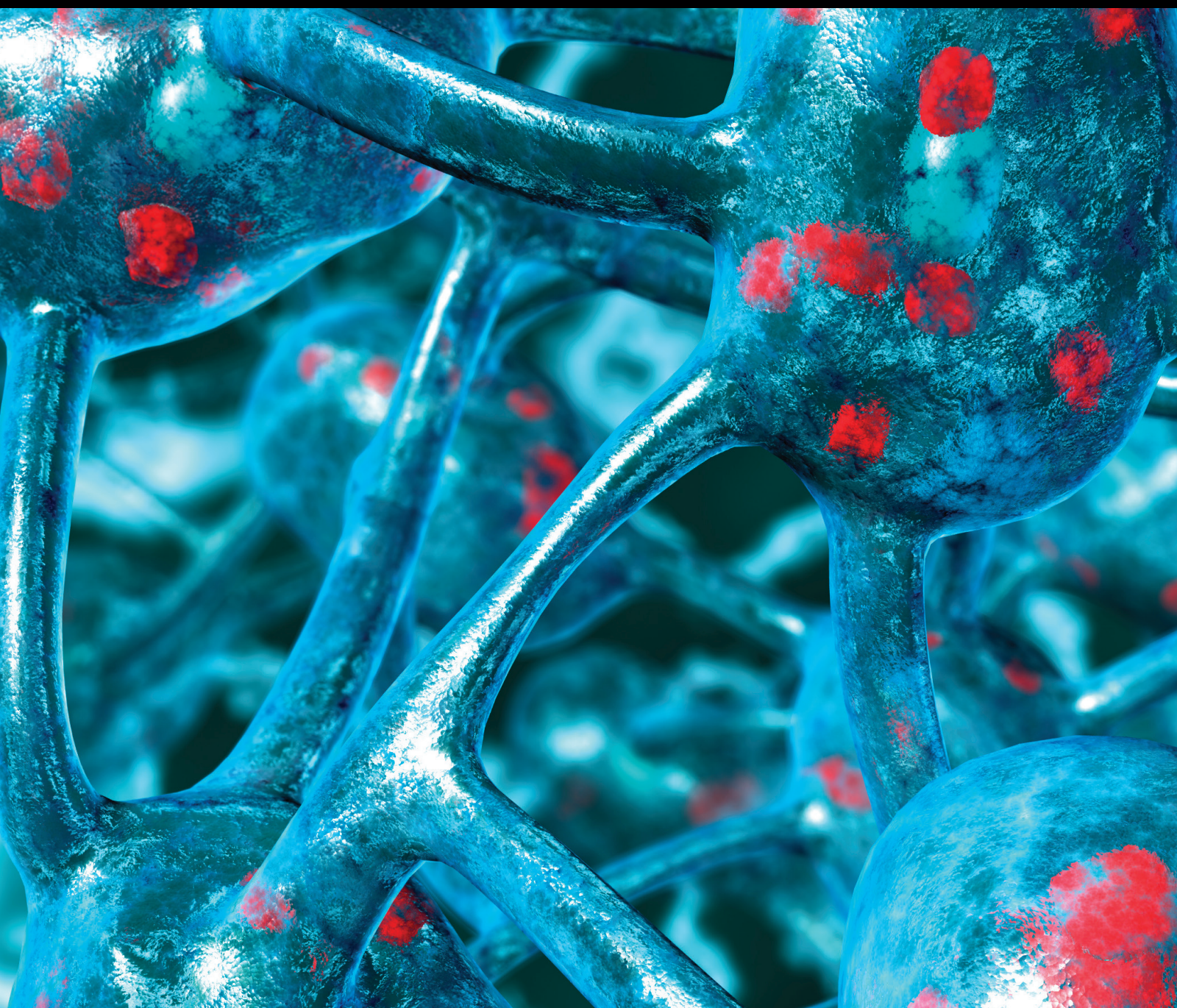
Parkinson's Disease

Mitochondria: Key Organelle in Parkinson's Disease

Guest Editors: Rubén Gómez-Sánchez, José M. Bravo-San Pedro, Rosa A. González-Polo, José M. Fuentes, and Matthew E. Gegg



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Editorial Board

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Editorial

Mitochondria: Key Organelle in Parkinson's Disease

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Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized pathologically by loss of dopaminergic neurons in the *substantia nigra pars compacta*. The etiology of PD is still unknown, involving genetic and environmental factors; however mitochondrial dysfunction plays a central role in PD pathogenesis. In this regard, several PD-related proteins (PINK1, Parkin, DJ-1, LRRK2, and α-synuclein) are linked to mitochondrial function. Mitochondria are highly dynamic organelles involved in essential cellular functions, including energy production, calcium homeostasis, metabolism of amino acids and lipids, mtDNA replication, and programmed cell death. Moreover, mitochondrial homeostasis is tightly regulated by several pathways, including mitochondrial biogenesis, remodeling (fusion/fission), and clearance of damaged mitochondria by autophagy (mitophagy), among others. Mitochondrial dysfunction and the engagement of calcium channels during autonomous pacemaking have been implicated in the increased susceptibility of dopaminergic neurons to cell death in the *substantia nigra*.

This special issue is comprised of two reviews and five articles, which provide new insights into the molecular and cellular pathways related to mitochondria that may influence the pathogenesis of PD.

In the first review ("Chaperone-Mediated Autophagy and Mitochondrial Homeostasis in Parkinson's Disease"), the authors summarize the current knowledge about autophagy and the relevance of this degradative pathway in the maintenance of mitochondrial function. Specifically, they highlight the link between mitochondrial dysfunction and impairment of chaperone-mediated autophagy activity in PD patients.

The second review, titled "Parkinson's Disease: The Mitochondria-Iron Link," is focused on the relationship between accumulation of redox-active iron and the development/pathogenesis of PD. It is well-known that mitochondria are involved in the exchange of iron with the cytoplasm, with evidence suggesting that dysfunction in PD-related proteins (i.e., α-synuclein, Parkin, PINK1, DJ-1, LRRK2, and ATP13A2) leads to iron dysregulation. Because of the neurotoxicity linked to iron accumulation, Y. Muñoz et al. suggest that iron chelation is a potential therapeutic approach to slow down the progression of the disease.

Related to the previous review, the first research paper included in this special issue, entitled "Protection against Mitochondrial and Metal Toxicity Depends on Functional Lipid Binding Sites in ATP13A2," examines the cytoprotective role of ATP132A and its consideration as a therapeutic target to reduce cellular toxicity. S. Martin et al. demonstrate that

ATP132A requires the signaling lipids phosphatidic acid and phosphatidylinositol 3,5-bisphosphate to mediate protection to toxic $Mn^{2+}/Zn^{2+}/Fe^{3+}$ concentrations and mitochondrial stress by the toxins rotenone and MPP^{+}.

In the second research article, "Methyl-Arginine Profile of Brain from Aged PINK1-KO+A53T-SNCA Mice Suggests Altered Mitochondrial Biogenesis," G. Auburger et al. use a powerful experimental model (PINK1-knockout with overexpression of A53T-SNCA double-mutant mice) to elucidate the polygenic etiology of PD. Based on quantitative global proteomics focused on methyl-arginine modifications, they report upregulation and downregulation of this specific posttranslational modification in several proteins, including some related to mitochondrial biogenesis such as CRTC1 and PSF. Moreover, posttranslational alterations of other identified factors could be required in molecular events linked to PD or other neurodegenerative disorders.

The third research article, "Altered Mitochondrial Respiration and Other Features of Mitochondrial Function in Parkin-Mutant Fibroblasts from Parkinson's Disease Patients" by W. Haylett et al., investigates mitochondrial health in *Parkin*-mutant fibroblasts from PD patients. Their results show that mitochondrial respiration and cell growth are higher in these cells, suggesting a compensatory mechanism in the absence of Parkin. Identification of this response could be a therapeutic target to preserve mitochondrial function in PD patients with Parkin mutations.

The fourth research paper of this special issue, "A Feed-Forward Circuit of Endogenous PGC-1α and Estrogen Related Receptor α Regulates the Neuronal Electron Transport Chain," addresses the role of the key mitochondrial regulator PGC-1α in the activation of the nuclear-encoded mitochondrial electron transport chain (ETC) genes. R. Bakshi et al. show that PGC-1α regulates *ERR*α transcription. Interestingly, they report that pioglitazone treatment increases expression of endogenous *PGC-1*α, *ERR*α, and their ETC target genes. The modulation of the PGC-1α transcription network by drug administration could potentially be a clinical target for PD and other neurodegenerative diseases.

In the final review, "Activation Mechanism of LRRK2 and Its Cellular Functions in Parkinson's Disease," the authors discuss the cellular role of LRRK2 and the recent research linking LRRK2-mediated PD to mitochondrial dysfunction and aberrant autophagy. In this regard, PD-associated mutations in *LRRK2* lead to impaired kinase and decreased GTPase activity. Thus, development of kinase inhibitors, as well as characterization of substrates and regulators of LRRK2, is essential in understanding LRRK2 pathogenesis and identifying potential targets for therapy.

The main purpose of this special issue is to shed light on the relevance of mitochondria as an essential organelle in postmitotic cells such as neurons and how mitochondrial damage contributes to the PD pathogenesis. An accurate and comprehensive understanding of mitochondrial quality control processes is critical to prevent cell death and development of age-related neurodegenerative disorders like PD. Current therapeutic strategies in PD are based on slowing down disease progression; however, they are not successful. New therapeutic approaches should be based on early biomarkers of PD and mitochondrial dysfunction is one promising target to be investigated.

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Review Article

Chaperone-Mediated Autophagy and Mitochondrial Homeostasis in Parkinson's Disease

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Parkinson's disease (PD), a complex neurodegenerative disorder, is pathologically characterized by the formation of Lewy bodies and loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Mitochondrial dysfunction is considered to be one of the most important causative mechanisms. In addition, dysfunction of chaperone-mediated autophagy (CMA), one of the lysosomal proteolytic pathways, has been shown to play an important role in the pathogenesis of PD. An exciting and important development is recent finding that CMA and mitochondrial quality control may be linked. This review summarizes the studies revealing the link between autophagy and mitochondrial function. Discussions are focused on the connections between CMA and mitochondrial failure and on the role of MEF2D, a neuronal survival factor, in mediating the regulation of mitochondria in the context of CMA. These new findings highlight the need to further explore the possibility of targeting the MEF2D-mitochondria-CMA network in both understanding the PD pathogenesis and developing novel therapeutic strategies.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. It affects about 1% of people above 60 years of age [1]. Rigidity, bradykinesia, postural instability, and tremors are the four characteristic clinic features of PD. Parkinson's disease is caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), which leads to decreased dopamine (DA) levels in the dorsal striatum [2, 3]. Pathologically, the cytoplasm of SNc dopaminergic neurons in the brain of PD patients is characterized with the presence of Lewy bodies, which represents aggregation of proteins including α-synuclein [4]. Current treatment available for PD offers only symptomatic relief but cannot modify the disease progression. Nor can it slow down the progress of the disease. Although over 90% of PD cases are sporadic, the remaining 10% have a family inheritance [5]. Mutations in PARK 1 (SNAC) and PARK 8 (LRRK2) cause autosomal-dominant PD, while mutations in PARK2 (PARKIN), PARK6 (PINK1), and PARK7 (DJ-1) are responsible for autosomal recessive PD [5]. Neurons in both familial and sporadic PD cases display the same key pathophysiological features. Extensive research on PD pathogenesis has firmly established mitochondrial dysfunction, oxidative stress, and impaired protein clearance as the key cellular processes altered in both familial and sporadic PD [6–8]. Interestingly, mutations in PARKIN, PINK1, and DJ-1 all lead to mitochondrial dysfunction [9, 10].

Growing evidence shows recently that autophagy, a protein clearance pathway, is critical for maintaining mitochondrial homeostasis and is impaired in neurodegenerative disorders such as PD [11, 12]. Autophagy is a cellular self-eating process in which lysosomes degrade intracellular components including proteins and other organelles. Autophagy is active under the normal basal metabolic condition as well as activated upon stress such as starvation, both of which are important to maintain cellular homeostasis [13]. There are three types of autophagy, microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). These processes involve different mechanisms and may serve different cellular functions [14, 15]. Both macroautophagy, which is termed mitophagy when targeting mitochondria, and CMA are associated with mitochondrial function [16–20]. Many studies indicate that autophagy level is often

downregulated in various neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) [21–23], and impaired autophagic function may in varying degree contribute to the neurodegenerative process. For PD, increasing evidence shows that dysfunction of mitophagy and CMA appears to be particularly prominent and a key pathogenic theme common to multiple genetic risk factors associated with the disease [9, 17, 24, 25].

2. Mitophagy and Parkinson's Disease

Mitophagy selectively removes damaged mitochondria. The process of mitophagy needs two key proteins, PINK1 and Parkin. The genes encoding these two proteins are mutated in autosomal recessive parkinsonism [9], supporting the idea that dysfunction of mitophagy caused by PINK1 and PARKIN mutation may be an important mechanism in PD pathogenesis.

PINK1 is a key protein in the pathway of mitophagy [9]. Structurally, the C terminus of PINK1 is predicted to be a kinase domain while its N terminus contains a mitochondrial targeting sequence, which helps it locate onto mitochondrial outer membrane and transfers it into mitochondria [26]. PINK1 accumulates specifically on dysfunctional mitochondria and recruits Parkin from the cytosol [27, 28]. Parkin is an E3 ubiquitin ligase with an N-terminal ubiquitin-like domain and a C-terminal ubiquitin ligase domain [29, 30]. After being recruited to the damaged mitochondria, Parkin ubiquitinates substrates on the damaged mitochondria to instigate their elimination by autophagy [31, 32]. Mutations in both PINK1 and Parkin reduce their ability to eliminate dysfunctional mitochondria, indicating that PINK1 and Parkin are essential for mitochondrial quality control [33–35]. Accumulation of dysfunctional mitochondria causes stress to the SNc DA neurons. A reduction in mitophagic activity following a loss of either PINK1 or Parkin function may trigger or exacerbate the loss of homeostasis and viability of these neurons, contributing to PD pathogenesis [36–39].

Although mitophagy plays a crucial role in mitochondria quality control, it can only recognize mitochondria that have already been damaged and remove them. PINK1 and Parkin also regulate mitochondria via nonmitophagy processes. Mitochondria are cellular respiratory factory and have a high oxidative level. This oxidative environment can often damage individual mitochondrial proteins without causing irreparable mitochondrial damage. In such cases, it is best for cells to dispose the damaged individual proteins via proteolytic pathways to maintain mitochondrial homeostasis without the need for mitochondria removal and biogenesis. PINK1 and Parkin appear to also participate in these pathways. For example, an interesting in vivo study showed that the turnover of many mitochondrial respiratory chain subunits is impaired when Parkin and PINK1 are mutated [40]. Furthermore, this impairment caused by Parkin mutation seems to be greater than it is in Atg7 mutant. This finding cannot be simply explained by the model that Parkin acts upstream of Atg7 to promote mitophagy. Instead, it is more consistent with the notion that in addition to mitophagy PINK1-Parkin pathway may promote the selective turnover of mitochondrial proteins such as the respiratory complex subunits [40]. PINK1 and Parkin are also related to the biogenic pathway of mitochondria derived vesicles (MDVs), in which vesicles of budding off mitochondria with a specific repertoire of cargo proteins are ultimately targeted to lysosomes for degradation [41]. In this study, Parkin colocalizes with MDVs in a PINK1-dependent manner and stimulates their formation in response to antimycin A, a potent generator of reactive oxygen species (ROS). And once formed, the MDVs target to lysosomes for degradation in a manner independent of canonical mitophagy. These findings implicate that PINK1 and Parkin participate in a mitochondrial quality control pathway besides mitophagy. Moreover, Parkin mediates the ubiquitination of some outer mitochondrial membrane (OMM) proteins such as Mitofusins (Mfns) and Miro1 to regulate their function or degradation by proteasome [9, 42]. PINK1 may also be involved in this process by acting as ubiquitin kinase. Through Mfns, PINK1 and Parkin can potentially regulate mitochondrial fission and fusion.

