encoded proteins partake in oxidative phosphorylation (OXPHOS) for producing ATP. Hence, impaired OXPHOS machinery could lead to a lack of cellular energy. In particular, a deranged METC was identified as an important role player in the pathogenesis of PD [50,40]. Along the length of the METC, there are several sites where electrons may potentially 'leak'. For example, a defect at Co-I of the METC allows for 'leaked' electrons to combine with molecular oxygen to form ROS. Although the precise nature of the free radical species responsible for the cell death seen in PD remains enigmatic, accumulating evidence suggest that subspecies, such as superoxide (O^{2-}), hydroxyl radical (OH^-), peroxynitrate, nitric oxide and hydrogen peroxide (H_2O_2) may be significantly involved [51,52]. The accumulation of ROS severely damages macromolecules, by increasing the oxidisation of cellular proteins, rendering them more susceptible to aggregation or for undergoing proteolytic degradation [53].

Recent detection of specific gene mutations as causative for PD has further reinforced the relevance of oxidative stress and mitochondrial dysfunction in familial and sporadic PD. Although parkinsonism is not considered a hallmark of mitochondriopathies in general, a point mutation has been found in the mitochondrial 12S rRNA in a family pedigree that presented with clinical parkinsonism, deafness and neuropathy [54]. Other studies also found that the presence of certain mtDNA polymorphisms significantly increase the risk for developing PD [55,56], including a high PD susceptibility in patients containing a polymorphism of the human nuclear gene, *NDUFV2*, which encodes for a METC Co-I subunit [57]. Reports of increased mtDNA deletions observed in the SN dopaminergic neurons of PD patients, accompanied by lowered cytochrome oxidase activity compared with age-matched controls, served to further substantiate claims of an underlying mitochondrial pathology in PD [58].

Disruption of mitochondrial transcription factor A (*Tfam*), which regulates mtDNA transcription and copy number [59] reportedly produces a parkinsonian phenotype in mice. PD-like features include adult onset, slowly progressive loss of SNpc dopaminergic neurons, accompanied by the formation of intraneuronal inclusions that contained mitochondrial protein and membrane components, L-dopa responsive motor deficits, reduced mtDNA expression and METC defects in midbrain dopaminergic neurons [59].

Mutations in pol γ, encoded for by the nuclear-encoded gene, polymerase γ 1 (*POLG1*) was also recently shown to associate with a parkinsonian profile in humans, including a loss of SNpc dopaminergic neurons, a reduced DA uptake in the striatum and a good response to L-dopa and DA agonists [60–62]. The relation between POLG1 and mitochondria was demonstrated by *POLG1*'s role in regulating mtDNA synthesis, replication and repair [63], following its successful import into the mitochondrial inner membrane.

Point mutations in the genes encoding for the mitochondrial-related proteins PINK1, parkin, DJ-1 and Omi/HtrA2 associate with autosomal recessive and sporadic PD [64–66]. For example, phosphatase and tensin homolog-induced putative kinase (*PINK1*) encodes for a mitochondrial kinase (PARK6) that regulates mitochondrial dynamics and function. A working model was recently proposed by Chu to explain the role of *PINK1* loss-of-function in PD pathogenesis [67]. Here, disablement of

PINK1's functions is thought to result in the excessive production of ROS, cristae/respiratory dysfunction and destabilization of calcium homeostasis, which trigger compensatory fission, autophagy and biosynthetic repair pathways. All these pathological changes dramatically alter mitochondrial structure and may contribute to PD pathogenesis. Knock-out (KO) studies performed in mice and *Drosophila* also indicate a mitochondrial function for the ubiquitin ligase parkin (*PARK2)*, by inducing fragmented and apoptotic mitochondria, with compromised structural integrity [68]. The finding that *PINK1* loss-of-function mutant phenotype in *Drosophila* was reversed by overexpressing *Parkin* indicates that the two PD-related genes share a common biochemical pathway [69]. In addition, *Parkin* KO mice displayed decreased mitochondrial respiration, decreased metabolic drive and increased lipid and protein phosphorylation, indicative of functional mitochondrial impairment [70].

Wild-type (W/T) *PARK7* (DJ-1) seemingly protects against oxidative stress, with the protein localizing to mitochondria under conditions of excessive cellular stress [71]. DJ-1 was seen to disperse widely throughout the brains of mice and in human neuroblastoma cells, while the use of cell fractionation and immunogold electron microscopy revealed an endogenous pool of DJ-1 in the mitochondrial matrix and intermembrane space [72]. The same study also reported that although human neuroblastoma cells transfected with mutant (L166P) *DJ-1* reduced protein levels, the pathogenic mutations did not prevent the distribution of DJ-1 to mitochondria [72].

Several animal and human studies provided evidence that DNA deletions or point-specific mutations of mtDNA reduce capacitance for oxidative phosphorylation. This includes heteroplasmic mutations in *ND5*, a mitochondrial gene encoding for a METC Co-I subunit, which was identified in PD patients [73]. The nuclear-derived mitochondrial protein HtrA2/Omi ordinarily protects against mitochondrial cellular stress. However, disruption of its gene modulates proteolytic activity, leaving cells less resistant to the effects of mitochondrial stress [74,75]. Strauss *et al.* reported a heterozygous mutation (G399S) and a polymorphism (A141S) in *HtrA2/Omi*, detected in four patients who presented with typical, late-onset, sporadic PD [76]. Animal experiments confirmed these results with HtrA2/Omi KO mice revealing a striking parkinsonian phenotype with symptom onset at a young age, while the animals also died prematurely [75]. Other groups confirmed and extended these results further by revealing that HtrA2/Omi protease activity by the Ser276Cys mutation in mice associates with neurodegeneration, muscle wasting and motor dysfunctions and increased susceptibility for cells to undergo stress-induced apoptosis [74]. It was furthermore revealed that expression of the HtrA2/Omi mutation associates with reduced mitochondrial membrane potential and increased sensitivity to apoptosis [76]. However, the role of HtrA2/Omi mutations as a risk factor for developing PD has recently been challenged by results from a large-scale screen that detected the *HtrA2/Omi* gene variations in neurologically healthy patients also [77]. Another study that questioned whether *HtrA2/Omi* can be regarded as a true PD gene, found no pathogenic mutations in a large proband of autosomal-dominant PD patients [78]. This issue, therefore, remains to be explored further by future scientific studies.

Currently, the factors responsible for causing abnormalities in mtDNA are unclear, as is the question whether alterations in mtDNA or of mitochondrial proteins comprise a primary or secondary event in the pathogenesis of sporadic PD. Moreover, as with toxin-evoked mtDNA damage, several reports contradict claims of inheritable defects in mtDNA. For example, investigators reported that no evidence was found of homoplasmic mtDNA single-nucleotide point-mutations in exons of DNA isolated from white blood cells [79] or in post-mortem SN samples taken from PD patients [80]. It is hoped that modern technologies, such as proteomics, will serve to elucidate on such controversial reports.