3. CMA and Cellular Homeostasis in Parkinson's Disease

Chaperone-mediated autophagy, one of the lysosomal proteolysis pathways, is characterized by its specificity in selective degradation of substrate proteins. This process can be divided into four steps: (1) recognizing substrate proteins and targeting them to the lysosomes; (2) binding to lysosomal receptor and unfolding of substrate proteins; (3) translocating substrates into lysosomes; and (4) degrading substrates in the lysosomal lumen [25]. The substrate proteins of CMA are recognized in the cytosol by the chaperone protein heat shock-cognate protein of 70 KDa (Hsc70) via a pentapeptide motif similar to KFERQ in sequence [43, 44]. This motif is not in strict conformance with a specific amino acid residue sequence but is like a pattern recognition motif related to the charge and hydrophobicity of amino acid residues [45, 46]. Posttranslational modifications such as phosphorylation and acetylation can facilitate an imperfect motif acquiring more effective recognition [47–49]. It is predicted that almost 30% of cytosolic proteins have a KFERQ-like motif, but only a few of them have been experimentally confirmed as CMA substrates [50].

Once recognized by Hsc70, the substrate is targeted to the surface of lysosomal membrane and bound to the cytosolic tail of the lysosome-associated membrane protein type 2A (LAMP2A) [51]. LAMP2A exists as monomer at the lysosome membrane. During CMA, it oligomerizes to form a multiprotein complex to facilitate the translocation of substrates into the lysosomal lumen [52]. Before substrate protein translocation, it needs to be unfolded. This is mediated by Hsc70 and its co-chaperones [53]. Translocation of the substrate proteins into the lysosomal lumen requires a lysosomal resident form of Hsc70 (lys-Hsc70) [54]. The mechanism by which lys-Hsc70 facilitates substrate protein translocation remains unknown. After substrate translocation, LAMP2A rapidly

disassembles from the translocational multimer-complex into monomers to which substrates can bind again [52]. Thus, the rate of CMA is modulated by the level of LAMP2A and the rate of assembly/disassembly of the translocation complex [25].

With its highly selective mechanism, CMA is especially suited for removing misfolded, oxidized, or damaged cytosolic proteins under both physiological and pathological conditions [50, 55]. This removal is not only an amino acid recycling pathway, but also a mechanism of balancing cellular homeostasis. Under moderate oxidative stress, which is related to and can cause mitochondrial dysfunction, CMA is often activated to accelerate the elimination of proteins damaged by oxidative stress [56]. Consequently, blocking the upregulation of CMA under those conditions leads to the accumulation of proteins damaged by oxidative stress and impairs mitochondrial function and cellular viability [57]. CMA is also activated under other stress conditions such as exposure to prolonged starvation [58] and hypoxia [59]. It appears that CMA is an essential stress response mechanism that is required to maintain cellular homeostasis through the removal of damaged proteins under various conditions.

There are multiple lines of evidence for the impairment of CMA activity in both familial and sporadic PD [60, 61]. Two critical proteins mutated in familial PD, α-synuclein and leucine-rich repeat kinase 2 (LRRK2), are both degraded by lysosomes via CMA [62–64]. α-synuclein is a key factor in PD pathogenesis. The accumulation of both wild-type and mutant α-synuclein, which is caused by the dysfunction of autophagy-lysosome pathways including macroautophagy and CMA, causes SNc neuron loss [64–66]. One proposed pathogenic mechanism by which mutant α-synuclein may exert stress is interference of cellular protein homeostasis through the blockade of CMA process [62]. Mutation in LRRK2 is the most common cause of familial PD. Although wild-type LRRK2 itself is degraded by CMA, the most common pathogenic mutant form of LRRK2, G2019S, is poorly degraded by this pathway. Moreover, LRRK2 mutants or high levels of WT LRRK2 bind to the lysosomal membrane and inhibit the assembly of the CMA translocation complex [63]. Furthermore, mutation of UCHL1, which is also associated with familial PD, has also been shown to inhibit CMA process [67, 68]. Thus, CMA controls the turnover of several proteins whose mutation is linked to familial PD. Inhibition of CMA appears to be a key common mechanism through which multiple proteins associated with familial PD exert their toxic effect.

4. MEF2D, CMA, and Oxidative Stress in Parkinson's Disease

Myocyte enhancer factor 2 (MEF2) is initially identified as a transcription factor vital for muscle cell differentiation [69]. There are four isoforms of MEF2, MEF2A-MEF2D. MEF2s share a highly homologous sequence of the first 86 amino acids at the N-terminus, which participates in MEF2s hetero- or homodimerization and their binding to an A/T rich cis-acting DNA element. The C-terminal sequence of MEF2s is diverse and responsible for MEF2-mediated transcription activation. MEF2s can be modulated by posttranslational modifications such as phosphorylation at the C-terminus and interaction with other cofactors [70].

Although MEF2s are first identified in muscle cells, recent studies have revealed that MEF2s play an important role in several cellular pathways in neurons including neuronal survival [70–72]. It has been shown that neuronal activity activates MEF2 by p38-mediated phosphorylation. Inhibition of MEF2s blocks the neuronal activity-induced survival of cerebellar granule neurons, leading to their apoptosis.

The connection between MEF2D and Parkinson's disease started with the finding that cyclin dependent kinase 5 (Cdk5) directly phosphorylates MEF2D at Ser444 under stress conditions [73], which leads to an impairment of MEF2D transcriptional function. It was shown subsequently that phosphorylation of MEF2D at Ser444 promotes its degradation by caspases, leading to a sharp reduction of MEF2D level and neuronal death [74]. It was late demonstrated that this Cdk5-mediated inhibition of MEF2D is involved in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) induced loss of DA neurons in a mouse model of PD [75, 76]. Therefore, MPTP-induced loss of SNc DA neurons in vivo may be in part due to Cdk5-mediated modulation of MEF2D [77].

In addition to being modulated by Cdk5, MEF2D is also regulated by CMA [78]. MEF2D has several imperfect KFERQ-like motifs at the N-terminus, mediating its interaction with Hsc70 and degradation by CMA. The blockage of CMA leads to an increase of cellular MEF2D level, and the accumulated MEF2D shows a decrease in DNA binding ability. Thus, the inhibition of CMA significantly impairs MEF2D function. Furthermore, degradation of MEF2D by CMA can be blocked by both wild-type and mutant α-synuclein. Though efforts were made to understand how the increase in the level of wild-type α-synuclein causes PD, the pathogenic mechanisms underlying it toxicity remain unclear. The finding that increased level of wild-type α-synuclein interferes CMA-mediated degradation and homeostasis of MEF2D provides a mechanism by which aberrant increase in wild-type α-synuclein induces neuronal death.

SNc DA neurons show increased oxidative stress in PD. Oxidative stress is considered to be a key mechanism that either triggers or exacerbates the pathological process in PD. How oxidative stress modulates neuronal activity is an intense and important area of investigation. Recent studies show that a short-term exposure to 6-hydroxydopamine (6-OHDA), a neurotoxin used to model PD in rodents, leads to oxidization of MEF2D [79]. MEF2D can be oxidized at cysteine residues and carbonylated. Oxidized MEF2D loses its DNA binding ability as well as gene transcription control and is preferentially removed by CMA, which is activated by moderate oxidative stress as a protective response [79]. High levels of α-synuclein, neurotoxins, and excitotoxicity are known to cause excessive oxidative stress [80–82], which not only oxidizes MEF2D but also inhibits CMA. Therefore, the combination of losing both survival factor such as MEF2D and CMA protection may underlie, at least in part, the toxic effects of those diverse stress conditions. Oxidative stress is widely considered to be one of the key mechanisms that trigger or

exacerbate the pathological process in PD [83–85]. Given the vital role of MEF2D in the survival of SNc DA neurons [78] and CMA-mediated protection, enhancing MEF2D or CMA should be explored further as a therapeutic strategy for PD.

5. MEF2D and Mitochondrial Function in Parkinson's Disease

MEF2s have been studied as a nuclear factor for its dynamic roles in many cellular processes. For example, previous research of MEF2A in muscle indicates that MEF2A regulates mitochondrial function through controlling nuclear gene expression [86]. However, this nuclear centric role of MEF2s has been expanded with the unexpected finding of MEF2 function outside the nucleus in mitochondria [87].

In an elegant study [87], researchers showed that a portion of MEF2D is localized in mitochondria in neuronal cells. This localization is mediated by the N-terminal 33 amino acid residues of MEF2D and requires mitochondria heat shock protein 70 (mtHsp70). Functionally, MEF2D regulates mitochondrial DNA (mtDNA) expression. mtDNA is a circular DNA, containing 16,569-base-pair-length genome that encodes 13 genes for subunit components of oxidative phosphorylation and its own tRNAs and rRNAs [88]. It has a heavy (H) strand and a light (L) strand determined by buoyant densities. The L strand encodes a single polypeptide, NADH dehydrogenase 6 (ND6), an essential component of complex I [89, 90]. Mutations in the *ND6* gene or changes of its transcriptional level are associated with PD [91, 92]. Mitochondrial MEF2D binds to mtDNA in the coding region of the *ND6* gene through a MEF2 site ($5'$-$C^{73}CTATTTATG^{82}$-$3'$) to directly control the transcription of *ND6* gene [87]. Inhibition of mitochondrial MEF2D activity reduces the levels of ND6 mRNA and protein. Reduction of ND6 level, triggered by the loss of mitochondrial MEF2D function, decreases complex I level and activity, reduces the level of ATP, and increases the level of H_2O_2. These findings demonstrate that mitochondrial MEF2D directly regulates ND6 and affects mitochondrial function. Increasing mitochondrial MEF2D level promotes the survival of SNc DA neurons under MPTP-induced toxicity. In both postmortem PD patient brains and MPTP model of PD, MEF2D colocalization with mitochondria is reduced, which correlates with and accounts for the reduced transcription of *ND6* gene in these specimens.

Complex I deficiency has been shown to occur in the mitochondria in PD [93, 94]. The discovery of MEF2D-ND6 axis demonstrates clearly that loss of mitochondrial MEF2D contributes to the mitochondrial dysfunction and may underlie part of the pathogenic process in PD. Since the level and activity of mitochondrial MEF2D are also regulated by oxidative stress and CMA [79], together, these findings provide another pathway by which CMA may closely modulate mitochondrial activity via regulation of MEF2D.

6. CMA and New Targets in Mitochondrial Homeostasis

As mentioned previously, CMA maintains cellular homeostasis in basal conditions through a highly selective protein degradation mechanism. It is predicted that almost 30% of cytosolic proteins have a KFERQ-like motif. Although only a few of them have been experimentally confirmed as CMA substrates, it highlights the huge potential for CMA being involved in regulating many important cellular processes. We investigated our previous unpublished data showing many possible CMA substrates are correlative with mitochondria function. By comparing the levels of proteins by mass spectrum analysis following up- or downregulation of LAMP2A, we identified the proteins whose levels changed sharply in a LAMP2A sensitive manner following activation of CMA. Among these proteins, a majority of them are involved in mitochondrial function (unpublished data). Furthermore, our analysis revealed that a decrease in CMA activity is correlated closely with a significant increase in the level of ROS and decrease in mitochondrial membrane potential. Identifying and validating the individual protein substrates of CMA that are directly involved in mediating these mitochondrial changes should provide us with mechanistic insights into how CMA specifically regulates mitochondrial homeostasis under both physiological and pathological conditions.

To this end, our recent work on regulation of DJ-1 by CMA provides such an example. DJ-1, also known as PARK7, is a mitochondria related protein that regulates the organellar function and morphology and antioxidative response [95]. Mutation of *DJ-1* gene leads to mitochondrial defects and is associated with autosomal recessive familial PD [96]. Our study showed that DJ-1 is a direct CMA substrate [97]. Moreover, CMA preferentially degrades the nonfunctional and oxidatively damaged DJ-1 and protects cells against neurotoxin-induced mitochondrial damage and stress. Thus, our identification of CMA in maintaining mitochondrial homeostasis via regulation of DJ-1 serves to highlight and strengthen the notion that there is a strong and critical link between CMA and mitochondria.

7. Conclusion

Mitochondrial dysfunction is an important cellular feature in PD pathogenesis. In addition to the evidence of mitophagy failure as a cause for mitochondrial damage, recent studies support a strong link between CMA and mitochondria and a role for loss of CMA activity in mitochondrial dysfunction.

CMA has the potential to regulate many cellular pathways and maintains cellular homeostasis. Furthermore, it protects cells from diverse stress conditions and promotes cellular viability, especially neurons. Although CMA has been shown to participate in some cellular processes, few studies have investigated whether and how CMA directly regulates mitochondrial function. Since CMA plays a vital role in protecting neurons from stress and SNc DA neurons are especially sensitive to mitochondrial dysfunction, it is essential for us to clarify the role of CMA in mitochondrial dysfunction in PD pathogenesis.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Parkinson's Disease: The Mitochondria-Iron Link

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Mitochondrial dysfunction, iron accumulation, and oxidative damage are conditions often found in damaged brain areas of Parkinson's disease. We propose that a causal link exists between these three events. Mitochondrial dysfunction results not only in increased reactive oxygen species production but also in decreased iron-sulfur cluster synthesis and unorthodox activation of Iron Regulatory Protein 1 (IRP1), a key regulator of cell iron homeostasis. In turn, IRP1 activation results in iron accumulation and hydroxyl radical-mediated damage. These three occurrences—mitochondrial dysfunction, iron accumulation, and oxidative damage—generate a positive feedback loop of increased iron accumulation and oxidative stress. Here, we review the evidence that points to a link between mitochondrial dysfunction and iron accumulation as early events in the development of sporadic and genetic cases of Parkinson's disease. Finally, an attempt is done to contextualize the possible relationship between mitochondria dysfunction and iron dyshomeostasis. Based on published evidence, we propose that iron chelation—by decreasing iron-associated oxidative damage and by inducing cell survival and cell-rescue pathways—is a viable therapy for retarding this cycle.

1. Introduction

Parkinson's disease (PD) is the most frequent neurodegenerative movement disorder worldwide. Despite substantial amount of research, its founding causes remain elusive. Hence, while the initial causes of PD are not clearly determined, factors like aging, mitochondrial dysfunction, oxidative stress, and inflammation, are thought to have a pathogenic role in the disease [1–8]. PD is characterized by degeneration of dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) and the presence of proteinaceous cytoplasmic inclusions, called Lewy bodies [9, 10]. Loss of dopaminergic neurons in the SNpc produces a decrease in dopamine levels in the *corpus striatum* generating a deregulation of basal ganglia circuitries that leads to the appearance of motor symptoms including resting tremor, rigidity, bradykinesia, and postural instability. In addition, nonmotor symptoms such as depression, cognitive deficits, gastrointestinal problems, sleep disturbances, and smell loss have been identified. Sporadic cases represent more than 90% of total PD patients, but there are several inherited forms caused by mutations in single genes. Although sporadic and familial PD cases have similar outcomes, inherited forms of the disease usually begin at earlier ages and are associated with atypical clinical features [11].

Mitochondrial dysfunction is a plausible cause of PD neurodegeneration. Endogenous and exogenous mitochondrial toxins like nitric oxide, 4-hydroxynonenal, aminochrome, paraquat, rotenone, and others have been linked to sporadic forms of the disease [7, 12–16], and mitochondrial defects have been described in SNpc mitochondria of PD patients [17, 18]. Additionally, as discussed below, several PD-associated proteins, including α-synuclein (α-syn), Parkin, PTEN-induced putative kinase 1 (PINK1), protein deglycase DJ-1, leucine-rich repeat kinase 2 (LRRK2), and P-type ATPase A2 (ATP13A2), point to a role for mitochondria in the development of the disease.

In another aspect of PD neurodegeneration, a large body of literature strongly indicates that excess redox-active iron is involved in the pathogenesis of PD [19–34]. Iron, in its ferrous (Fe^{2+}) and ferric (Fe^{3+}) states, is present in Lewy bodies as well as in many other amyloid structures [35–37]. Iron content in the SNpc is higher than in other areas of the brain [38] and is even higher in PD patients [39]. Here, we

review the evidence that points to mitochondrial dysfunction and the subsequent iron accumulation as early events in the development of PD.