Defective mitochondrial permeability transition pore as a contributing cause of PD

Numerous studies have highlighted a prominent role for the mitochondrial permeability transition pore (mPTP), which controls the permeability of the inner mitochondrial membrane. In PD, pathologically high levels of Ca^{2+}, which trigger apoptosis tho lead to neuronal death was seen, relating to defective opening and closing of the mPTP [81]. Ca^{2+}-induced mitochondrial dysfunction was first included as a possible mechanism by which PD-related cell death occurs, when it was observed that exposure to the mitochondrial toxins MPTP and 6-OHDA stimulated the release of Ca^{2+} from mitochondria and hydrolysis of intra-mitochondrial pyridine nucleotides [82]. MPTP exposure also associates with mitochondrial swelling, the release of cytochrome c [83], calcium efflux and mitochondrial membrane depolarization [84]. These effects were reversed by addition of the weak mPTP inhibitor, promethazine [85], which also prevented the loss of MPP^+-induced SN dopaminergic neurons [86]. Since MPP^+ exerts its toxic effects by inhibiting Co-I of the METC, this finding raises the possibility that Co-I may be intimately involved in modulating the open–close state of the mPTP [87].

Since the mitochondrial channel in the opened state renders mitochondria incapable of producing ATP, it was postulated that excessive opening of the mPTP severely damages mitochondrial ultrastucture. This has received support from *in vitro* cell models, which showed that mPTP opening results in mitochondrial swelling, an obligatory decrease in the membrane potential [88] and the eventual loss of matrix solutes, such as NAD [89]. These events, in turn, impair mitochondrial respiration and stimulate ROS production [90]. Although the voltage-dependent anion channel (VDAC)2 was shown to facilitate opening of the mPTP, its main function is thought to be activity regulation of the pro-apoptotic molecule Bak [91]. This suggests that VDAC2 may be a major role-player in mitochondrial-mediated apoptosis [92].

However, claims that the mPTP fulfills a causative role in PD remain poorly substantiated on [93]. For example, studies reporting an increased generation of ROS in PD-related mPTP

dysfunction has been criticized for only making use of exogenously applied oxidants, while the role of endogenous ROS in mPTP activation remains unknown [94]. However, reports that faulty mPTP opening stimulates ROS production in isolated mitochondria [95], with similar findings reported in intact cells [96], suggests that endogenous ROS could potentially be important in the neurodegenerative changes associated with chronic opening of the mPTP.

Possible candidates for the protein that constitutes the mPTP include the voltage-dependent anion channel, adenine nucleotide transporter and the mitochondrial phosphate translocator protein [97]. Novel proteome platforms, such as high-throughput proteomics promise to deliver information on these and related aspects. This may lead to answers with regards to what protein components regulate mPTP dynamics in health and disease, to help establish the role of the mPTP mechanism in PD's etiology. Additionally, such applications could result in developing therapeutic compounds targeting this faulty molecular mechanism.

Contributions made by proteomics to elucidate the role of mitochondria in PD pathology

Emerging technologies, such as proteomics, are capable of generating data of high throughput, which may serve as a viable platform for investigating molecular aspects of PD. Moreover, proteomic tools allow for the global analysis of structural and functional changes at the proteome level, without the restrictions imposed by more traditional methods, such as antibody-based targeting of suspected candidate proteins [98]. It has been estimated that at least a thousand different polypeptides reside in mitochondria [99]. Mitochondrial prefractionation using standard ultracentrifugation facilitates identification of mitochondrial-specific low-abundant proteins as well as hydrophobic proteins using proteomics techniques [100]. This is an essential experimental step because the dynamic range of protein abundance within cells is estimated to be as high as 10^7 [101]. See Figure 2 for an overview of the mitochondrion-based proteomic approaches.

Neuroproteomic techniques used on isolated brain mitochondria have revealed and confirmed deficits in energy production, protein degradation, antioxidant protein function and cytoskeletal regulation associated with neurodegenerative diseases, such as AD and PD. Results from studies that have applied sensitive proteomic techniques for detecting and characterizing mitochondrial markers in the CNS of PD post-mortem specimens or in animal models of PD are discussed below.

Proteomic analysis of human tissue samples

Several proteomics-based studies have reported intriguing results, which could potentially enhance understanding of the mechanisms underlying the destruction of dopaminergic destruction seen in PD and treatment strategies aimed at combating relentless cell death.

Basso and colleagues analyzed human SN using standard 2-DE protein separation techniques and subsequently identified using mass fingerprinting [102]. The results were compared with those seen in healthy, age-matched controls. Of the 44 proteins identified by peptide mass fingerprinting, nine expressed differentially in PD patients. Peroxiredoxin II, mitochondrial Co-III, ATP synthase D chain, complexin I, profilin, L-type calcium channel δ subunit and fatty-acid binding protein were overrepresented in PD samples. The abundant presence of mitochondrial and ROS-scavenging proteins in PD brain samples consolidates with the view that oxidative stress is involved in PD pathogenesis [102]. Also of interest were L and M neurofilament chains, which expressed less abundantly in PD specimens than in control brains. The presence of accumulated neurofilament aggregates in LBs have previously been identified in PD

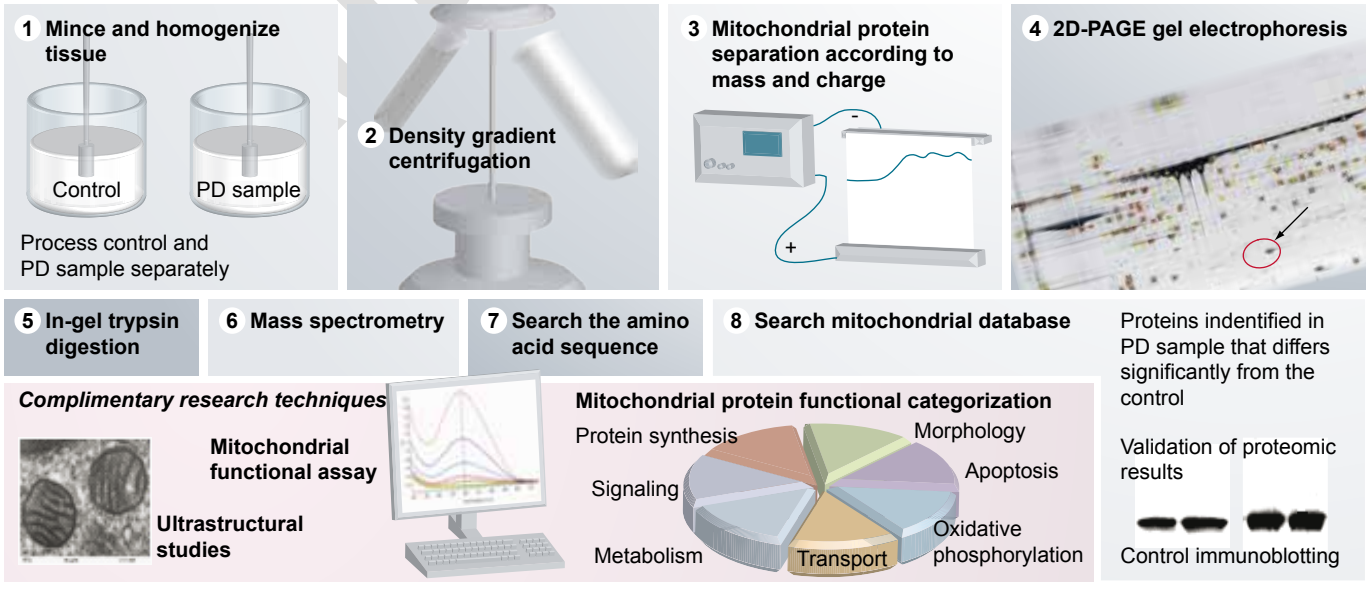

Figure 2. Overview of the experimental steps required for applying proteomics to isolated mitochondria. Complimentary methodologies are also shown that could reciprocally inform on the role of mitochondrial proteins in Parkinson's disease pathogenesis.

patients [103]. In addition, others have shown that oxidative stress can alter the structure and stability of neurofilament proteins, possibly by evoking proteolysis and/or structural changes in neurofilaments [104]. Although the relevance of this finding in the context of impaired mitochondrial function in PD remains unclear, it is hoped that further proteomics-based investigations, combined with neurotoxin and/or genetic models of PD will elucidate these aspects.