2. Cell Iron

Iron has been described as an important cofactor in many proteins involved in crucial biological processes, including cellular respiration, nitrogen fixation, photosynthesis, DNA synthesis and repair, oxygen transport, metabolism of xenobiotics, and neurotransmitter synthesis [40–49]. In most proteins iron is present in iron-sulfur clusters (ISCs), either as [2Fe-2S], [4Fe-4S], or [3Fe-4S] clusters [50, 51]. The main feature of iron as prosthetic group resides in its high redox flexibility. Thus, iron has the capacity to exchange one electron, either by oxidation ($Fe^{2+} \rightarrow Fe^{3+}$) or by reduction ($Fe^{3+} \rightarrow Fe^{2+}$). This flexibility is very important in biological processes such as cellular respiration, where the transport of electrons depends on 12 ISCs present in complex I to complex III and on 5 heme-containing proteins transporting electrons through complexes III and I [52].

Increases in redox-active iron directly associate with increased reactive oxygen species (ROS) generation and with changes in the intracellular reduction potential due to glutathione oxidation [53, 54]. Within the cell, most iron is associated with proteins, as either iron oxy-hydroxy crystals in ferritin or forming part of ISCs and heme prostetic groups. Around 1% of cell iron is in a redox-active form called the labile iron pool or labile cell iron [55–58]. The predominant component of this pool is Fe^{2+}-glutathione, but iron is also bound weakly to phosphate, citrate, carboxylates, carbohydrates, nucleotides, polypeptides, and other molecules [59, 60]. Through the Fenton reaction, reactive iron catalyzes the production of hydroxyl radical ($^{\bullet}OH$) in the presence of H_2O_2, in a self-renewed cycle caused by the presence of oxygen as an electron acceptor and intracellular reductants such as glutathione (GSH) and ascorbate as electron donors [28]. These characteristics of the intracellular environment demand a tight regulation of the reactive iron pool to decrease hydroxyl radical production.

Redox-active iron mediates GSH consumption [54]. After exposure to increasing concentrations of iron, SH-SY5Y dopaminergic cells undergo sustained iron accumulation and produce a biphasic change in intracellular GSH levels, increasing GSH levels at low iron concentrations and decreasing them thereafter. Indeed, cell exposure to high iron concentrations markedly decreases the GSH/GSSG molar ratio and the GSH half-cell reduction potential, with the associated loss in cell viability [54].

Iron levels in the SNpc increase significantly with age, and PD patients present an even greater increase that correlates with clinical PD status [64–69]. Experimental evidence shows that iron is crucial to the degeneration of SNpc dopaminergic neurons in the model of PD caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Mice fed for 6 weeks with a low iron diet before the administration of MPTP present neuronal protection, normal striatal dopamine levels, and no changes in motor behavior when compared with control animals fed a normal iron content diet [70]. Furthermore, increased iron levels in the brain aggravate dopaminergic cell death and motor impairment after MPTP treatment and this condition is attenuated by treatment with the iron chelator desferrioxamine (DFO) [71].

Clinical studies have not provided an evident correlation between dietary iron intake and risk of Parkinson's disease in humans [72–75]. Nevertheless, some reports point to a higher incidence of PD in hereditary hemochromatosis patients [76–79] although other reports found no correlation between these two diseases [80–82]. It is possible that under normal conditions the iron homeostasis system protects the brain from iron accumulation due to dietary variations. This homeostasis is most likely lost in iron-overload disease states yet.

Overall, these antecedents suggest that increased redox-active iron in the SNpc is part of the neurodegenerative process in PD, possibly due to increased oxidative stress and oxidative damage.

3. Iron Homeostasis in Mitochondria

Mitochondria consume about 90% of cellular oxygen and transform 1–5% of this oxygen into superoxide anion ($O_2^{\bullet -}$), due to the leaking of electrons that takes place in their passage through complexes III and I [83–86]. During aging, the activity of these complexes decreases, leading to higher oxidant production of $O_2^{\bullet -}$ and H_2O_2 [86, 87]. The superoxide anion generated in this process dismutates into hydrogen peroxide, either spontaneously or following catalysis by superoxide dismutase (SOD) [88, 89]. Proteins containing ISCs in mitochondria are significantly vulnerable to oxidative stress, participating in redox sensing and signaling reactions [90, 91].

The mitochondrion has an active exchange of iron with the cytoplasm, as required for the mitochondrial synthesis of heme and ISCs (Figure 1(a)) [92–94]. Kinetic experiments show that extracellular iron is readily incorporated into mitochondria. Indeed, iron incorporation into mitochondria apparently has a kinetic preference over incorporation into the cytoplasm (Figure 1(b)) (also see [94, 95]). Possible mechanisms for this preferential delivery include siderophore-mediated iron transport from the plasma membrane to the mitochondrion [96, 97], the entrance of iron into the cell by fluid-phase endocytosis with subsequent delivery to mitochondria without passing through the cytoplasmic labile iron pool (cLIP) [98], and iron delivery to mitochondrion by direct interaction with transferrin-containing endosomes [99].

Mitoferrin-2, a protein located in the inner mitochondrial membrane, represents the main pathway of mitochondrial iron uptake, whereas the ABCB7 and ABCB8 transporters are involved in ISC export [100–103] (Figure 1). Inward transport of iron by mitoferrin-2 apparently is regulated. Studies with the mitoferrin Mrs3p and Mrs4p yeast homologs revealed that inner mitochondrial membrane vesicles show rapid uptake of Fe^{2+} in response to iron starvation [104]. There is no reported evidence as to how cell or mitochondrial iron levels could regulate mitoferrin-2 levels. Additionally, mitoferrin

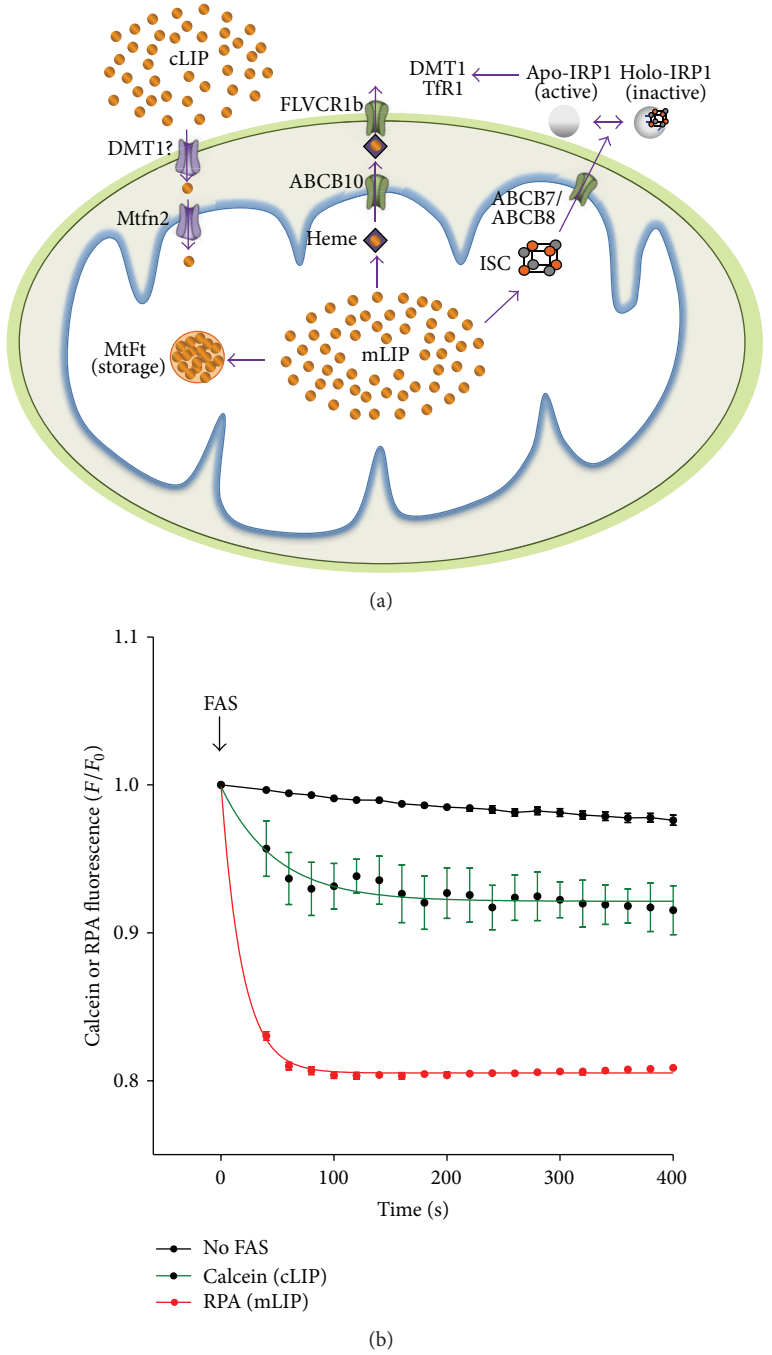

Figure 1: (a) Mitochondrial iron traffic. Iron enters mitochondria from the cLIP in a process mediated by the inner mitochondrial iron transporter Mtfn2 and probably by DMT1 located in the outer membrane. Upon entering, iron incorporates into the mLIP from where it distributes for heme and ISC synthesis or for storage in mFt. Heme leaves the mitochondrion through ABCB10 and the mitochondrial heme exporter FLVCR1b, located in the inner and outer mitochondrial membranes, respectively. ISCs are transported out of the mitochondrion by the ABCB7 transporter and probably by the ABCB8 transporter as well. In the cytoplasm, ISCs bind to the corresponding apoproteins. IRP1 binds a 4Fe-4S cluster; the holoprotein is inactive to induce the transcriptional regulation of cell iron-import proteins like DMT1 and TfR1. In contrast, apo-IRP1, normally abundant under low cell iron conditions, upregulates the expression of iron-import proteins like DMT1 and TfR1. ABC: ATP-binding cassette transporter; cLIP: cytoplasmic labile iron pool; DMT1: divalent metal transporter 1; FLVCR1b: feline leukemia virus subgroup C receptor 1B transporter; ISC: iron-sulfur cluster; mFt: mitochondrial ferritin; mLIP: mitochondrial iron pool; Mtfn2: mitoferrin-2; TfR1: transferrin receptor 1. (b) Kinetic determination of iron entrance into the cLIP and mLIP. SH-SY5Y cells preloaded with the mitochondrial iron sensor rhodamine B-[(1,10-phenanthroline-5-yl)aminocarbonyl]benzyl ester (RPA) and the cytoplasmic iron sensor calcein were challenged with 40 μM ferrous ammonium sulfate (Fe) and changes in RPA and calcein fluorescence were followed in a multiplate fluorescence reader [61, 62]. Iron binding quenches RPA and calcein fluorescence; thus, a decrease in RPA or calcein fluorescence is directly proportional to iron entrance into the mLIP or cLIP, respectively. Note that the initial rate of iron entrance into the mLIP ($K = 0.0536 \pm 0.0021\Delta(F/F_0)$/sec) is larger than the rate of iron entrance into the cytoplasmic LIP ($K = 0.0206 \pm 0.0070\Delta(F/F_0)$/sec). Values represent mean ± SD of quadruplicates; $P = 0.004$.

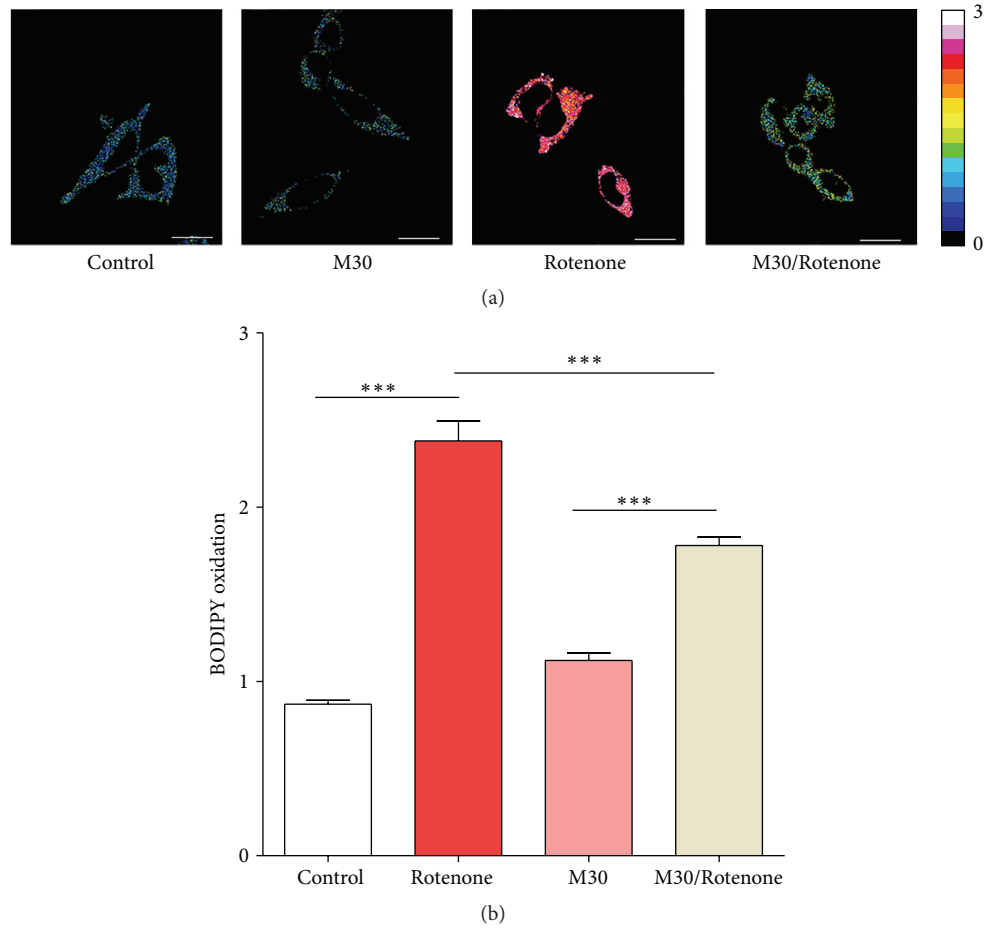

Figure 2: The iron Chelator M30 protect SH-SY5Y cells from rotenone-induce lipid peroxidation. (a) Mitochondrial lipid peroxidation was evaluated by green/red fluorescence changes of C11-BODIPY$^{581/591}$ (ThermoFisher Scientific-Molecular Probes) as described [63]. Oxidation of C11-BODIPY$^{581/591}$ results in a shift of the fluorescence emission peak from 590 nm (red, nonoxidized) to 510 nm (green, oxidized). SH-SY5Y cells were preincubated or not for 24 hours with 500 nM of M30 in DMEM-10% FCS medium and then loaded for 15 minutes at 37°C with 1 μM C11-BODIPY$^{581/591}$. Confocal images were obtained 15 minutes both before (Control, M30) and after (Rotenone, M30/Rotenone) applying 80 μM rotenone to the cells. Representative images are shown, where the ratio of green over (green + red) fluorescence was converted into a pseudothermal scale using the ImageJ program. (b) Changes in C11-BODIPY$^{581/591}$ oxidation quantified by the thermal scale. Values represent the mean ± SD of 40–52 individual cell measures per experimental condition. Significance between mean differences was determined by one-way ANOVA and Tukey *post hoc* test. $^{***}P < 0.001$.

dysregulation under pathological conditions promotes mitochondrial iron accumulation [100, 104].

A recent report described a role for mitoferrin-2 in the development of Friedreich's ataxia, by showing that mitoferrin-2 downregulation improved many of the conditions of frataxin deficiency whereas its overexpression exacerbated them [105]. Similarly, loss-of-function mutations in *ABCB7* produce a sideroblastic anemia condition called X-chromosome-linked sideroblastic anemia, in which patients show iron accumulation in mitochondria [101, 102].

A fraction of the intramitochondrial iron is redox-active. Petrat et al. demonstrated presence of a chelatable iron pool, which renders mitochondria sensitive to iron-mediated oxidative damage [106]. Evidence from our laboratory shows that complex I inhibition generates mitochondrial lipid peroxidation as determined by C11-BODIPY$^{581/591}$ oxidation [63], which is probably caused by redox-active iron since it is inhibited by coincubation with the iron chelator M30 (Figure 2).