Using 2-DE-PAGE proteomics, Werner and colleagues analyzed PD-affected SNs compared with healthy age-matched control brains [105]. In total, 221 differentially regulated proteins were detected in the PD-affected brains. Of the 37 proteins identified, 16 were found differentially expressed. These included elements of iron metabolism (H-ferritin) and glutathione-related redox metabolism (GST M3, GST P1, GST O1), including novel redox proteins (SH3BGRL). Many glial or related proteins were also found to be differentially regulated in PD (GFAP, GMFB, galectin-1, sorcin), as well as proteins belonging to metabolic pathways, such as adenosyl homocysteinase (methylation), aldehyde dehydrogenase 1 and cellular retinol-binding protein 1 (aldehyde metabolism). Other differentially regulated proteins included annexin V, β-tubulin cofactor A, co-actosin-like protein and V-type ATPase subunit 1. Proteins that were similarly expressed in healthy and diseased SN tissue included the housekeeping proteins COX5A, Rho GDI α, actin γ1, creatin-kinase B, lactate dehydrogenase (Ldh2), disulfide isomerase ER-60, Rab GDI β, methyl glyoxalase 1 (AGE metabolism) and glutamine synthetase. A prominent finding to emerge from this work is that proteins involved in familial PD (i.e., DJ-1 and UCH-L1) were not found differentially regulated in idiopathic PD compared with controls.

Thus far, few studies have combined highly purified mitochondrial fractions, taken from PD patients, with a proteomics methodology. Reports of mitochondrial pathologies detected in the brains, muscles and platelets of PD patients [43] call for the application of more specific subproteomic approaches to elucidate on the extent of mitochondrial changes seen in human PD.

Mutations in the ubiquitin-protein ligase (E3) *Parkin* cause juvenile-onset, autosomal recessive PD, with resultantly reduced enzymic activity. By contrast, overexpressed W/T *Parkin* seemingly protects against mitochondrial dysfunction and neurodegeneration [106]. A study by Davison and colleagues [107] aimed to elucidate the mechanisms by which increased parkin levels may induce such an anti-apoptotic effect, and therefore, how decreased parkin content may increase cells' vulnerability to mitochondrial dysfunction, eventually leading to cell death. *Parkin* was overexpressed in human embryonic kidney 293 cells (HEK293) and the effects on protein expression investigated using 2-DE and mass spectrometry (MS) proteomic techniques applied to the whole cell HEK293 lysates. A total of nine proteins were found to be differentially expressed at a statistically significant level. MS identified these proteins as ACAT2, HNRNPK (confirmed by western blot analysis), HSPD1, PGK1, PRDX6, VCL, VIM, which all revealed reduced expression, while TPI1 and IMPDH2 were upregulated. In addition, using tandem affinity purification and MS, 14 potential parkin interactors were identified, including CKB, DBT, HSPD1, HSPA9, LRPPRC, NDUFS2, PRDX6, SLC25A5, TPI1, UCHL1, UQCRC1, VCL, YWHAZ and YWHAE. The involvement of the majority of these in mitochondrial energy metabolism and glycolysis has previously been described [108]. Overall, this study provides further evidence for a role for parkin in regulating mitochondrial activity within cells.

Mitochondrial proteomic studies on toxin-based animal models of PD

As discussed, several chemicals that induce aspects of human PD in animals predominantly act by impairing mitochondrial function in cells. However, the lack of coherence between results from different animal models of PD that make use of mitochondrial targeting toxins and PD patient post-mortem sample analyses has led to increasing acknowledgement that Co-I inhibition as a sufficient mechanism for inducing the selective dopaminergic cell death seen in PD, offers an oversimplified view of PD. In this regard, proteomic technologies applied to the study of mitochondrial alterations in PD is increasingly regarded as a useful research tool by which to expand on the insights gained so far on the mitochondrial contribution towards PD pathology.

Although the mitochondrial toxins MPTP and methamphetamine (METH) both induce parkinsonism in mice, MPTP results in the demise of neurons projecting to the striatum, whereas METH inhibits cell function and DA production without resulting in dopaminergic neuron death [109]. This difference in effect is believed to stem from a different mechanism of action between the two neurotoxins. The combination of proteomics (high-density liquid chromatography [LC] coupled to MS) with microarray analysis of the pooled left and right striata from mice previously exposed to MPTP compared with METH exposure culminated in a large descriptive data set on proteomic changes induced by these mitochondrial poisons [109]. Several of the proteins identified (n = 912) imply mitochondrial dysfunction and a cellular response to excessive oxidative stress, misregulated protein degradation, increased apoptosis and eventual cell death. These results are consistent with previous findings that implicate similar factors in the manifestation of parkinsonism in both the MPTP [110] and METH [111] mouse models of PD.

In the study reported by Chin and colleagues, the downregulation of several anti-apoptotic and upregulation of pro-apoptotic genes and proteins were detected in both models [109]. For instance, the classic glycolytic protein glyceraldehyde-3-phosphate dehydrogenase, previously identified as a mediator of one or more apoptotic cascades and as a role-player in promoting LB formation [112], was found upregulated in both PD models. The anti-apoptotic mitochondrial heat-shock protein 10 kDa (Hspel) was also seen to be significantly reduced in terms of levels of abundance in both animal models, suggesting increased susceptibility for neuronal cells to undergo cell death.

However, the study also revealed striking differences in the protein expression patterns between the two models. This includes the neuronal pro-apoptotic heme protein cytochrome c, which was seen upregulated in MPTP-treated mice, but not in METH-treated ones. This protein was previously shown to increase in response to Co-I deficiency [113]. Since this result could not be replicated in METH-treated animals, it was suggested that MPTP generates a potentially higher degree of oxidative damage and cell death than that of METH. The study also reported a sixfold decrease in abundance for the protein product of *aldehyde dehydrogenase* (*Aldh1a1*) that expresses specifically in dopaminergic neurons in MPTP-mice [114], while it was only decreased to a threefold level in METH-treated animals. Also of importance was the finding that glutathione peroxidase 4 (Gpx4) expressed more abundantly in METH-treated mice (at both the transcriptome and proteome level) than in the MPTP model. This antioxidant enzyme reportedly protects against oxidative stress-induced apoptosis [115].

This multimodal analysis allowed for discovering induced changes brought on by two neurotoxins, the active metabolite of MPTP, MPP^+ as well as METH. Moreover, the experimental approach allowed for identifying the pathways activated specifically by these toxins. For instance, a more pronounced decrease in protein levels was observed in the MPTP mice than in the METH model [109]. The majority of the proteins identified in this study functionally associate with apoptosis, which is likely to be due to mitochondrial dysfunction and oxidative damage. However, the study failed to pinpoint the exact isoform(s) contributing to the observed changes, while a low correlation rate between the proteomic and microarray data was reported, with only Gpx4 and Gfap that showed overlapping results. This may be due to translational and post-translational regulations, the difference between the two technology's abilities to accurately quantify and detect genes present in low abundance, or because of the neurotoxins' different mechanism(s) of action. Future proteomic technology should include modifications to its aminomethyltransferase tag database in order to overcome this problem [116].

Overexpressed L1cam has been shown to improve learning and memory, emphasizing the role of the molecule in synaptic plasticity [117]. By contrast, in transgenic mice, impaired L1cam expression was shown to impair long-term potentiation, resulting in reduced learning and memory abilities [118]. Moreover, *in vitro* experiments have demonstrated L1cam's capacity for promoting neuronal survival and dopaminergic neurogenesis [119]. To elucidate the role of L1cam in PD pathogenesis, a proteomic profile was compiled of the acute phase of MPTP toxicity (1 day after MPTP injection) in transgenic C57BL/6 L1cam mouse midbrain samples, compared with W/T controls [120]. The results showed that, acutely, proteins inherent to the METC are downregulated in MPTP-treated W/T mice. Following a period of recovery (up to 7 days post-MPTP), the same proteins reverted to normal levels. In L1cam transgenic mice, however, a greater number of proteins were altered during the acute phase, with this number increasing post-recovery. Taken together, the results from this study show that L1cam induces lasting impairment in energy systems, including the mitochondrial OXPHOS system in a parkinsonian mouse model.

Using the unihemispheric 6-OHDA rat model of PD, De Iuliis and colleagues combined 2-DE-PAGE with matrix-assisted laser desorption/ionizing time-of-flight (MALDI-TOF) MS for separating protein mixtures, visualizing protein patterns and analyzing differentially expressed protein spots [121]. An upregulation in several proteins' relative level of abundance was observed in the SN. This included α-enolase (ENO1), which was previously shown to undergo increased oxidation in PD and AD-affected brains [122]. The results from the proteomics study by De Iuliis and coworkers suggest that glycolytic changes occur as a compensatory effect in reaction to 6-OHDA-induced oxidative stress seen in rats, which, in rats, replicates the defective METC mechanism proposed to underlie human PD [121].

Proteomics have also allowed for elucidating on the therapeutic mechanisms underlying currently used anti-PD drugs or those being developed. Such drugs include *N*-propargyl-1R-aminoindan (rasagiline) and L-deprenyl (selegiline), which act as potent inhibitors of MAO-B [123], the major enzymatic step in the brain for converting DA to its inactive catabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid (HVA). Inhibition of MAO-B retards the otherwise rapid turnover of striatal DA. This results in DA accumulating in the brain, and enhanced striatal DA activity, providing the principal rationale for the symptomatic motor benefits seen in PD patients prescribed the drug [124]. Recent work demonstrates that rasagiline protects against MPTP [125] and 6-OHDA-induced neurotoxicity [126], possibly by preventing neurotoxin-induced opening of the mPTP. Consistent with this, a recently published, delayed-start clinical trial that included 1176 patients suffering from untreated PD, showed that rasagiline at a daily dosage of 1 mg taken early in the course of the disease, slowed worsening of PD symptoms more so than when the drug was taken at a later stage of disease [127].

The observation that the neuroprotective benefits of rasagiline are only observed at concentrations below the MAO inhibition threshold in preclinical animal models, while the same was reported in cellular systems not containing MAO-B [128,129], suggests that the neuroprotection provided by the drug is not entirely attributable to MAO-B inhibition and that multiple mechanisms, in particular mitochondria-based, may be involved. This includes rasagiline-induced increased expression of the anti-apoptotic proteins Bcl-2 and BclxL and downregulating the expression of Bax and Bad, the gateway proteins to the mitochondrial pathway of apoptosis [130,131]. The drug was furthermore proposed to suppress oxidative stress in dopaminergic neurons by increasing expression of the anti-oxidant enzymes SOD and catalase [128,132]. Also, pre-administration of the drug was shown to protect against MPTP- [125] and 6-OHDA-induced [126] opening of the mPTP, rupture of the mitochondrial outer membrane and a decline in mitochondrial membrane potential. In this regard, rasagiline was seen to bind directly to the mPTP, thereby stabilizing mitochondrial membrane potential [129].

Another MAO-B inhibitor, selegiline, differs from rasagiline molecularly and pharmacologically. Selegiline undergoes extensive hepatic first-pass metabolism to L-METH and L-amphetamine [133]. It's been suggested that these metabolites may neutralize the neuroprotection offered by the drug [134]. For instance, in rats, exposure to amphetamine and METH may cause destruction of striatal dopaminergic nerve fibers [135] and of depleted striatal DA content in human METH users [136]. However, large-scale trials of selegiline-use by PD patients failed to confirm these adverse effects [137]. Despite these controversial if not contradictory claims, preclinical experimental evidence of selegiline's neuroprotective properties [134,138] and data showing that rasagiline holds anti-oxidant and anti-apoptotic properties, which potentially translates into long-term neuroprotective benefits [26,125,134,138,139], argues in support of current use of these drugs as either monotherapy or taken as adjunctive with L-dopa for treating PD patients [139,140].

The midbrains of mice pretreated with rasagiline and selegiline, followed by MPTP infusion, were dissected for proteomic and genomic analyses [141]. Results demonstrated that both drugs could induce significant molecular changes at the protein and transcriptional level. The changes associated with neuronal differentiation, cell survival and death pathways, metabolism/oxidation stress, signaling system and biomarkers of neurodegenerative disorders. It was previously demonstrated that rasagiline and selegiline provide neuroprotection by activating the PKC-MAP kinase pathways [142,143], while upregulating levels of growth-derived nerve factor, brain-derived nerve factor and NGF [144], which promotes neuronal survival in animal models of PD [145]. This finding holds potential clinical significance, since reduced brain-derived nerve factor expression has also been demonstrated in the SN of PD patients [146]. Upregulation of neurotrophin levels is seemingly mediated by Ras and downregulated by Fas and Fas ligands, which interact with Bad and Bax on the outer mitochondrial membrane [147]. It is anticipated that future proteomic analyses will help clarify the inter-relationship between rasagiline-induced brain-derived nerve factor, growth-derived nerve factor and other trophic substances. In addition, such studies are likely to add to the growing body of knowledge concerning the interaction this drug is likely to have with the PKC signaling pathway and Bcl-2-related protein family.