4. Mitochondrial Dysfunction in PD

Mitochondrial dysfunction and oxidative stress have long been implied as pathophysiological mechanisms underlying PD [17, 107]. Mitochondria not only have a key role in electron transport and oxidative phosphorylation but also are the main cellular source of ROS and they are involved in calcium homeostasis and in the regulation and initiation of cell death pathways [1]. Mitochondria isolated from human brain tissues and peripheral cells of sporadic PD patients exhibit reduced mitochondrial complex I activity [108] and postmortem SNpc tissues from idiopathic PD patients display decreased number of complex I subunits [107, 109, 110]. Mitochondrial complex I activity is reduced in the SNpc [111]

and the frontal cortex [112] in patients with PD. However, total protein and mitochondrial mass from SNpc of patients with PD are similar to controls [111]. The main consequences of mitochondrial complex I inhibition in humans and experimental models are decreased ATP levels [113, 114], decreased glutathione levels, and increased oxidative damage [115–118]. Other reported effects are reduction in the concentrations of DA accompanied with decreased density of DA receptors and diminished activity of TH (reviewed in [119]), increased total SNpc iron content [120], increased redox-active iron [121, 122], decreased Fe-S cluster synthesis [61, 123], and calcium dysregulation [124–126]. Any one of these events may result in cell death once the homeostatic mechanisms are surpassed.

The first evidence of mitochondrial dysfunction as a causal source of PD was obtained in the 1980s when four students developed marked Parkinsonism after intravenous injection of an illicit drug contaminated with MPTP. Because of the striking Parkinson-like features and additional pathological data, it was proposed that MPTP selectively damaged dopaminergic neurons in the SNpc causing the Parkinson syndromes [127]. Later studies showed that MPTP causes an irreversible destruction of the dopaminergic nigrostriatal pathway that results in symptoms of Parkinsonism in primates and mice [128–130].

In animal models of PD, inhibition of complex I by MPTP or 6-hydroxydopamine (6-OHDA) results in iron accumulation in the SNpc [131, 132]. Importantly, iron chelators effectively abrogate this neurodegenerative process (see below). Thus, with all probability redox-active iron mediates the degenerative process of SNpc neurons induced by inhibition of complex I.

5. IRP1: The Link between Mitochondrial Dysfunction and Iron Dyshomeostasis

Iron Regulatory Proteins 1 and 2 (IRP1 and IRP2) are largely responsible for maintaining cytoplasmic iron levels through the translational regulation of iron homeostasis proteins. IRPs bind to RNA stem loops called iron responsive elements (IREs), which are found in untranslated regions of target mRNAs that encode proteins involved in iron metabolism. Binding of IRPs to IREs in the $5'$-untranslated region inhibits the translation of mRNA, as is the case for the iron-storage protein ferritin. Binding of IRPs to IREs present in the $3'$-untranslated region increases the stability of mRNAs, thus increasing the translation of DMT1 and the transferrin receptor [133, 134].

Importantly, IRP1 activity depends on the protein having or not a 4Fe-4S cluster. Binding of the 4Fe-4S cluster to IRP1 renders the protein inactive to bind to mRNA [135]. Low cell iron induces the dissociation of this 4Fe-4S cluster activating IRP1 and inducing the expression of iron uptake proteins like the transferrin receptor 1 (TfR1) and dimetal iron transporter 1 (DMT1) [136]. Furthermore, IRP1 is sensitive to several oxidative stress stimulus: hydrogen peroxide, nitric oxide, and peroxynitrite all activate IRP1 by induction of ISC disassembly [137, 138], while superoxide inhibits aconitase activity [139].

IRP1 is deregulated in PD tissue, since postmortem brain tissue from PD patients displays increased IRP1 activity when compared to tissue from control individuals. Increased IRP1 activity was found also in the ipsilateral ventral mesencephalon of 6-OHDA-treated rats [140]. Studies performed in our laboratory showed that in SH-SY5Y cells inhibition of complex I by rotenone results in decreased Fe-S cluster synthesis and increased IRP1 mRNA binding activity, accompanied by increased cLIP [61]. Therefore, inhibition of complex I and the subsequent activation of IRP1 lead to increased DMT1 and TfR1 expression, increased iron uptake, and increased ROS generation.

6. Environmental Toxicants, Mitochondrial Dysfunction, and Iron Dyshomeostasis

A considerable body of evidence epidemiologically links exposure to environmental toxicants like paraquat and rotenone to the generation of PD in rural workers [141–144]. The herbicide paraquat is a free radical generator that inhibits mitochondrial electron-transport activity [145–147] and causes dopaminergic neuron loss, α-synuclein aggregation, and motor deficits in rodents, with a dramatic increase in free radical formation [148–150]. Moreover, systemic application of paraquat reduces motor activity and induces dose-dependent loss of striatal tyrosine hydroxylase positive (TH+) fibers and SNpc neurons in mice [151–154]. Paraquat has been proposed to cause Parkinsonism in humans. However, the clinical and epidemiological evidence in this regard is still inconclusive [1, 144, 155, 156]. In fact, paraquat remains one of the most widely used herbicides in developing countries [157, 158].

Although its association with PD is not firmly established, emerging evidence links paraquat exposure to brain iron accumulation. Patients from acute paraquat poisoning displayed excessive brain iron deposition [159]. Similarly, incubation of rat primary mesencephalic cultures with paraquat resulted in increased production of H_2O_2 and Fe^{2+} at times preceding cell death [160]. Mechanistic studies identified m-aconitase from astrocytes as the main mediator in ROS production, although neurons were identified as the primary dying cell type, and death was attenuated by addition of catalase and/or a cell permeable iron chelator [160]. We propose that these results are consistent with a mechanism whereby paraquat affects mitochondrial activity resulting in increased ROS production and increased iron content, a combination that induces neuronal death by hydroxyl radical-mediated damage.

Rotenone is a classic complex I inhibitor [161, 162]. Both rotenone and MPP+ inhibit complex I NADH dehydrogenase, shutting off mitochondrial respiration and causing selective injury of SNpc neurons [128, 163–166]. Rotenone and MPP+ also produce superoxide anion in submitochondrial particles [167–169]. Chronic rotenone administration to mice reproduces Parkinson-like syndromes that include death of SNpc neurons, complex I inhibition, and Lewy bodies-like fibrillar cytoplasmic inclusions containing ubiquitin and α-synuclein [141, 170].

Treatment with rotenone induces iron accumulation in animal and cell models [61, 171]. Rats treated with rotenone evidence iron accumulation in the SNpc, the striatum, the globus pallidus, and other brain areas and treatment with iron chelating agents significantly reduces iron deposition and the loss of dopaminergic neurons in these areas [171]. Similarly, treatment of SH-SY5Y dopaminergic neuroblastoma cells whit rotenone results in mitochondrial iron accumulation and oxidative damage [172]. The mitochondria-tagged iron chelator Q1 abolishes both effects [94]. Overall, these data are consistent with the notion that inhibition of complex I results in the dysregulation of iron homeostasis in dopaminergic cells.

In summary, although the epidemiological evidence that links paraquat or rotenone exposure with PD still needs consolidation, increasing evidence shows that inhibition of mitochondrial activity by these compounds results in iron accumulation. The mechanisms causing this accumulation are unknown. Considering the previous *in vitro* evidences discussed above, iron accumulation may be mediated by activation of IRP1 due to decreased ISC synthesis.

7. PD Genes Associated with Mitochondrial Dysfunction and Iron Accumulation

As detailed below, a wealth of reports indicate that the product of a number of PD-associated genes, including α-syn, Parkin, PINK1, DJ-1, LRRK2, and ATP13A2, disrupts mitochondrial function. Moreover, this disruption is generally associated with increased iron load. Here we will review the evidence that links mitochondrial dysfunction and iron accumulation in familial cases of PD.

7.1. α-Syn. The function of wild type α-syn is still an open issue [173, 174]. There is consensus, however, that misfolding and aggregation of α-syn underlie its toxicity in both PD and Lewy body-associated dementia [173]. Accumulation of cytosolic α-syn can render toxic endogenous dopamine [175] and acts as a seed promoting the formation of cytosolic inclusions [176]. If degradation pathways do not clear these aggregates promptly, neurodegeneration can ensue.

There is a reciprocal relationship between α-syn activity and mitochondrial function; thus, α-syn overexpression in dopaminergic cell lines results in mitochondrial alterations accompanied by increased levels of ROS [177–180]. The N-terminal sequence of α-syn contains a cryptic mitochondrial targeting signal, and α-syn has been localized into mitochondria after acidification of the cytosol or α-syn overexpression [181, 182]. Mitochondrial α-syn decreases the activity of complex I, increases ROS production [183], causes cytochrome c release, increases mitochondrial calcium and nitric oxide levels, and induces oxidative modification of mitochondrial components [184]. Moreover, mice that overexpress α-syn A53T exhibit dysmorphic mitochondria with evidence of DNA damage [185], while administration of MPTP to mice that overexpress α-syn leads to swollen and morphologically abnormal mitochondria [186]. An open issue is whether α-syn aggregation promotes mitochondrial dysfunction or vice versa. Probably both phenomena are interrelated: α-syn induces mitochondrial dysfunction and mitochondrial dysfunction induces α-syn aggregation [187].

Recent evidence suggests that α-syn aggregation induces iron accumulation. In PD patient brains, neurons containing α-syn deposits also display increased iron concentrations and upregulated levels of Nedd4 Family Interacting Protein 1 (Ndfip1), an adaptor for the neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4) family of E3 ligases [188]. Similarly, rat midbrain neurons and PC12 cells overexpressing human α-syn accumulate increased levels of iron and show iron redistribution from the cytoplasm to the perinuclear region within α-synuclein-rich inclusions [189].

Interactions between iron and α-syn most probably contribute to the process of neurodegeneration [190]. Further work indicated that divalent metals, including Fe^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}, bind to the C-terminal of α-syn, and the N-terminus residues 119–124 were recognized as the main binding site of divalent metal ions [191]. Incubation of wild type and mutant α-syn with Fe^{3+} resulted in the formation of short thick fibrils [192]. In BE(2)-M17 cells overexpressing wild type or mutant α-syn (A30P and A53T), treatment with Fe^{2+}, dopamine, and hydrogen peroxide generated α-syn-positive inclusions, which also contained ubiquitin [193]. Similarly, Fe^{2+}-treated BE(2)-M17 cells were more susceptible to Fe^{2+}-induced DNA damage when overexpressing mutant α-syn [194]. In contrast, Mg^{2+} inhibits both spontaneous and Fe^{2+}-induced aggregation of wild type but not A53T α-syn [195], and dopamine suppresses the Fe^{3+}-induced fibrillation of α-syn [196].

Interestingly, α-syn aggregation in turn produces oxidative stress, in a process mediated by metal ions like Fe and Mn, thus generating a vicious cycle between oxidative stress and α-syn aggregation [197–201]. Moreover, pesticides such as rotenone, paraquat and dieldrin, and metal ions (iron, manganese, copper, lead, mercury, zinc, and aluminum) induce a conformational change in α-syn and directly accelerate the rate of formation of α-syn fibrils *in vitro* [202–204]. In addition, the simultaneous presence of metal ions and pesticides leads to synergistic effects on the rate of fibrillation [205].

In summary, there seems to be a cyclic association between α-syn and iron in which α-syn induces iron accumulation and iron induces α-syn aggregation. This cycle is aggravated by α-syn-induced mitochondrial dysfunction. These associations may originate a sequence of events in which α-syn aggregation induces mitochondrial dysfunction, which in turn results in iron accumulation and further α-syn aggregation and hydroxyl radical-mediated damage.

7.2. Parkin. Various mutations in Parkin, an E3 ubiquitin ligase of the ubiquitin-proteasome system, lead to an autosomal recessive PD form, which also is seen in some young-onset sporadic PD cases [206, 207]. Abundant evidence links Parkin to mitochondrial function. Cultured fibroblasts from patients carrying Parkin mutations present longer and more branched mitochondria than controls [208] and leukocyte mitochondrial complex I and IV activities are reduced in PD

patients who are homozygous for Parkin mutations [209]. Parkin-deficient mice have decreased levels of mitochondrial complexes I and IV in the striatum, together with increased protein and lipid peroxidation [210]. In addition, Parkin-null *D. melanogaster* mutants develop muscle degeneration with mitochondrial pathology and display decreased resistance to oxidative stress [211, 212]. Moreover, overexpression of Parkin attenuates the dopaminergic neurodegeneration induced by MPTP through protection of mitochondria and reduction of α-syn in the nigrostriatal pathway [213]. After chronic MPTP administration, Parkin overexpression prevents motor deficits and dopaminergic cell loss in mice [214].

Published observations linking Parkin mutations and iron accumulation are scarce. In an initial study, PD patients carrying Parkin mutations as well as mutation carriers without clinical manifestations of the disease showed increased echogenicity of the SNpc, which in asymptomatic Parkin mutation carriers was associated with abnormal nigrostriatal F-dopa positron emission tomography [215, 216]. Recently, a $R2^*$ relaxometry study in the SNpc of genetic and idiopathic PD patients reported that $R2^*$ values, indicative of iron deposition, were increased in idiopathic PD patients and in patients carrying Parkin and LRRK2 mutations when compared to control subjects [217].

Overall, the bulk of the evidence points to a relationship between Parkin and mitochondria structural functionality. Further investigations are needed to assert if PD Parkin mutations also result in iron dyshomeostasis.

7.3. PINK1. Mutations in PINK1, a serine-threonine protein kinase localized to the mitochondrial membrane via an N-terminal mitochondrial targeting sequence [218], lead to a rare autosomal form of PD. It is generally accepted that PINK1 has a physiological role in mitochondria maintenance, suppressing mitochondrial oxidative stress, fission, and autophagy [219]. PINK1 KO mice exhibit age-dependent moderate reduction in striatal dopamine levels, accompanied by low locomotor activity [220–222]. These mice show no loss of dopaminergic neurons in the SNpc region but display decreased striatal innervations [223, 224], together with decreased mitochondrial respiration and mitochondrial aconitase activity in the striatum [220].

Fibroblasts from patients homozygous for the G309D-PINK1 mutation have reduced complex I activity and evidence oxidative damage compared with cells from control individuals [225]. In flies, PINK1 deficiency results in loss of dopaminergic cells, enhanced susceptibility to oxidative stress, reduced mitochondrial mass with disorganized morphology, and decreased ATP levels [226]. Parkin and PINK1 work in a common pathway, with Parkin acting downstream of PINK1 [226–228]. Under conditions of severe mitochondrial damage, PINK1 and Parkin act to induce mitophagy and mitochondrial membrane depolarization [229]. PINK1 also regulates mitochondrial dynamics through interaction with the fission/fusion machinery [230]. Further genetic studies in *Drosophila* revealed that the PINK1/Parkin pathway regulates mitochondrial morphology by tipping the balance of mitochondrial fission/fusion dynamics toward fission in dopaminergic and hippocampal neurons [230, 231] and muscle cells [232–234].

In SNpc dopaminergic neurons, PINK1 is required to maintain normal mitochondrial morphology and membrane potential, exerting this neuroprotective effects by inhibiting ROS formation [235]. In human dopaminergic neurons, PINK1 deficiency produces mitochondrial dysfunction and marked oxidative stress. These defects result in reduced long-term cell viability, with neurons dying via cytochrome c-mediated apoptosis [236]. Additionally, PINK1 knockdown SH-SY5Y cells show decreased resistance against thapsigargin-induced apoptosis, while PINK1 overexpression restores it [237].

Evidence linking PINK1 and iron is scarce. Patients carrying a PINK1 mutation display a significantly larger area of SNpc echogenicity assessed with transcranial ultrasound relative to healthy controls [238]. In a *Drosophila* model, PINK1 mutants present increased superoxide levels, which induce 4Fe-4S cluster inactivation and increased iron levels in the mitochondrion [239]. As discussed above, decreased ISC synthesis can lead to iron accumulation through IRP1 activation [61].

Overall, published data indicates that under conditions of PINK1 deficiency mitochondrial quality control mechanisms are compromised, resulting in increased ROS production and apoptotic cell death. Up to date, evidence of a relationship between PINK1 loss of function and iron dyshomeostasis is discrete but enticing. The observation of decreased mitochondrial aconitase activity, indicative of a possible decrease in ISC synthesis, and the observed link between PINK1 mutations and superoxide-mediated iron accumulation in mitochondria are powerful incentives to study possible changes in iron homeostasis under PINK1 deficiency and to assess how these changes impact on cell death.