Although various animal models of PD are capable of generating nigrostriatal neuronal loss, only the rotenone [37] and MPTP models have so far been reported to produce LB-like cytoplasmic inclusions, although this has not consistently been reported [148]. Jin and colleagues employed a 'shotgun' proteomic strategy combined with isotope-coded affinity tag (ICAT) for studying the processes potentially involved in nigral neurodegeneration and LB formation in PD [149]. In this study, analysis was performed on mitochondrial-enriched fractions taken from the SN of mice treated chronically (5 weeks) with MPTP and the adjuvant, probenecid (prob), a uricosuric agent that potentiates MPTP's effects by inhibiting the rapid clearance and excretion of MPTP from the brain and kidney [150]. Of the more than 300 proteins identified, the majority being mitochondrial, over 100 displayed significant differences in relative abundance in the parkinsonian mice compared with vehicle-injected controls. This included DJ-1, the presence of which was validated by using western-blot analysis. When the gene encoding for this protein (*PARK7*) is mutated, the resultant mutation may be instrumental in familial PD. Co-localization analysis further revealed that DJ-1 not only co-localized with SNCA in dopaminergic neurons but also to cytoplasmic inclusions in the mouse model of PD. Ultrastructural analysis by the same authors further revealed that DJ-1 localizes to the halo, but was absent inside the core of these LB-like inclusions, in the brains of these mice [149]. Although investigators have shown that the protein product from the *PINK1* gene mutation, which associates with PARK6-linked PD localizes to LBs in human PD brains [151], a lack of post-mortem material makes it currently unknown whether patients with DJ-1 mutations and who display parkinsonian symptoms, form LBs or Lewy neurites. The study by Jin and co-workers informs on this by suggesting that DJ-1 might play a significant part in mitochondrial dysfunction and in promoting LB formation in PD [149]. A recent report of the successful generation of a mouse that contains the functional mutation of *DJ-1* provides the opportunity to further explicate the relationship of DJ-1 to SNCA and its possible role in LB formation [152].

Dopamine's oxidation to its reactive metabolites, ROS and DA quinone (DAQ), may also contribute to the oxidative stress and mitochondrial dysfunction seen in PD [153,154]. As an unbiased approach to identify mitochondrial proteins susceptible to DA oxidation, isolated rat brain mitochondria exposed to reactive DAQ were exposed to 2-DE-DIGE combined with cysteine- and lysine-reactive fluorescent dyes [155]. Detection of the loss of specific mitochondrial proteins, encompassing a range of mitochondrial functions, including structural maintenance, transport and metabolism, was reported. In particular, the relative fluorescent intensity of mitochondrial creatine kinase was found to be significantly decreased in this study. This protein generates phosphocreatine that is critical for maintaining homeostatic levels of ATP in cells [156]. As an octameric protein, it also plays a critical role in mitochondrial morphology by forming and stabilizing the contact sites that exist between the inner and outer mitochondrial membrane [157,158]. Previous studies have shown that oxidative stress may dissociate mitochondrial creatine kinase's octameric structure into dimers [159,160], which potentially disrupts the inter-membrane contact sites and, in turn, potentiates opening of the mPTP [161].

The study also detected decreased abundancy of mortalin, a mitochondrial inner membrane-associated chaperone protein [155] important for importing and folding mitochondrial proteins [162], following DAQ exposure. Mitofilin also showed decreased fluorescence labeling in animals exposed to DAQ, compared with non-exposed control animals. Although the specific role of mitofilin is currently undefined, a recent study suggests it may be critical for maintaining mitochondrial cristae structure [163]. It may also fulfill a supplementary role in importing

mitochondrial proteins through interaction with the mitochondrial proteins Sam50 and metaxins 1 and 2 [164]. Significant losses of mitochondrial creatine kinase, the 75-kDa subunit of NADH dehydrogenase and SOD2 were also reported here. Overall, these results add to compelling evidence that DA oxidation impairs the function of specific mitochondrial proteins.

Jin and coworkers compared nigral mitochondrial proteins of PD patients with age-matched controls and subsequently validated the results using a cell model of PD, consisting of dopaminergic (MES) cells treated with rotenone versus vehicle-treated controls [165]. The investigators combined stable isotope labeling by amino acids in cell culture (SILAC) with multidimensional protein identification technology (MudPIT), a multidimensional LC and tandem MS (MS/MS), for separating and fragmenting peptides to allow for protein identification [166,167].

For quantifying and comparing relative protein abundance in the SNpc of healthy control patients with PD patient samples, this proteomic technique was combined with nonbiased quantitative ICAT labeling. ICAT is one of the most employed chemical isotope-labeling methods for quantifying relative protein abundance in a complex mixture consisting of two differentially labeled protein samples [168]. The technique overcomes many of the shortcomings inherent to 2-DE, including offering improved detection of insoluble and low-abundant proteins [169]. A potential limitation pertaining to ICAT is that certain mitochondrial proteins, in particular those lacking cysteine residues (e.g., cytochrome oxidase polypeptide Vic) may be transparent to ICAT detection. However, a method has been proposed for overcoming this, namely covalent labeling of cysteine residues of the two related protein isolates with isotopically normal and heavy versions of the same reagent, respectively [170].

Here, a total of 119 proteins showed altered levels of abundance between the rotenone-exposed and the control group, including the mitochondrial stress protein mortalin (mthsp70/GRP75) [165]. In subsequent experiments, the investigators induced overexpression versus silencing of mortalin in dopaminergic cells in the presence of rotenone. Rotenone significantly influenced cell viability, suggesting that mortalin acts as a major mediator of oxidation-induced neurotoxicity. However, although rotenone exposure reduced mortalin levels in isolated mitochondria, silencing mortalin produced only an offset to rotenone-mediated toxicity [165]. This result implies that a delicate balance of mortalin and its associated proteins may determine the sensitivity of dopaminergic cells to rotenone, and not necessarily the absolute level of mortalin present in tissue. The investigators identified a total of nine candidate mortalin-binding partners as potential mediators in PD pathology. Interestingly, hsp60 was left unaffected by rotenone in the cellular model, although it's relative abundance was significantly increased in human PD samples compared with patient controls [165].

Purification of mitochondria prior to proteomic analysis, as opposed to preparing mitochondria-enriched fractions, as used in this study, is expected to produce a lower yield of confounding elements. Despite this potential limitation, the study yielded several important finds, which may guide future investigations. This includes the finding that a number of mitochondrial subunit complexes, including those inherent to Co-I, were found to be substantially decreased in the PD patients. Since these enzymatic complexes provide protein substrates for mitochondrial dysfunction during PD pathogenesis, these observations both confirm previous findings and contribute to growing knowledge concerning the role of mitochondrial deficits in PD pathogenesis. Moreover, this study supports other work that mortalin is capable of influencing PD pathogenesis, while also demonstrating that mortalin's effects are achieved via its mitochondrial and proteasomal functions, as well as its ability to provoke oxidative stress [165]. Planned follow-up work by the authors to explore whether post-translational modification of mortalin exists in the rotenone PD model is expected to yield important insights for better understanding PD pathogenesis.

Mitochondrial proteomic studies of genetic animal models of PD

Several elegantly designed studies have applied a proteomics protocol to genetically modified model organisms that mimic aspects also seen in human PD to reveal insights into the contribution made by several mitochondrial proteins in the pathogenesis of PD. Some of the most important ones in the context of PD are described below.

The discovery that missense mutations (A30P, A53T and E46K) in *SNCA* associate with PD and that the SNCA protein is a major component of LBs spurred attempts at creating *SNCA* transgenic *Drosophila*, by which to study aspects of the human disease. Although *Drosophila* lacks endogenous *SNCA* and also does not possess the *SNCA* ortholog [171], Feany and Bender demonstrated that flies expressing both W/T or mutated (A30P/A53T) human *SNCA* display symptoms resembling those seen in human PD patients [172]. Features seen in these PD-like flies include gradually progressive degeneration of dopaminergic neurons, the formation of LB-like inclusions, and impaired climbing ability.

In a series of investigations led by Xun and colleagues, the proteome of the *SNCA* transgenic *Drosophila* model of PD was analyzed in an effort to address issues relating to the underlying molecular mechanisms of SNCA-mediated neurotoxicity in PD [173,174]. In the first of these studies to be discussed, LC coupled to MS and database analysis techniques were employed for analyzing the proteome of an A30P *SNCA Drosophila* model of PD. The proteome analysis was performed at three different disease stages (presymptomatic, early and advanced) and the results compared with changes detected in the gene expression profiles of the same animals [173]. Approximately 44% of transcriptional changes compared favorably with those seen at the proteome level, although the pattern of change in protein expression varied substantially compared with that seen in the corresponding transcripts. A total of 19 genes expressed differentially, but only at the proteome and not at the transcriptome level. Eight of these 19 proteins were mitochondrial-associated, the perturbance that were detected as early as day 1 to represent

the presymptomic stage of the human disease [173]. The identified mitochondrial proteins are encoded for by CG3011, CG4685, CG6439, CG3731, CG6543, ATP synthase-γ chain, CG11015 and ATP synthase-β, while proteins related to the mitochondrial OXPHOS system, namely ATP synthase-γ chain, CG11015 and ATP synthase-β were upregulated.

Drosophila expressing the A53T *SNCA* point mutation develop similar human PD-like symptoms as *Drosophila* expressing A30P *SNCA* [172]. In other work performed by the same group, the proteome changes in a A30P *SNCA Drosophila* model of PD were compared with age-matched controls at seven different ages across the adult lifespan [174]. Data were captured using a shotgun proteomic approach, involving multidimensional LC coupled to MS. Moreover, proteins were labeled using an isotopic-labeling strategy that incorporates global internal standard technology, as previously described by Chakraborty and Regnier [175]. The study detected a total of 24 proteins that expressed differentially between the A53T transgenics and W/T flies. Subsequent gene ontology analysis indicated that the dysregulated proteins primarily associate with cellular membranes, endoplasmic reticulum, actin cytoskeleton, mitochondria and ribosomes. Changes in the mitochondrial proteome were most dominant in the youngest (presymptomatic and early disease stage) flies. This indicates that future research should focus particularly on understanding molecular changes that occur at the presymptomatic stage, in efforts to address what factors cause the disease. It is anticipated that this may provide valuable insights to help develop diagnostic tools and find ways by which to intervene in the disease progression of PD.

For understanding how mutations in *SNCA* contribute to the pathophysiology of PD, proteomic analysis was conducted on parkinsonian transgenic mice that overexpress human mutant A30P *SNCA* [176]. In this regard, the A30P mutation was previously shown to accelerate SNCA aggregation [177–180]. In turn, this step could increase mitochondrial vulnerability to oxidative insult [181]. The aim of this study was to increase understanding concerning what proteins associate with impaired energy metabolism and determine whether mitochondria are particularly vulnerable to oxidative stress in mice expressing mutant A30P *SNCA*.

Results from the proteomic analysis, generated by 2DE followed by MS were subsequently compared with the brain proteins from W/T mice. All the brain proteins that displayed oxidatively modified changes in the brains of the transgenic mice associated with mitochondria. This provides strong evidence for the notion that mitochondrial dysfunction contributes to PD pathology, while the study also implicates mitochondrial pathology in aggregated SNCA toxicity. Subsequent functional analysis of the identified proteins revealed impaired energy metabolism in the brains of these A30P transgenics compared with controls, indicating that A30P-mutant *SNCA*-associated proteins are particularly vulnerable to oxidative stress. The authors affirmed that oxidative stress impaired energy metabolism and found that mitochondrial dysfunction seen in these animals may be brought on by oxidative inactivation of the metabolic enzymes α-enolase, Ldh2 and Car2.

Carbonyl levels of the glycolytic enzyme enolase, which associates with the intermembrane space/outer mitochondrial membrane fraction [182], to place it in a position to contribute to mitochondrial function, was significantly increased. Its enzyme activity was decreased in the brains of the transgenic mice compared with W/T controls. This result suggests that oxidative inactivation may alter normal glycolysis and mitochondrial function in the brain, as well as contribute to altered energy metabolism in PD.

The Zn^{2+} metallo-enzyme Car2, which reversibly catalyzes hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) was found significantly oxidized in the brains of A30P *SNCA* mutant mice. Car2 shares high (68%) amino acid sequence similarity to its mitochondrial counterpart carbonic anhydrase 5a (Car5a) and 5b (Car5b). It was proposed that they interact to maintain metabolic processes, cellular transport, gluconeogenesis and mitochondrial metabolism [183]. In the study by Poon and colleagues, mentioned previously, Car2 activity was also decreased significantly in the brains of A30P *SNCA* transgenic mice compared with W/T controls [176]. The authors suggested that the inactivation for Car2 may underlie the resulting aggregation of synuclein and subsequent neurodegeneration seen in A30P-mediated parkinsonism. Another oxidatively modified protein was the glycolytic protein Ldh2 for catalyzing the reversible NAD-dependent interconversion of pyruvate to lactate. The study showed that Ldh2 was significantly modified and inactivated by oxidative insults in A30P *SNCA* transgenic mice brains. An assay to determine the enzymatic activity of Ldh2 also showed Ldh2 inactivation due to oxidative insult in the brains of A30P *SNCA* transgenic mice, suggesting that oxidative inactivation of Ldh may contribute to mitochondrial dysfunction seen in PD. The insights gained from this work could aid understanding of what mechanisms underlie the loss of antioxidant capacity by overexpressed A30P mutant *SNCA* and how this may contribute to cell death.

Parkin mainly serves as a ubiquitin ligase that is essential for the ubiquitin-proteasomal system. Subcellular fractionation has demonstrated parkin's association with the outer mitochondrial membrane [184]. Evidence was also given that parkin localizes to mitochondria in dividing SH-SY5Y neuroblastoma cells [185]. Moreover, mitochondrial export of parkin has been described, with parkin translocating to the golgi apparatus and the nucleus in dividing cells, while a similar phenomenon was described when cells were treated with METC inhibitors, such as rotenone, METC uncouplers as well as cell cycle couplers [186]. Data suggesting that parkin is involved in mitochondrial transcription and replication also support a potential role for parkin in mitochondria [186].

Mutations in the *Parkin* gene (PARK2) associate with an early-onset form of autosomal recessive parkinsonism [187]. Early clinical studies have gained support from genetic animal models, such as *Drosophila* and mice lacking the gene encoding for parkin in which mitochondrial defects have been described [68,70]. *Parkin* deficient mice resemble the biochemical and behavioral changes observed in presymptomatic PD

patients [188], although intriguingly, neuronal degeneration is absent in these mice. The same is true for *Drosophila parkin*-null mutants, which show no apparent dopaminergic cell loss, but do present with a neurodegenerative phenotype, including shrinkage of the dorsomedial dopaminergic cell body, impaired flight and climbing ability, reduced longevity and male sterility [68]. Morphological and physiological mitochondrial pathology detected in such *Parkin* knock-out animals include swollen mitochondria, severely fragmented cristae, decreased abundance of several protein subunits of METC Co-1 (NADH dehydrogenase) and IV, as well as decrements in mitochondrial respiratory capacity [189]. Further support for a correlation between mitochondrial defect and PD was shown, such as locomotor defects and male sterility in *Parkin* KO flies due to a mitochondrial dysfunction, which manifests already during early development [68].