7.4. DJ-1. DJ-1 is a multitask protein that participates in the protection of cells from oxidative stress-related death [240–243]. DJ-1 *null* mice show decreased locomotor activity, a reduction in the release of evoked dopamine in striatum but no loss of SNpc dopaminergic neurons [223, 224]. A relationship between DJ-1 and mitochondrial function has long been suspected [244]; however, DJ-1-null mice show no apparent mitochondrial defects [223, 224]. In contrast, ROS production, mitochondrial structural damages, and complex I deficit are significantly higher in DJ-1-null cultured dopaminergic neurons [245].

To date, the evidence linking DJ-1 and iron is scanty. PD patients carrying DJ-1 mutations have an area in the SNpc of significantly larger echogenicity than in healthy controls [238]. As SNpc hyperechogenicity is related to increased iron content, these findings suggest that DJ-1 mutations may result in iron accumulation.

7.5. LRRK2. LRRK2 is a cytosolic serine-threonine-protein kinase, with a fraction of about 10% associated with the outer mitochondria membrane. Overall, LRRK2 mice models display mild or no functional disruption of nigrostriatal dopaminergic neurons of the SNpc [246]. Recently, a new

LRRK2 knock-in mice evidenced profound mitochondrial abnormalities in the striatum of older homozygous mice, which are consistent with mitochondrial fission arrest described previously [247]. In skin biopsies from human LRRK2 G2019S carriers, however, mitochondrial function and morphology are perturbed, as demonstrated by reduced mitochondrial membrane potential, reduced intracellular ATP levels, mitochondrial elongation, and increased mitochondrial interconnectivity [248]. LRRK2 mutations reduce the activity of peroxiredoxin 3, an antioxidant enzyme located within mitochondria. This effect appears to be phosphorylation-dependent [249, 250].

To date, just a few studies have shown a relationship between LRRK2 dysfunction and iron accumulation. In a recent study determining $R2^*$ relaxometry rate, high nigral iron deposition in LRRK2 mutation carriers was demonstrated [217]. In a small cohort of patients, it was found that $R2^*$ values in the SNpc were increased in idiopathic PD patients and LRRK2 mutation-carrying patients as compared with controls, with LRRK2 mutation patient having larger $R2^*$ values than idiopathic PD patients [217]. Similarly, studies using transcranial sonography showed that LRRK2-associated PD patients had increased iron levels in the SNpc [238, 251]. These evidences support the notion that PD resulting from a variation in the LRRK2 allele has an iron accumulation component that affects neurodegeneration via increased oxidative damage. Further analysis will be required to evaluate this hypothesis.

7.6. ATP13A2. ATP13A2 is a lysosomal P-type 5 ATPase. Mutations in its gene are associated with a juvenile-onset, levodopa-responsive PD type named familial Kufor-Rakeb syndrome [252, 253]. ATP13A2 *null* mice display late-onset sensorimotor deficits and deposition of α-syn aggregates without changes in the number of dopaminergic neurons in the SNpc or in striatal dopamine levels [254]. Arguably, ATP13A2 may help prevent neurodegeneration both by inhibiting α-syn aggregation and by supporting normal lysosomal and mitochondrial function [253].

A relationship between ATP13A2 and mitochondrial function is emerging. Reduced activity of ATP13A2 mutants may lead to mitochondrial defects [255] and higher ROS levels [256]. Fibroblasts from Kufor-Rakeb syndrome patients show lower mitochondrial membrane potential and lower ATP synthesis rates than fibroblast from controls [257]. In addition, overexpression of ATP13A2 inhibits cadmium-induced mitochondrial fragmentation, while silencing ATP13A2 expression induces mitochondrial fragmentation [258]. It remains to be elucidated if ATP13A2-associated mitochondrial dysfunction is due to a primary effect of on mitochondria integrity or is secondary to other event(s), like increased α-syn aggregation.

Two recent studies report neurodegeneration with brain iron accumulation in one Pakistani [259] and one Chilean [257] Kufor-Rakeb syndrome patients. Both patients showed abnormal bilateral hypo intensity in the putamen and caudate nuclei on $T2^*$ diffuse MRI images. In the Pakistani patient case, the clinicians attributed the abnormal MRI hypo intensity to iron deposition [259]. In the Chilean patient, the clinicians attributed the hypo intensity to ferritin deposits though they did not perform tests to exclude the possibility of deposition of other metal ions [257]. However, another study reported opposite results in an adolescent Brazilian patient with homozygous ATP13A2 mutation [260]. It is possible that brain metal ion accumulation only occurs very late in the course of the disease or in cases in which ATP13A2 mutations lead to a total loss of protein function, such as the Pakistani patient described by Schneider et al. [259]. Additional studies in patients with pathogenic ATP13A2 mutations are needed to clarify this point.

In summary, the activities of several PD genes, namely, α-syn, Parkin, PINK1, DJ-1, LRRK2, and ATP13A2, are involved in the maintenance of mitochondrial function and integrity. Mutations in these genes that result in familial PD are accompanied by decreased mitochondrial activity and increased oxidative stress. Emerging evidence points to iron dyshomeostasis as a direct or indirect consequence of decreased mitochondrial activity. There is much to learn regarding the mechanisms linking particular mitochondria-associated PD proteins with iron dyshomeostasis.

The question arises on the reasons why dopaminergic neurons from SNpc are more sensitive to neurodegeneration than similar neurons in the midbrain. Neurons from SNpc have increased IRP1 activity [61, 123, 261] and increased DMT1 expression [262–264] coupled to decreased ferritin expression [265–267], which most probably results in increased redox-active iron and oxidative damage. Similarly, intrinsic L-type calcium channel pace-marker activity and the associated tendency to elevated calcium levels [268, 269] put a metabolic burden in these neurons. Both aspects, iron and calcium burden, are particular factors in SNpc neurons that could be augmented by mitochondrial dysfunction.

8. Iron, Mitochondrial Dynamics, and Mitophagy

Mitochondria are highly dynamic organelles that continuously fuse and divide through the processes of fusion and fission, respectively. Increases in the fission events generate fragmented mitochondria whereas fusion events produce elongated mitochondria. A balance between mitochondrial fusion and fission is important in cellular function [270] and an imbalance can promote neuronal dysfunction and cell death [269, 271]. In neurons, mitochondrial fission is crucial for axonal transport of the organelles into areas of high metabolic demand, whereas mitochondrial fusion supports substitution and regeneration of mitochondrial proteins, mitochondrial DNA repair, and functional recovery. Indeed, enhanced mitochondrial fragmentation was associated with induction of neuronal death triggered by oxidative stress [272].

Dynamin-related protein 1 (Drp1) is a key regulator of mitochondrial fission and it has been associated with neuronal cell death induced by glutamate toxicity or oxygen-glucose deprivation *in vitro* and after ischemic brain damage *in vivo* [273]. Many studies have demonstrated that post-translational modification of Drp1 (phosphorylation, ubiquitination, S-nitrosylation, and others) affects Drp1 activity

and contributes to altered mitochondria dynamics and neurodegeneration in cell culture systems [274–278]. Recently, it was shown that ferric ammonium citrate (FAC) decreased cell viability and promoted cell death of HT-22 cells [279]. The FAC-induced iron overload triggered mitochondrial fragmentation and Drp1(Ser637) dephosphorylation by calcineurin. Iron chelation and pharmacological inhibition of calcineurin prevented mitochondrial fragmentation and apoptotic death. These findings suggest that, under iron-induced toxicity, calcineurin-mediated dephosphorylation of Drp1(Ser637) mediates neuronal cell loss by modulating mitochondrial dynamics [279].

As mentioned above, several groups observed that a deficiency in Parkin and PINK1 leads to mitochondrial pathology [211, 234, 280, 281]. PINK1 overexpression suppressed the translocation of Drp1 from the cytosol to the mitochondria, maintaining mitochondrial function [282]. In Drp1-deficient cells the Parkin/PINK1 knockdown phenotype did not occur, indicating that mitochondrial alterations observed in Parkin- or PINK1-deficient cells are associated with an increase in mitochondrial fission [281]. Moreover, Drp1 seems to activate autophagy/mitophagy pathways for morphologic remodeling of mitochondria in PINK1-deficient neuroblastoma cells [283]. Currently, the inhibition of Drp1 has been proposed as a strategy of neuroprotection in many neurodegenerative diseases because the altered Drp1 activity promotes exacerbated mitochondrial fragmentation.

Iron induces calcium release from intracellular stores, increase that is mediated by the ryanodine receptor (RyR) calcium channel [284]. A recent study showed that in hippocampal neurons iron induced a RyR-dependent increase in mitochondria-associated Drp1 together with increased mitochondrial fragmentation [285]. These results suggest that iron accumulation contributes to mitochondrial fission and, presumably, to the impairment of neuronal function by a mechanism that involves RyR activation, calcium release, and Drp1 activation.

9. Iron Chelation as a Therapeutic Approach for the Treatment of PD

Iron chelators are molecules from different origins with the ability to coordinate iron ions. In general, three distinct groups are identified: siderophores isolated from lithotrophic bacteria, phytochemicals, and synthetic molecules. Historically, the clinical use of these chelators has been focused on the treatment of iron-overload syndromes such as hemochromatosis, β-thalassemia, myelodysplastic syndrome, and other blood transfusion-requiring diseases [286, 287]. As discussed above, however, during the last years a growing set of evidences has demonstrated that many neurodegenerative disorders, prominently PD, present an iron accumulation component in the affected brain areas [7, 288–292]. Desferrioxamine (DFO) in 6-OHDA intoxicated rats provided the first evidence of neuroprotection by iron chelation. Injection of DFO in one cerebral ventricle of rats previously intoxicated showed partial protection from depletion of DA in the striatum and improvement in behavioral tests with respect to the intoxicated rats without DFO administration [293]. Recently, intranasal administration of DFO to the α-syn rat model of PD decreased Fe^{+3} content and the number of α-syn inclusions but did not protect dopaminergic neurons from death [294]. Administration of DFO to endotoxin-shocked mice attenuates the inflammatory response by suppressing the activation of mitogen-activated protein kinase (MAPKs) and NF-κB [295], suggesting an anti-inflammatory effect of DFO. This is a potentially important observation given that inflammation is associated with the dysregulation of iron homeostasis [296–298].

Given the positive effects of DFO and other chelators like clioquinol and deferiprone (DFP) in PD and other models of neurodegeneration [290, 299–301], a series of new 8-OH-quinoline-based chelators was developed, which include VK-28, HLA-20, M30, and VAR. VK-28 [302], HLA-20 [299], M30 [303], and VAR [304] were shown to protect TH+ cells in murine MPTP and 6-OHDA intoxicated models and increase DA content in the striatum. In addition to the 8-hydroxyquinoline chelator moiety, HLA-20, M30, and VAR also have the monoamine oxidase (MAO) inhibitor group propargyl, conforming bifunctional iron chelator/MAO inhibitor drugs. These molecules were demonstrated to chelate iron, decrease DA breakdown, and induce prosurvival factors through putative interactions with signaling components. Indeed, M30 was shown to upregulate protein levels of hypoxia inducible factor 1α (HIF-1α), through decreasing the activity of HIF-degrading enzyme HIF prolyl hydrolase [305–307]. As a consequence, many prosurvival genes controlled by HIF-1α were upregulated after M30 administration, including vascular endothelial growth factor, erythropoietin, enolase-1, transferrin receptor 1, heme oxygenase-1, inducible nitric oxide synthase, and glucose transporter 1 [307]. In addition, mRNAs for brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, and three antioxidant enzymes (catalase, superoxide dismutase-1, and glutathione peroxidase) were also upregulated by M30 administration [307, 308]. Possibly, these later genes are activated through the propargyl moiety via induction of increased phosphorylation of protein kinase C, mitogen-activated protein kinase (MAPK/ERK), protein kinase B, and glycogen synthase kinase-3βs [304]. In addition, Naoi and Maruyama suggested that the propargyl moiety might stabilize the mitochondrial membrane through direct interaction with protein components of the mitochondrial permeability transition pore, leading to increasing levels of antiapoptotic Bcl-2 and Bcl-xL proteins [309]. Supporting the prosurvival effects of iron chelators, a recent study showed that M30 and other hydroxyquinoline-based iron chelators regenerate the neuritic tree in cultured DA neurons treated with sublethal concentrations of MPP+; in addition, M30 given orally regenerated nigrostriatal fibers mouse model after MPTP intoxication [310]. Following the multifunctional approach in iron chelation, others studies tested iron chelators with D2/D3 dopamine receptor agonists to attack the motor symptoms and the oxidative stress simultaneously in the MPTP and lactacystin PD models. Interestingly, the authors found that activation of D3 dopamine receptors was important for the protective effect of these molecules [311, 312].

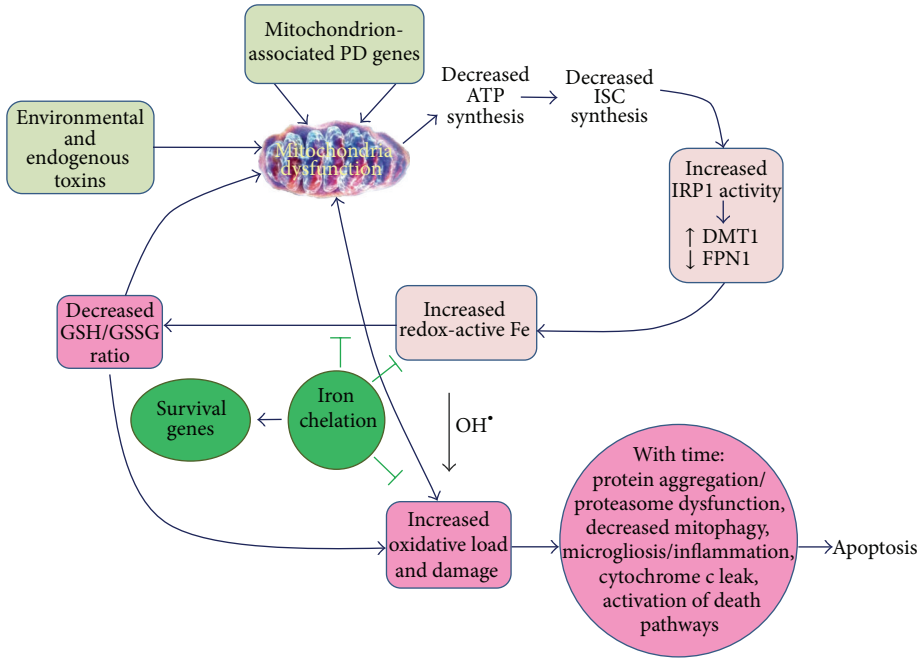

Figure 3: Mitochondrial dysfunction leads to iron accumulation and cell death. Mitochondrial dysfunction in PD, caused either by environmental or endogenous toxins or by genetic dysfunctions, results in decreased ATP and ISC synthesis. The lack of ISCs results in a false low iron signal and the spurious activation of IRP1. Activation of IRP1 results in increased redox-active iron levels mediated by increased expression of DMT1 and TfR1 and decreased expression of FPN1. Because of hydroxyl radical generation through the Fenton reaction, increased redox-active iron results in a decreased GSH/GSSG ratio and an increased oxidative load. The decrease in GSH further affects mitochondrial activity. With time, the increased oxidative load induces protein aggregation and saturation of the ubiquitin-proteasome system, further mitochondrial dysfunction, an inflammatory microenvironment, increased cytochrome c leak, and activation of death pathways. Iron chelation has been demonstrated to slow this cycle by decreasing iron-associated oxidative damage and by induction of cell survival and cell-rescue pathways. Environmental and endogenous toxins: paraquat, rotenone, MPTP, nitric oxide, 4-hydroxynonenal, advanced glycation end products, and aminochrome. Mitochondria-associated PD genes with mitochondrial dysfunction component: α-Syn, Parkin, PINK1, DJ-1, LRRK2, and ATP13A2.