Using 2-DE gel electrophoresis followed by MS, Palacino and colleagues aimed to determine whether mice engineered to express a *Parkin* loss-of-function mutation, which serves as a prevalent cause of familial PD, resulted in altered levels of abundance and/or modified protein levels in the ventral mesencephalon [70]. Although electron microscopic analysis revealed no gross morphological changes in striatal mitochondria harvested from *Parkin* KO mice, functional analysis of the MS-identified proteins complimented the proteomic findings. Proteomic analysis revealed decreased abundance of various proteins involved in mitochondrial function or oxidative stress, including consistent reductions in subunits of METC Co-I and IV. These findings were consistent with those derived from functional analysis, which detected reduced respiratory capacity of the striatal mitochondria in these animals. Accompanying these deficits, the animals also exhibited delayed weight-gain, suggesting for an underlying metabolic abnormality. Moreover, the animals revealed decreased serum antioxidant capacity and increased protein and lipid peroxidation, in line with proteomics results showing that the levels of proteins that fulfill a protective role against oxidative stress were significantly decreased in *Parkin* KO mice. Therefore, by making use of a recombinant approach, which utilized genetic, physiological and proteomic analysis techniques, evidence was provided that mitochondrial dysfunction and oxidative damage are major mechanisms underlying the deficits seen in the *Parkin*-KO mouse model of PD.

Periquet and colleagues conducted a study, for which the objective was to gather clues as to the pathogenic mechanisms underlying the preclinical stages of parkin-related parkinsonism, as well as the compensatory mechanisms that might be functioning at the proteome level [188]. Application of MS identified 87 proteins that differed in relative abundance between W/T and *Parkin* KO mice (45%). Functional classification of the differentially regulated proteins revealed that a large proportion of these are involved in energy metabolism, including being prominent role-players in the METC. Several are also known for the part they play in detoxification, their role as stress-response-related chaperones and as components of the ubiquitin-proteasomal system. The authors of this study proposed that the changes in protein abundance, in the absence of a neurodegenerative phenotype might reflect the activity of adaptive mechanisms that serve to regulate cellular energy, necessary due to the induced parkin deficiency. Evidence for this was seen in the altered abundance of enzymes involved in glycolysis and changes in proteins involved in energy regeneration, such as subunits of the mitochondrial ATP synthase [188].

Multidimensional LC, including offline strong cation exchange chromatography and reversed-phase LC was coupled with MS/MS and database searching techniques [190] for analyzing adult (1-day-old) *Drosophila Parkin* null mutants. The results were matched with age-matched controls. For relative protein quantification, a label-free peptide hits technique, based on extracted ion chromatogram peak area (XICPA33, with addition of 350 mM potassium chloride) [191] and an isotope-labeling strategy, based on global internal standard technology [175] was utilized. A total of 253 proteins were identified, 24 of which showed differential expression compared with W/T controls. This included the mitochondrial antioxidant SOD and the mitochondrial enzymes ATP synthase subunit b and ATP synthase γ chain. Similar upregulations were also reported following the proteome analysis of human PD-affected SN to possibly reflect an underlying energy deficiency [102]. Furthermore, an upregulation of cytochrome c was seen in these *Parkin* null mutants, which possibly reflects an attempt at counterbalancing the energy deficiency caused by the abundance of ATP synthase [190].

This investigation was conducted on flies within a narrow age-range. Since the *Drosophila Parkin* PD model may encompass more human-like PD symptoms at advanced ages, the investigators suggested that differential analysis of the proteome of aged *Drosophila* (e.g., at 40, 50 and 60 days postfertilization) might provide a more comprehensive view of protein expression changes that underlie Parkin-induced parkinsonism.

Genes inherent to the nematode *Caenorhabditis elegans* display high conservation with human genes (especially noticeable for transporter proteins [192] and the anti-apoptotic chaperone 14–3–3 protein family [193], which earns the organism a unique position in the genetic and genomic arsenal available to investigators, who work in diseases as diverse as cancer and PD.

The enzymes synthetase, protease, catalase, hydrolase, dehydrogenase, oxidase and isomerase, metabolically process amino acids, carbohydrates, lipids, nucleotides and co-factors. This class of proteins also shows a high degree of overlap between nematode and mammalian metabolic pathways. Li and colleagues recently conducted a study, where the generated information is expected to make a valuable contribution towards characterizing the role of mitochondria in nematodes, while extending our understanding of mitochondrial dynamics, mitochondrial disease, aging and life span [194]. For this study, the BeadBeater, described by Grad and colleagues, was used in an effort to overcome the technical barriers for isolating intact mitochondria in *C. elegans* [195]. Here, purified mitochondria were harvested from W/T (N2) worms and subjected to shotgun proteomics (2D-LC-MS/MS), to compile a complete protein composition of *C. elegans*. A total of 1117 proteins were

identified, including two unique peptides. The majority of these had middle-to-low molecular weights, consistent with the finding that most mitochondrial proteins are synthesized in the cytoplasm, from where they are transported into mitochondria. Hence their effective transmembrane transfer requires relatively light proteins [72]. By subjecting the dataset to a disease gene-identifying protein database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) [196], a total of 75 proteins were assigned a PD-related role.

Representing a series of tightly related mitochondrial-regulated processes, OXPHOS is largely responsible for cell respiration and energy metabolism. The dysfunctional management of these processes has been implicated in PD pathology [197]. The importance of OXPHOS in the physiological and pathological processes of *C. elegans* was emphasized in this study, with over 90% of identified proteins confirmed as belonging to METC subunits.

In an effort to create an animal model of endogenous oxidative stress, Hinerfeld and colleagues generated *SOD* KO mice, with W/T *SOD* that serve as the primary defense mechanism against mitochondrial superoxide [198]. In addition to having a survival-time of only 3 weeks, *SOD2* KO mice develop a severe neurological phenotype presenting as degeneration of neurons in the basal ganglia and brainstem, progressive motor disturbances characterized by rapid fatigue, exaggerated circling behavior, severe spongiform encephalopathy and decreased levels of mitochondrial aconitase activity [199,200]. Proteomic analysis entailing 2-DE electrophoresis and MALDI-TOF-MS were performed on the cortices of these animals. Application of this approach led to detecting seven proteins that displayed differences in terms of relative abundance. Moreover, a number of enzymes showed a differential display between *SOD* KO and W/T controls, including the two tricarboxylic acid cycle enzymes ketoglutarate dehydrogenase and succinate dehydrogenase. Two enzymes with a role to play in maintaining redox balance, peroxiredoxin 5 (Prx5) and glutathione *S* transferase class µ1 (GST class-µ1), as well as cyanide-detoxifying enzyme 3-mercaptosulfurtransferase and glycolytic enzyme triosephosphate isomerase (TPI) were also found upregulated in the *SOD* KO mice, as opposed to W/T mice [198]. Using this genetic animal model, the study therefore generated insight into the specific protein targets of endogenously generated mitochondrial oxidative stress. This knowledge holds implications for designing and developing anti-PD therapeutics aimed at preventing damage being incurred to proteins, through exposure to excessive mitochondrial-generated oxidative cellular stress.