Other studies reported that some phytochemicals evaluated in their capacity to confer neuroprotection in PD models acted through iron chelation [313]. Curcumin, a lyphenolic compound from *Curcuma longa* decreases the iron content in the SNpc of 6-OHDA lesioned rats and partially protects them from the decrease in the number of TH+ cells [314]. Moreover, ginkgetin, a biflavonoid from *Ginkgo biloba*, showed neuroprotection and attenuated the decrease in mitochondrial membrane potential in dopaminergic cell cultures [295]. In addition, ginkgetin enhanced the performance in the rotarod test and attenuated SNpc neuron lost in the MPTP mouse model [295].

Despite the promising character of the field, only the relatively old iron chelator deferiprone (DFP) has been tested in clinical trials for the treatment of PD. DFP is a small lipophilic molecule that is orally active since it crosses the intestinal and blood-brain barriers. DFP also permeates the cell and mitochondrial membranes, interchanging iron between mitochondria, cytoplasm, and extracellular apotransferrin, that is, not only chelating iron but also redistributing it [315]. The ability to "move" iron out of mitochondria is a very important property because, as discussed earlier, the mitochondrion has a prominent reactive iron pool and is the major ROS producer in the cell [28, 94, 316].

A pilot clinical trial of DFP in PD patients, tested with a design comparing the progression in iron content trough MRI and behavior alterations by the Unified Parkinson's Disease Rating Scale, was successful. Comparison between groups that began the treatment with a six-month difference ("early start" and "delay start" groups) showed significant improvement in the parameters in the "early start" group compared with the "delay start" group [317].

A possible drawback of putative iron chelating therapy is that chelators may facilitate the depletion of systemic iron, with severe consequences for other organs like the heart, the liver, and the hematopoietic system [286, 287]. The detected undesirable effects of iron chelation include neutropenia in a small percent of DFP-treated patients [317] and the possibility of high blood pressure resulting from the selective inhibition of peripheral MAO-A by the propargyl moiety of M30 and VAR [304]. Maneuvers designed to counteract these undesirable effects of iron chelation should be sought-after in futures studies.

Clioquinol, recently evaluated in clinical trials [318, 319], presented apparently neurotoxic properties at high doses. Indeed, clioquinol was indicated like the causative agent of subacute myelo-optic neuropathy (SMON) [320], DNA

double-strands breaks induction [321], superoxide dismutase 1 inhibition [322], and nerve growth factor-induced Trk receptor autophosphorylation inhibition [323]. In addition, the clioquinol derivative PBT2 showed low effectiveness and in some cases adverse effects in a recently phase-2 trial for Huntington's disease [324].

Overall, the above evidence shows that iron chelation is a promising therapeutic approach to slow or rescue the neurodegenerative process of PD. The development of new chelators should consider characteristics to make them specific for cell type and effective at lower concentration than those actually in use. A high affinity for iron seems not to be relevant for neuroprotection [325] but as Mena et al. showed [172], mitochondrial targeting should enhance mitochondrial protection and neuroprotective capacity. In summary, the neuroprotective effects of iron chelation reported up to date are a stimuli for the development of new multifunctional iron chelators with blood-brain barrier permeability and mitochondrial targeting, with significant activity at pharmacological concentrations and devoid of noxious side effects.

10. Concluding Remarks

The mitochondrion is the main intrinsic ROS producer in the cell and has an intensive traffic of iron due to the synthesis of ISCs and heme prosthetic groups. Because of the Fenton reaction, mitochondrial levels of ROS and iron need to be tightly regulated to avoid generation of the damaging hydroxyl radical. In both idiopathic and familial cases of PD, mitochondrial dysfunction, iron accumulation, and oxidative damage are commonly found in defective neurons. We propose that these three occurrences are causally linked (Figure 3). Mitochondrial dysfunction, product of endogenous or exogenous toxins, or genetic predisposition results not only in increased ROS production but also in decreased ISC synthesis and IRP1 activation. In turn, IRP1 activation results in iron accumulation and hydroxyl radical-mediated damage. These three events—mitochondrial dysfunction, iron accumulation, and oxidative damage—generate a positive feedback loop of increased iron accumulation and oxidative stress. Intervention at some of these three levels may retard the progression of the disease. Pharmacologically, this effect could be achieved with the use of multifunctional molecules with iron chelation capacity, since iron chelation has been linked to the protection against oxidative damage and the activation of prosurvival pathways.

Disclosure

FONDECYT had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

Competing Interests

The authors have declared that no competing interests exist regarding the publication of this paper.

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Review Article

Activation Mechanism of LRRK2 and Its Cellular Functions in Parkinson's Disease

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Human LRRK2 (Leucine-Rich Repeat Kinase 2) has been associated with both familial and idiopathic Parkinson's disease (PD). Although several LRRK2 mediated pathways and interaction partners have been identified, the cellular functions of LRRK2 and LRRK2 mediated progression of PD are still only partially understood. LRRK2 belongs to the group of Roco proteins which are characterized by the presence of a Ras-like G-domain (Roc), a C-terminal of Roc domain (COR), a kinase, and several protein-protein interaction domains. Roco proteins exhibit a complex activation mechanism involving intramolecular signaling, dimerization, and substrate/effector binding. Importantly, PD mutations in LRRK2 have been linked to a decreased GTPase and impaired kinase activity, thus providing putative therapeutic targets. To fully explore these potential targets it will be crucial to understand the function and identify the pathways responsible for LRRK2-linked PD. Here, we review the recent progress in elucidating the complex LRRK2 activation mechanism, describe the accumulating evidence that link LRRK2-mediated PD to mitochondrial dysfunction and aberrant autophagy, and discuss possible ways for therapeutically targeting LRRK2.

1. Introduction

Parkinson's disease (PD) is a progressive motor disorder that is caused by the degeneration of dopaminergic neurons in the midbrain. The prevalence of PD increases with age, with 2% of individuals over the age of 80 being affected thereby representing the second most common neurodegenerative disorder worldwide [1–3]. Causations are various and mostly divided into a sporadic form without a clear trigger and a familial form in which a genetic factor is involved. The monogenic form of PD is caused by a single mutation in a recessively or dominantly inherited gene. It has been found in sporadic as well as familial PD and accounts for approximately 3–5% and 30% of the cases, respectively [4, 5]. Mutations in SCNA and LRRK2 (Leucine-Rich Repeat Kinase 2) are a specific subset of familial PD as they are autosomal-dominant with LRRK2 representing the most common cause of inherited PD [5]. It belongs to the Roco family of proteins, which constitutes a novel family of Ras-like G-proteins being conserved in almost all kingdoms of life [6–8].

LRRK2 is a large (286 kDa) and complex protein with a unique multiple-domain architecture (Figure 1), consisting of Armadillo repeats (ARM), Ankyrin repeats (ANK), leucine-rich repeats (LRR), a Ras of complex proteins (Roc), a C-terminal of Roc (COR), a kinase domain, and WD40 repeats [2, 6, 7].

Over 40 LRRK2 mutations have been identified representing risk factors for PD [9–11]. Most of the verified pathogenic PD-linked LRRK2 mutations are accumulated around the central core of the protein; one is found in the LRR, one in the Roc domain (with multiple substitutions), one in the COR domain, and two in the kinase domain (Figure 1). The multiple disease-linked mutations in LRRK2 represent a unique opportunity to explore the activation mechanism of the protein, its misregulation in PD, and the underlying molecular mechanisms of genetic and sporadic PD.

In this review, we will focus on the recent progress in elucidating the complex LRRK2 activation mechanism, highlight the evidence for a role of LRRK2 in the mitochondrial

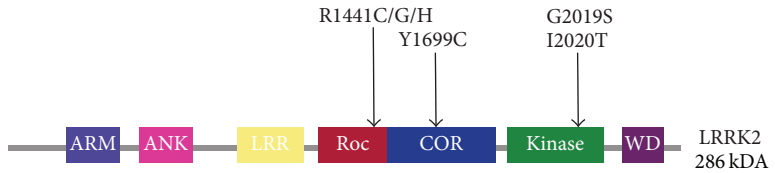

Figure 1: Schematic diagram of the domain architecture of LRRK2. Above, the segregating mutations of LRRK2 in Parkinson's disease are shown (arrows). ARM: Armadillo repeats, ANK: Ankyrin repeats, LRR: leucine-rich repeats, and WD: WD40 repeats.

and autophagy pathways, and discuss possible ways to therapeutically target LRRK2-mediated PD.

2. LRRK2 Kinase and GTPase Activity

LRRK2 has two bona fide enzymatic activities via its Roc (GTPase) and kinase domain. Several studies have shown that the Serine/Threonine specific kinase activity is responsible for LRRK2-mediated PD symptoms, including the degeneration of nigrostriatal dopaminergic neurons and the formation of Lewy bodies [2, 4, 12–14]. While PD-mutated LRRK2 triggers increased inclusion body formation in SH-SY5Y and cell death in primary rat cortical neurons, both of these phenotypes were diminished upon introduction of a LRRK2 kinase dead mutation [15]. For a long time, the function of the kinase domain has been considered as the main output of LRRK2. However, only for the G2019S PD mutation, representing the most common pathogenic point mutation, an increased phosphorylation activity has been reported [16–18]. For other pathogenic mutations, inconsistent, modest, or no effect on kinase activity has been shown [16–18]. Furthermore, PD mutations in LRRK2 probably have different defects in its activation mechanism and it is unclear if all pathogenic effects are mediated via the kinase domain [17–19]. Also the enzymatic activity of the Roc domain is affected in LRRK2-mediated PD-mutants and recent data strongly suggest that PD mutations in both Roc and COR domains result in decreased GTP hydrolysis [18, 20–24]. The Roc domain of LRRK2 belongs to the family of small G-proteins which are GTP binding proteins switching between an active GTP- and inactive GDP-bound state (Figure 2) [25]. Studies with both LRRK2 and an amoebic homologue revealed that a functional Roc domain is essential for kinase activity and disruption of Roc or the kinase domain by a single point mutation leads to the complete inactivation of the protein [15, 22, 23, 26]. *In vivo* studies with LRRK2 G2019S showed that primary neurons possess a lower level of toxicity after the GTPase function was abolished [27]. Further studies confirmed that GTPase activity is central for neuronal toxicity and LRRK2 pathobiology in human cell lines and model organisms [20–22]. However, the data prove the involvement of both enzymatic activities in the onset of PD and imply a present cross-talk between the two domains.

3. LRRK2 Activation Mechanism

The exact molecular mechanism by which the catalytic activity of LRRK2 is regulated remains unknown; however,

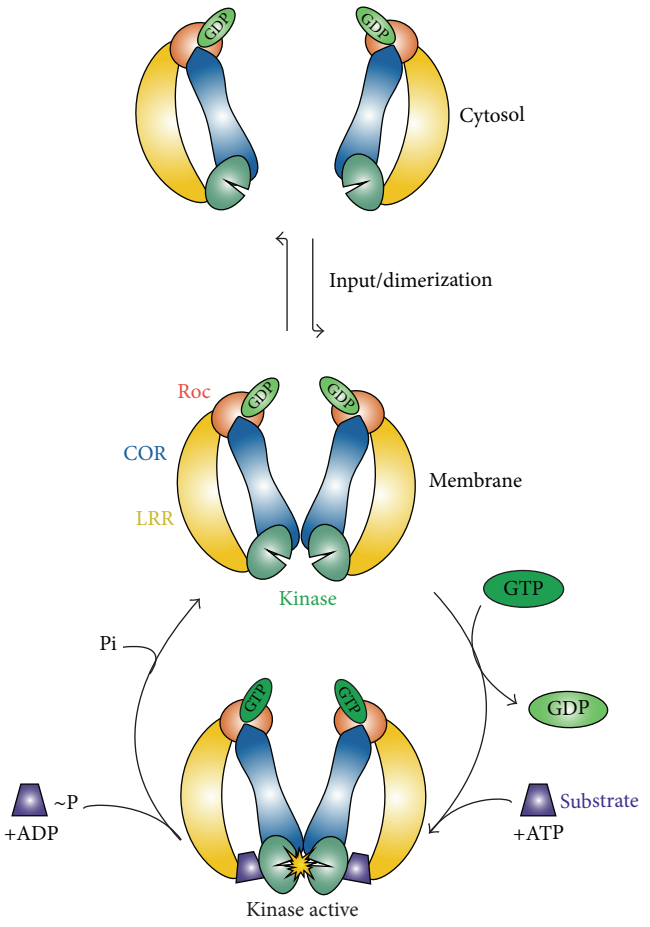

Figure 2: Proposed model of the activation mechanism of LRRK2. LRRK2 activation is at least regulated by three different mechanisms: cycling between (1) an almost inactive monomer and active dimer at the membrane, (2) intramolecular activation, and (3) binding of input/substrate to the N- and C-terminal domains.

accumulating evidence suggests the involvement of at least three different mechanisms: dimerization in close association with localization, intramolecular activation, and binding of input/substrate to the N- and C-terminal domains (Figures 2 and 3).

LRRK2 is monomeric and almost inactive in the cytosol, while it is predominantly dimeric and active when localized at the membrane [28–32]. Membrane enriched LRRK2 displayed an enhanced molecular mass as well as a 8.4 times

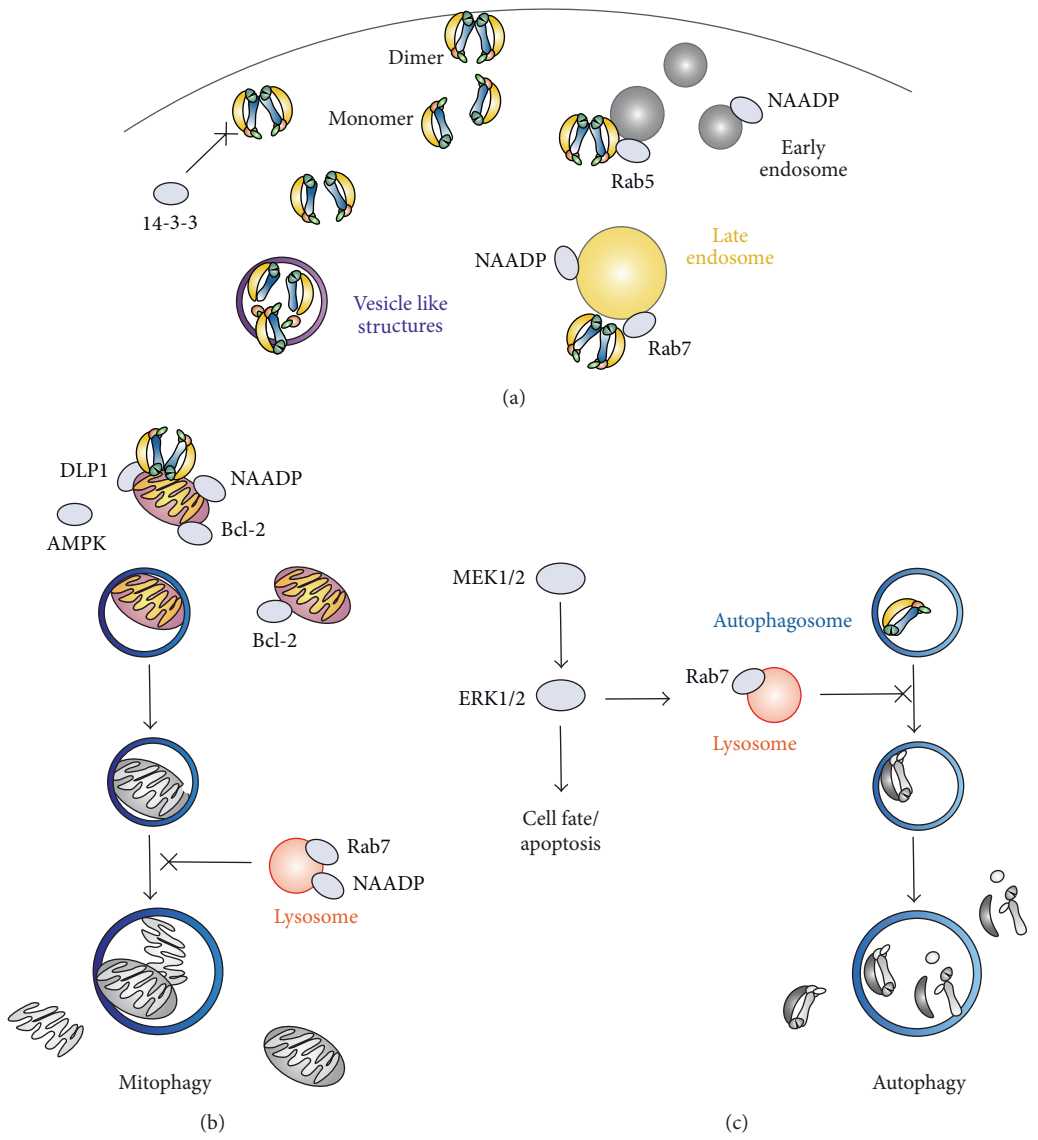

Figure 3: Proposed pathways regulating LRRK2-mediated mitochondrial homeostasis and autophagy.

higher kinase activity in comparison to the cytosolic LRRK2 suggesting that localization is dependent on and affects phosphorylation [31, 33, 34]. Structural studies with bacterial Roco proteins have revealed that the COR domain functions as an essential dimerization device [35]. During dimerization, the catalytic machinery for the GTPase reaction is being formed by complementation of the active site of one protomer with the other protomer [33, 35]. COR truncated proteins that are not able to dimerize have a drastically lower (700 times) GTPase activity. Interestingly, abolishing dimerization also alters autophosphorylation levels, indicating that both enzymatic activities are critically dependent on dimerization [35–38]. In this way, the intramolecular GTPase reaction functions as a timing device for the activation and biological functions of Roco proteins. Interestingly, a recent study has shown that mutations of known phosphorylation sides in the G-domain affect both kinase and GTPase activity [30]. Together the data suggest that the Roc-COR tandem is regulating kinase activity, the kinase is regulating the GTPase activity of Roc, and both events are critically involved in LRRK2 cellular distribution.

LRRK2 dimerization and activation is regulated by the N- and C-terminal LRRK2 protein-protein interaction domains. Cellular studies with LRRK2 and related Roco proteins lacking the N- or C-terminus suggested their essential role for signaling *in vivo* [7, 39].

Deletion of the WD40 repeats led to impaired dimer formation accompanied with diminished kinase activity and aberrant protein localization [40]. Recent data suggest that the N-terminus inhibits LRRK2 kinase activity, since deletion of the terminus resulted in increased LRRK2 autophosphorylation levels when expressed in human cell lines [32]. On the contrary, LRRK2 G2019S PD mutation displayed increased kinase activity with a lower level of autophosphorylation of

the N-terminus (S910/935~P) [32, 41]. Although the N- and C-terminus of LRRK2 have an essential role *in vivo*, they are not required for kinase activity *in vitro* [7, 39]. This might suggest that the N- and C-terminal protein-protein interaction domains regulate LRRK2 activity by binding to upstream and/or downstream effectors. In this perspective, it has been shown that the N-terminal segment of LRRK2 interacts in a phosphorylation dependent manner with the ubiquitous regulatory protein 14-3-3. Disruption of the phosphorylation sides S910 and S935 blocks 14-3-3 binding and leads to the delocalization of LRRK2 from the membrane and its accumulation in the cytosol (Figure 3) [34, 41]. Recently, members of the Rab family of small GTPases have been identified as valid LRRK2 interactors and substrates [42–44]. *In vivo* studies confirmed direct binding, most likely mediated via the N-terminus, and colocalization of LRRK2 with Rab5 and Rab7, suggesting an involvement in degradative and endocytic membrane trafficking (Figure 3). Strikingly, the PD mutation G2019S disrupted molecular trafficking and colocalization with Rab7, resulting in the formation of aberrant endosomal structures and endosomal/lysosomal localization thus interfering with the cellular degradative trafficking pathway of organelles [45, 46]. Furthermore, LRRK2 binding to Rab32 is regulating its localization to lysosomes as well as mitochondria [47].

4. LRRK2-Mediated Mitochondrial Dysfunction, Autophagy, and Cell Death

Numerous potential LRRK2 mediated pathways have been identified; however, much about its cellular functions and LRRK2 mediated progression of PD remains unknown. Accumulating evidence links LRRK2-mediated PD to mitochondrial dysfunction and aberrant autophagy (Figure 3) [48–51]. LRRK2 transfected HEK-293T cells showed a 10% enhanced localization of LRRK2 to the outer but not inner mitochondrial membrane [51]. The morphology and interconnectivity of mitochondria in skin samples of G2019S carrier patients were detected to be abnormal, most likely due to dysregulated fission and fusion events [50]. Analysis of the substantia nigra of patients with idiopathic PD revealed a glutathione depletion and mitochondrial complex-I deficiency, both representing known indicators of oxidative stress [52]. Furthermore, polymorphism in mtDNA (mitochondrial DNA) and aberrant levels of the neurotoxin MPP+ (1-methyl-4-phenylpyridinium) and its precursor MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) were found in patient samples, various model organisms, and human culture cell lines [53]. In neurons it was shown that LRRK2 colocalizes with the Dynamin like protein 1 (DLP1), a known mitochondrial fission factor (Figure 3). Expression of LRRK2 G2019S and R1441C in neurons induced mitochondrial fragmentation and increased their interaction rate with DLP1 which also displayed higher phosphorylation levels, resulting, among others, in an enhanced level of reactive oxygen species (ROS). All these defects could be rescued by silencing of DLP1, suggesting a LRRK2/DLP1 pathway regulating mitochondrial fission events and their clearance [51, 54]. Localization of LRRK2 is not limited to mitochondrial structures but was found at a variety of additional membranes, including multivesicular bodies (MVBs) representing autophagic vacuoles (AVs) [55]. Consistently, being involved in the regulation of the endosomal-autophagic pathway, expression of PD-mutated LRRK2 triggered the accumulation of (abnormal) MVBs and AVs via misbalancing the induction of macroautophagy and maturation of AVs to lysosomes (Figure 3) [55]. Furthermore, expression of LRRK2 G2019S in human cell lines led to the shortening of neurite length and an increase in autophagic vacuole levels [15, 56].

The pathways regulating and linking LRRK2 PD-mediated mitochondrial dysregulation and abnormal autophagy are only partly identified but most likely include the activation of the autophagy regulating protein 5′ AMP-activated protein kinase (AMPK) [45, 57]. The abnormal kinase activity of LRRK2 G2019S in human cell lines leads to an increased level of phosphorylated AMPK, which subsequently results in enhanced levels of autophagosomes [58]. The mitogen-activated protein kinases (MAPK) cascade may represent another important pathway regulating LRRK2-mediated autophagy. In addition to enhanced autophagic activity and cell death, cells expressing LRRK2 G2019S also showed a threefold increase in protein turnover and a higher level of phosphorylated MAPK/ERK. Incubation with a specific inhibitor of MEK1/2 (U0126) was sufficient to rescue the aberrant phenotypes of the LRRK2 G2019S cells [56, 59]. It was suggested that LRRK2 induces autophagy via the activation of NAADP (nicotinic acid adenine dinucleotide phosphate) receptors, which are involved in the calcium efflux from endosomes [58]. The mitochondrial antiapoptotic protein, Bcl-2, might represent the connection between LRRK2-induced dysregulated mitochondrial homeostasis and autophagy. Expression of phosphorylated Bcl-2 rescues both the mitochondrial and autophagy defects of LRRK2 G2019S cells [60].

Several other PD associated proteins, including α-synuclein, Parkin, DJ-1, PINK1, and HtrA2, have been linked to similar defects in mitochondria regulation and autophagy [37, 61, 62]. Parkin, a known regulator of mitochondrial clearance, and AMPK seem to be directly involved in an alternative or parallel pathway as overexpression acted protectively against cellular toxicity in fly dopaminergic neurons expressing mutated LRRK2 [61]. Mutations in Parkin and PINK1 (PTEN-induced kinase 1), both mitochondria regulating proteins, have been found in sporadic as well as autosomal recessive PD and result in severe mitochondrial abnormalities and cell death [63]. The parallel expression of PD-LRRK2 in PINK1 and DJ-1 deficient fly cells or mice neurons with abnormal α-synuclein activity leads to an increase of respective pathogenic phenotypes [64, 65]. Deletion of LRRK2 acts in a neuroprotective way towards α-synuclein mediated effects in mouse models [65]. DJ-1 is only partially able to rescue the phenotypes of PINK1 mutated neurons but, vice versa, overexpression of both Parkin and PINK1 restores the abnormal mitochondrial morphologies of DJ-1 deficient cells, suggesting a present connection between the involved pathways [66, 67]. Altogether it might suggest the presence of common PD-pathogenic pathways that result in mitochondrial dysfunction and autophagy.

5. Therapeutic Targeting of LRRK2

The major focus of academia and industry is the development of kinase inhibitors as potential therapeutics for LRRK2-mediated PD. Almost all clinical kinase inhibitors are used for short-time treatment in the cancer field and for immunological, neurological, and infectious diseases, where side effects caused by high dosage are tolerated [68]. In contrast, for the long-term treatment of chronic diseases such as LRRK2-associated PD no potential toxic side effects can be present. Several highly specific and brain penetrant LRRK2 kinase inhibitors were identified but have yet to be optimized in order to qualify as drug candidates for therapeutic treatment [69–72]. Our structures of a humanized *D. discoideum* Roco4 kinase domain bound to the common inhibitors LRRK2-IN-1 or Compound 19 revealed a highly similar binding mechanism and gave important information for potential optimization [73]. However, accumulation in peripheral tissues, especially kidneys and lungs, and related drug induced toxicity are still a major and common problem for all LRRK2 kinase inhibitors [72, 74, 75]. In rodent models, enhanced dosages of the recent highly specific and brain penetrant LRRK2 kinase inhibitors GNE-7915 and GNE-0877 are well tolerated over a longer time period; however, they induced the cytoplasmic accumulation of lysosome-related organelles in the lungs of nonhuman primates [76].

Understanding how other domains of LRRK2 modulate its activity is an important but rather neglected field in LRRK2 research and not a focus of industries. However, the PD causing mutations are found in nearly all domains of LRRK2 leading to the same well described symptoms. Furthermore, as recent data suggest that different PD mutations have diverse defects with regard to the activation mechanism, they might require specified ways of inhibition for the purpose of drug development [72, 74, 77].

Alternative approaches targeting further LRRK2 domains and sites of its complex activation mechanism, including the N- and C-terminus, the catalytic GTPase activity of Roc, LRRK2 localization, dimerization, or allosteric modulation of the kinase domain, might significantly improve therapeutic benefits (Figure 2).

The LRRK2 mutations in the Roc (R1441C/G/H) and COR (Y1699C) domain have a decreased GTPase activity and a functional LRRK2 G-domain is essential for LRRK2 activation, suggesting GTPase activity forms an interesting therapeutic target [20, 78–80]. Targeting the G-domain could be done by using small compounds that bind and interfere with nucleotide binding, resemble the GDP-bound off-state, or increase the GTPase cycle. Recently, the first LRRK2 GTP binding inhibitors, compounds 68 and 70, were identified and proved to inhibit both GTPase and kinase activity *in vitro* as well as *in vivo* and thereby attenuated neuronal degeneration in human cell lines/rodent tissues [14]. Importantly, FX2149, a novel analog of 68, even displayed an around two times higher brain inhibition efficiency in a rodent model organism [81].

The N- and C-terminal segments of LRRK2 contain several protein-protein interaction domains which are involved in regulating kinase activity, oligomerization, and/or localization. As described above, LRRK2 cycles between a low active monomeric cytosolic state and a high active dimeric membrane bound state. Importantly, since LRRK2 activation is dependent on membrane localization and dimerization, inhibiting either of these properties may be a good therapeutic approach.

6. Summary

Recent studies have shed light on the complex activation mechanism of LRRK2 and revealed highly precise and exact timed interactions on both intra- and intermolecular levels. These multiple layers of regulation and enzyme activities within one protein make LRRK2 an interesting therapeutic target. To further explore these therapeutic targets, it will be essential to completely characterize the molecular activation mechanism. Biochemical and structural characterization of LRRK2 and/or related Roco proteins can give important information about the dimerization mechanism, how the kinase domain regulates GTPase activity, how LRRK2 activity is regulated by binding of input or substrate to the LRR and WD40 domains, and how the PD mutations influence the complex regulatory mechanism. Recent data strongly suggest that LRRK2 dysfunction in PD results in mitochondrial defects and autophagy. However, the precise underlying mechanisms are still not well understood and many questions about the cellular function of LRRK2 remain to be addressed, including at which (inter)cellular membrane LRRK2 is activated and if common underlying pathways of familial PD are existing. To answer these questions, it will be crucial to identify physiological kinase substrate(s) and upstream and downstream regulators.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Protection against Mitochondrial and Metal Toxicity Depends on Functional Lipid Binding Sites in ATP13A2

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The late endo-/lysosomal P-type ATPase ATP13A2 (PARK9) is implicated in Parkinson's disease (PD) and Kufor-Rakeb syndrome, early-onset atypical Parkinsonism. ATP13A2 interacts at the N-terminus with the signaling lipids phosphatidic acid (PA) and phosphatidylinositol (3,5) bisphosphate (PI(3,5)P2), which modulate ATP13A2 activity under cellular stress conditions. Here, we analyzed stable human SHSY5Y cell lines overexpressing wild-type (WT) or ATP13A2 mutants in which three N-terminal lipid binding sites (LBS1–3) were mutated. We explored the regulatory role of LBS1–3 in the cellular protection by ATP13A2 against mitochondrial stress induced by rotenone and found that the LBS2-3 mutants displayed an abrogated protective effect. Moreover, in contrast to WT, the LBS2 and LBS3 mutants responded poorly to pharmacological inhibition of, respectively, PI(3,5)P2 and PA formation. We further demonstrate that PA and PI(3,5)P2 are also required for the ATP13A2-mediated protection against the toxic metals Mn^{2+}, Zn^{2+}, and Fe^{3+}, suggesting a general lipid-dependent activation mechanism of ATP13A2 in various PD-related stress conditions. Our results indicate that the ATP13A2-mediated protection requires binding of PI(3,5)P2 to LBS2 and PA to LBS3. Thus, targeting the N-terminal lipid binding sites of ATP13A2 might offer a therapeutic approach to reduce cellular toxicity of various PD insults including mitochondrial stress.

1. Introduction

Mitochondria are organelles with a pivotal role in ATP production, intracellular Ca^{2+} signaling, the generation of reactive oxygen species (ROS), and apoptotic cell death [1–3]. Because of high energy demands at locations distant from the cell body, neurons in particular critically depend on healthy and dynamic mitochondria to fuel membrane excitability and to execute neurotransmission and plasticity [4, 5]. Not surprisingly, defective mitochondrial dynamics is implicated in various neurological disorders, including Parkinson's disease (PD), a common progressive movement disorder characterized by a severe loss of dopaminergic neurons in the *substantia nigra pars compacta* [6]. PD is hallmarked by the accumulation of aggregated α-synuclein into Lewy bodies in neurons of the *substantia nigra* and specific brain stem, spinal cord, and cortical regions [7, 8], but also mitochondrial defects are common [9–11]. Strong support for mitochondrial dysfunction in PD comes from the observations that 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), a potent mitochondrial complex I inhibitor, triggers a PD-like syndrome [12, 13]. Moreover, several PD-associated genes, mainly parkin and PINK1, play a role in mitochondrial dynamics and clearance via mitophagy, which strengthens

the concept that mitochondrial dysfunction and/or impaired mitochondrial clearance are tightly linked to PD onset [9, 10].