Expert commentary

Although the cause of PD is currently unknown, strong evidence exists to support the belief that it could involve a complex interplay between several factors including genetic susceptibility, environmental factors, abnormal protein handling, oxidative stress etc. Many of the molecular pathways implicated in PD etiology converge on the mitochondria, resulting in dysfunction of this key organelle, which could impact on neuronal survival. Such a hypothesis is supported by several of the experimental models, used for recapitulating the core aspects of human PD in animals. Proteomics have proved to be a key tool for confirming already implicated pathways and identifying new molecular pathways involved in PD pathogenesis and for highlighting the central role played by mitochondria in the neurodegenerative process. Unfortunately, most of the studies on human tissue performed thus far have utilized patients with very advanced PD, hence it has not been possible to distinguish between which pathways serve as the key triggers for neurodegeneration, as opposed to downstream pathways that become activated just prior to apoptosis. However, animal models of PD allow researchers to examine the early phases of neurodegeneration and proteomic approaches have been able to shed important light on the pathways involved. Genetic models utilizing known mutations found in familial PD cases have also been utilized to establish how such mutant proteins affect mitochondrial and ultimately cell function. Several drugs, including rasagiline and selegiline have also been proposed as potential neuroprotective therapies for PD and proteomic approaches in animal models have yielded critical data on their mechanisms of action, particularly in relation to how they may provide protection to mitochondria. Such studies have only become possible through great technological advances made in the field of proteomics. However, such studies are hampered by the fact that we only know the cellular function of approximately 50% of the mitochondrial proteins and that current techniques cannot accurately detect proteins of low abundance. However, in this rapidly advancing field, we anticipate that such technological hurdles will be overcome, thereby helping to reveal the mechanisms that cause PD, thereby placing us on the path towards developing more effective therapies for treating and/or preventing this debilitating disease.

Five-year view

With the exact role of mitochondria in the pathology of PD currently unresolved, the advantages of mitochondrial proteomics, including the ability to concomitantly detect a vast amount of proteins on single gels and identifying the presence of isoforms and post-translational modifications of disease-related protein species, may offer valuable clues regarding the molecular mechanisms underlying PD's disease processes. Recent proteomic studies that were either conducted in isolation or combined with RNA expression have highlighted several mitochondrial proteins of interest. However, their physiological function remains unclear and to be determined in future scientific work. The mitochondrial genome encodes for 13 polypeptides, two rRNAs and 22 tRNAs [201]. Mammalian cells typically contain 800–2500 mitochondria. Although the exact number of mammalian mitochondrial proteins remains unconfirmed, studies utilizing proteomic and genomic methods suggest for the presence of approximately 1200 mammalian mitochondrial proteins [101]. Thus far, the functional roles of only 600–700 of these have been defined [202], leaving scientists with the tremendous challenge of elucidating on the remaining ones.

Exposure to oxidative stress can result in proteins undergoing oxidative and nitrative changes. By evaluating the broad range of mitochondrial enzymes, the 'molecular fingerprint' of PD-specific modifications could be highlighted, including altered protein levels, increased carbonylation, hydroxynonenal modification, tryptophan oxidation reactions or altered nitrotyrosine levels of certain proteins. Although all enzymes can be modified by oxidative stress, each enzyme seems to present with so-called 'hotspots' that undergo more extensive oxidative stress compared with other enzyme complexes. It is anticipated that future attempts will focus on generating antibodies immunoreactive to these areas of high activity, which will allow oxidative stress levels to be monitored in high throughput, while also requiring minimal manipulation. Such insights are expected to elucidate on the role mitochondria play in cell degeneration, as seen in PD and aid in identifying novel drug targets for preventing and treating PD.

However, technical limits remain, such as the challenge to identify alterations in proteins of low-abundance, in the presence of more highly expressed ones. One approach for overcoming this is to pre-apply subcellular cell fractionation [203]. The advent of MS/MS, which applies the Q-TOF approach, has allowed for identifying proteins of low-molecular mass, not detectable by MALDI-TOF-MS [204]. However, so far the method has proved labor intensive and unable to deliver data in high-throughput mode.

Novel proteomic techniques are under development and are expected to fuel the field, promising to open avenues with which to pursue mechanism-based drug-targeting and treatment monitoring. This includes using an *in vitro* model of oxidative stress, which allows all proteins to be modified to some extent, followed by an immunocapturing assay and then further studying the protein complexes by means of SDS-PAGE to identify the sites of modification at the subunit level. For example, application of an *in vitro* model (which entails adding an oxidant to isolated mitochondria) showed remarkable similarity to *in vivo* models, particularly in terms of carbonyl modification of Co-I in parkinsonism [42].

With an ever-increasing number of studies published on the mitochondrial proteome, the number, size and complexity of datasets make them increasingly difficult to analyze. A fully defined and searchable catalog system called MitoMiner [205] has recently been launched [301]. The database combines experimental data derived from published studies with protein annotation from UniProt and genome projects, metabolic pathway data from KEGG, homology relationships from HomoloGene and disease phenotypes from Online Mendelian Inheritance in Man (OMIM) [206].

Information derived from mitochondrial proteomics is expected to provide a comprehensive overview of neurodegeneration-associated changes in protein expression, while it is anticipated that these insights could facilitate identification of novel biomarkers for detection at an early stage of the disease, when therapeutic interventions are most beneficial. A related challenge, therefore, entails the extraction of meaningful information from the large datasets that mitochondrial proteomics continuously produce.

A future objective should also include an attempt to associate the abundance of genomic-related information to actual protein levels present in mitochondria. A more advanced understanding of the degree of overlap between changes at the mitochondrial transcriptome and at the mitochondrial proteome level, which can be either experimentally induced or repressed or, alternatively, result from the disease process itself, may enable the development of more targeted and effective therapies against PD.

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Key issues

- Parkinson's is the second most common neurodegenerative disease, and developing effective therapies is hampered by our lack of understanding of its cause.
- Mechanisms implicated in neuronal death in Parkinson's directly impact on mitochondrial function.
- Many of the mutated proteins associated with the rarer familial forms of Parkinson's interact with the mitochondria.
- Proteomics has played a key role in both confirming the involvement of already implicated pathways and in identifying new pathways.
- Studies on donated human tissue have been unable to differentiate between the key pathways that initiate the neurodegenerative process from those that are downstream and become activated prior to cell death.
- Animal models have allowed us to investigate mechanisms in the early phases of neurodegeneration.
- Genetic models of rarer familial forms of Parkinson's have revealed how mutated proteins impact on mitochondrial and cell function.
- We still do not know the full function of approximately 50% of the mitochondrial proteins.
- Future technological advances in proteomics will assist us in identifying the true cause of Parkinson's and help develop effective therapies.

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Website

301 MitoMiner website http://mitominer.mrc-dunn.cam.ac.uk (Accessed 5 January 2010)

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