In the present study, we focus on *ATP13A2/PARK9*, encoding a late endo-/lysosomal membrane protein, which belongs to a poorly characterized subfamily of P-type ATPases, namely, the P5-type transporters with unassigned function and substrate specificity. So far, the three-dimensional structure of ATP13A2 is unknown but can be modelled based on the known structures of other P-type ATPases (i.e., SERCA1a, H^+-ATPase, Na^+/K^+-ATPase, and Cu^{2+}-ATPase). Mutations in *ATP13A2* are associated with PD and Kufor-Rakeb syndrome, which is a severe early-onset autosomal recessive form of PD with dementia [15]. ATP13A2 provides cellular protection against metal toxicity induced by Mn^{2+} [16, 17], Zn^{2+} [18], and Fe^{3+} [19], which are considered as environmental risk factors of PD. In addition, ATP13A2 provides protection in several models of α-synuclein toxicity [16, 18]. Although loss of ATP13A2 leads to lysosomal dysfunction [20], interestingly, a strong link between ATP13A2 and mitochondrial dysfunction/clearance is emerging. Fibroblasts of patients with nonfunctional ATP13A2 exhibit general mitochondrial dysfunction, including decreased ATP production, enhanced oxygen consumption rates, and fragmentation of the mitochondrial network [21]. Furthermore, knockdown (KD) of ATP13A2 in mouse cortical neurons or human neuroblastoma SHSY5Y cells triggers mitochondrial fragmentation and ROS production [22], whereas ATP13A2 deficiency in patient-derived olfactory neurosphere cultures results in Zn^{2+} dyshomeostasis, which contributes to mitochondrial dysfunction [18]. Finally, in a cellular PD model in which SHSY5Y cells were exposed to the mitochondrial complex I inhibitor rotenone to induce mitochondrial stress, ATP13A2 activity confers cytoprotection, since overexpression of WT ATP13A2, but not a catalytic dead mutant, protects against, whereas KD of ATP13A2 exacerbates cell toxicity [14, 23].

Of interest, the signaling lipids phosphatidic acid (PA) and phosphatidylinositol (3,5) bisphosphate (PI(3,5)P2) interact at the ATP13A2 N-terminus and stimulate the autophosphorylation reaction, which is a hallmark of its catalytic activity [14]. PA is a conical phospholipid that can alter membrane curvature or act as a local signaling lipid, for example, produced by phospholipase D (PLD). The phosphoinositide PI(3,5)P2 is formed by the PIKfyve lipid kinase and mainly resides in the late endo-/lysosomes where it functions as an organelle tag. Pharmacological inhibition of PLD (to prevent PA production) or PIKfyve (to prevent PI(3,5)P2 generation) counteracts the ATP13A2-mediated protective effect on rotenone-induced mitochondrial toxicity [14, 23]. Together, these results indicate that PA and PI(3,5)P2 are required for the activation of ATP13A2 in conditions of mitochondrial stress. Three putative lipid binding sites (LBS1–3) were previously identified in the ATP13A2 N-terminus via protein lipid binding assays with purified mutant and WT N-terminal protein fragments of ATP13A2 (Figure 1(a)) [14].

To test whether PA and PI(3,5)P2 are key mediators of the cytoprotective effect of ATP13A2 under conditions of mitochondrial stress by direct interaction with and activation of the full length ATP13A2, we here compared SHSY5Y cell lines stably expressing WT ATP13A2 or LBS1–3 mutants. We further explored whether a similar PA- and PI(3,5)P2-dependent ATP13A2 activation mechanism may also provide cellular protection against metal (Mn^{2+}, Zn^{2+}, and Fe^{3+}) induced cytotoxicity.

2. Materials and Methods

2.1. Cell Culture. SHSY5Y neuroblastoma cell lines stably expressing firefly luciferase (FLUC, control), WT ATP13A2, mutants of the three putative N-terminal LBS (LBS1: ^{65}FRWKP→FAWAP; LBS2: ^{74}RLRLR→ALALA; LBS3: ^{155}KRVLR→AAVLA; LBS1.2.3: combination of LBS1–3 mutations), or sh-ATP13A2 (KD [14, 23]) were generated via lentiviral transduction and maintained as described previously [14].

2.2. Drug Treatments. Cells were exposed to rotenone (Rot, 1 μM; R8875, Sigma), MPP+ (50 μM, DO48, Sigma), zinc ($ZnCl_2$, 150 μM; Z0152, Sigma), manganese ($MnCl_2$, 2 μM; 205891000, Acros Organics), and iron ($FeCl_3$, 1.5 mM; 157740, Sigma) for 24 hours (h). The final concentration of each agent was chosen from a dose response analysis to obtain a submaximal inhibition of cell viability. Prior to stressor addition, cells were pretreated for 1 h with YM-201636 (PIKfyve inhibitor, PIK, 200 nM; 524611, Millipore) or 5-fluoro-2-indolyl deschlorohalopemide (PLD inhibitor, FIPI, 100 nM; F5807, Sigma) to inhibit the production of PI(3,5)P2 or PA, respectively. To inhibit the proteasome, cells were incubated with MG-132 (100 μM; M7449, Sigma) for 6 h.

2.3. Fluorescence Microscopy. Cells were fixed with 4% paraformaldehyde at 37°C (30 min) and permeabilized with 0.1% Triton X-100 at room temperature (10 min). After blocking in PBS containing 1% BSA and 10% goat serum for 1 h at room temperature, samples were incubated with primary antibodies targeting ATP13A2 or LAMP-1 (Sigma) overnight at 4°C. Thereafter, samples were washed and exposed to Alexa Fluor 488 (green) or Alexa Fluor 647 (red) secondary antibodies. Cells were counterstained with DAPI (1 μg/mL) for 10 min, mounted using Prolong Gold antifade reagent, and cured overnight. Images were acquired with an Olympus IX73 fluorescent microscope using a 63x objective and dimension cellSens software. Scale bars represent 10 μM.

2.4. Cell Death. Cell death was determined by propidium iodide (PI) exclusion. Briefly, cells were trypsinized at the indicated time points and incubated with 1 μg/ml PI. PI-positive (dead) cells were quantified via flow cytometry (Attune Cytometer, Life Technologies).

2.5. Cell Viability. Cells were seeded at 5000 cells per well of a 96-well plate. Following treatment cells were washed with PBS and incubated with 0.01 mg/mL MUH (4-methylumbelliferyl heptanoate, Sigma) dissolved in PBS for 30 min at 37°C. Fluorescence was measured with a Flex Station plate reader (Molecular Devices) with excitation 355 nm, emission 460 nm, and cutoff value of 455 nm.

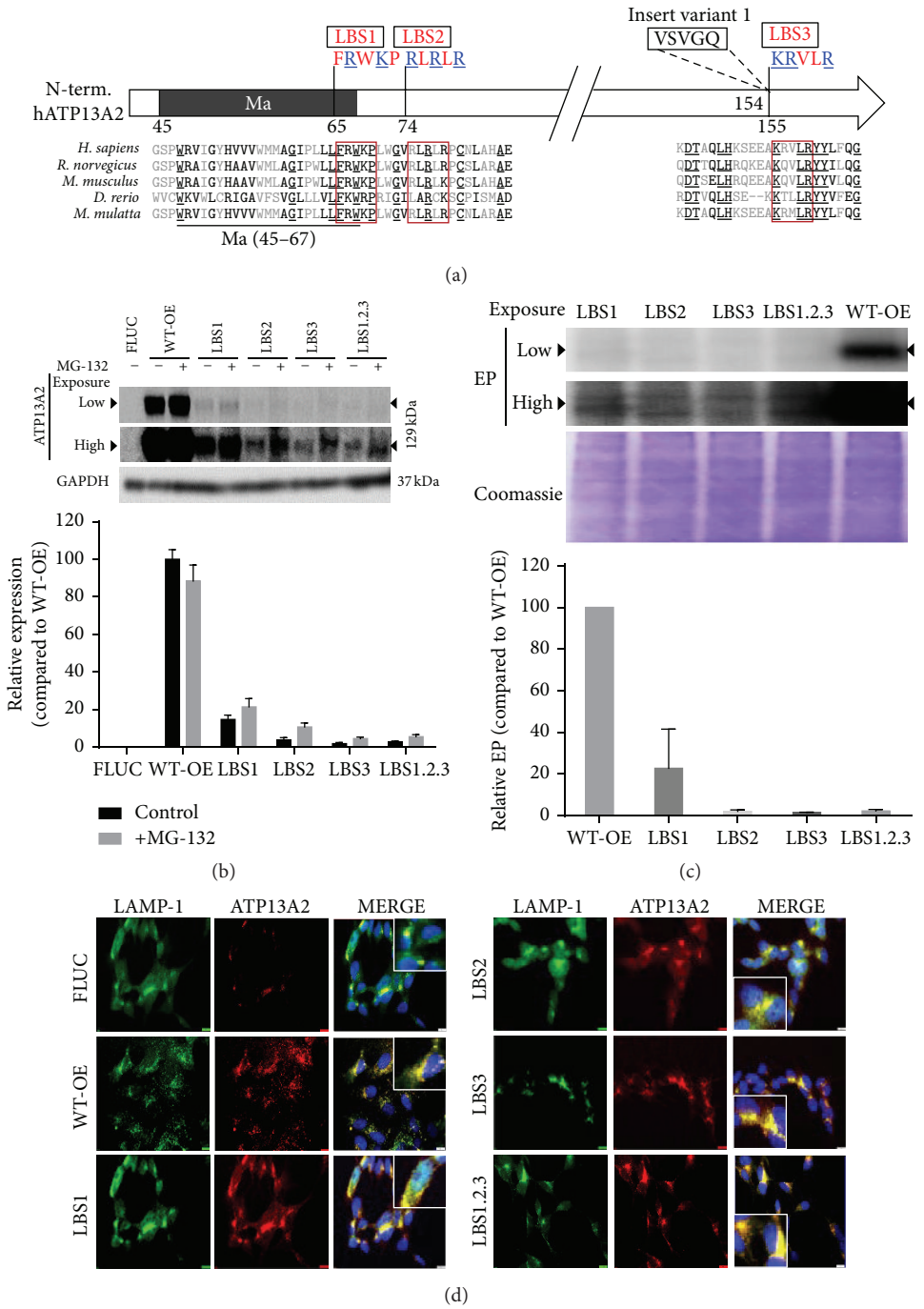

Figure 1: Mutations in the key lipid binding sites of ATP13A2 inhibit activity but not subcellular targeting. (a) Multiple positively charged residues in previously identified lipid binding sites [14] (blue, underlined) were substituted for Ala. LBS3 overlaps with an alternative splicing site rendering an insertion of five additional residues in splice variant 1 of ATP13A2. Ma, membrane-associated region. (b) Expression levels of ATP13A2 were analyzed by immunoblotting with a primary anti-ATP13A2 antibody in comparison to GAPDH as a loading control. Breakdown of LBS mutant proteins by the proteasome was assessed via treatment with the proteasome inhibitor MG-132 (100 μM) for 6 h. The same gel for the expression of ATP13A2 between cell lines has been provided at both low and high intensities. OE, overexpression. (c) Autophosphorylation assay (EP) on microsomes of ATP13A2 WT or LBS mutant expressing SHSY5Y cells. At the top, the same gel was depicted twice at different exposure times. Below, equal protein loading on the SDS-PAGE gel was confirmed by Coomassie staining. We consider that the double bands visible in LBS2 and LBS3 at longer exposure time are background levels while the actual ATP13A2 related EP band (visible in LBS1 and WT-OE) is located in between, but closer to the double band. Quantification of ATP13A2 expression ((b) ATP13A2/GAPDH) and autophosphorylation levels ((c) EP/Coomassie) are depicted. (d) Expression and localization of endogenous (FLUC), WT-OE, LBS1, LBS2, LBS3, or LBS1.2.3 ATP13A2 were confirmed by colocalization experiments with the lysosomal marker LAMP-1 and captured using Olympus IX73 fluorescent microscope. Representative enlarged images of colocalization for all cell lines have been provided as image inserts. Data are representative of 3 independent experiments. Scale bars represent 10 μM.

2.6. Cellular Fractionation. Cells were seeded in 15 cm dishes at a density of 1.5×10^6. After treatment, cells were harvested and resuspended in hypotonic buffer (10 mM Tris-HCl pH 7.5, 0.5 mM $MgCl_2$, and SigmaFast protease inhibitor cocktail (Sigma)) and a 10 min incubation period on ice. Cells were homogenized by applying 40 strokes in a Dounce homogenizer and after adding the 1 M buffer solution (0.5 M sucrose, 10 mM Tris-HCl pH 7.3, 40 μM $CaCl_2$, 0.23 μM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT)) 20 additional strokes were performed. The total cell lysate was subjected to differential centrifugation: the nuclear fraction (1,000 g; 10 min), the mitochondrial/lysosomal fraction (12,000 g; 20 min), and, lastly, the microsomal and cytosolic fractions (200,000 g; 35 min; pellet and supernatant, resp.). Microsomal pellets were resuspended in a 250 mM sucrose solution supplemented with protease inhibitor cocktail. All fractionation steps were carried out at 4°C. After solubilization, samples were aliquoted, flash-frozen in liquid nitrogen, and stored at −80°C. The protein concentration was determined by the Qubit fluorometric method (Life Technologies).

2.7. Immunoblotting. 10 μg protein of the microsomal fractions was separated on precast NuPAGE 4–12% BisTris gels using MOPS running buffer (Life Sciences), followed by transfer onto polyvinylidene fluoride membranes (Millipore) according to the manufacturer's instructions. After blocking in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) supplemented with 5% nonfat dry milk and 0.1% Tween-20 (Sigma), blots were incubated for 1 h with primary polyclonal anti-ATP13A2 (1/1,000 dilution; A3361, Sigma) and anti-GAPDH antibodies (1/5,000 dilution; G8795, Sigma) and 45 min with horseradish peroxidase conjugated IgG secondary antibodies (1/2,000 dilution; Bioke). Expression was detected using enhanced chemiluminescence substrate (Pierce) and the Bio-Rad ChemiDoc MP imaging system. Quantification was performed with ImageJ software (http://rsbweb.nih.gov/ij/).

2.8. Autophosphorylation Assay. 40 μg of the microsomal fraction was added to a final volume of 95 μL of reaction buffer (160 mM KCl, 17 mM Hepes, 2 mM $MgCl_2$, 1 mM DTT, and 5 mM NaN_3). The autophosphorylation assay was started by adding [γ-^{32}P] ATP (2 μCi) and stopped after 1 min with 400 μL of ice-cold stop solution (20% trichloroacetic acid, 10 mM phosphoric acid). Samples were incubated on ice for 30 min to precipitate protein and centrifuged at 20,000 g for 30 min at 4°C. The pellet was washed twice with 400 μL of ice-cold stop solution and finally dissolved in sample buffer (10% LDS, 10 mM NaH_2PO_4, 0.01% SDS, 10 mM 2-mercaptoethanol, and 0.15 mg/mL bromophenol blue). After loading the samples on NuPAGE 4–12% BisTris gels (Life Sciences), electrophoresis was conducted for 1.5 h (40 mA, 170 V) in running buffer containing 0.1% SDS and 170 mM MOPS (pH 6.3). Following fixation in 7.5% acetic acid, the gel was exposed to a PhosphorImager screen (GE Healthcare) and, the next day, radioactivity was visualized in a PhosphorImager scanner (Storm 860, GE Healthcare). Quantification was performed with Image QuanT (Molecular Dynamics) and ImageJ software packages (http://rsbweb.nih.gov/ij/).

2.9. Statistical Analysis. Data are presented as the average ± SD of three independent experiments. Statistical analysis was conducted by one-way analysis of variance (ANOVA) with either Dunnett's or Bonferroni post hoc corrections. Consider $^{*/\$}P < 0.05$, $^{**/\$\$}P < 0.01$, and $^{***/\$\$\$}P < 0.001$.

3. Results

3.1. LBS2-3 Mutations Prevent ATP13A2 Activation and Cytoprotection. Previously, using purified N-terminal fragments of ATP13A2 in protein lipid overlay assays, we described that LBS1 and LBS2 are mainly required for interaction with PI(3,5)P2 (PA to a lesser extent), whereas LBS3 is essential for PA interaction [14] (Figure 1(a)). Here, we tested whether the protective effect of ATP13A2 in conditions of rotenone-induced mitochondrial toxicity can be explained by a specific and direct interaction of PI(3,5)P2 and PA to the full length ATP13A2 protein. To that end, we generated stable SHSY5Y cell lines with ATP13A2 KD (75.4 ± 8.9% reduction in ATP13A2 mRNA levels as compared to FLUC [14]) or overexpression of FLUC (firefly luciferase, control), ATP13A2 WT, and four mutants in the putative N-terminal lipid binding sites (LBS1, LBS2, LBS3, and LBS1.2.3).

First, we confirmed by immunolocalization that, like WT ATP13A2 overexpression, the LBS1, LBS2, LBS3, and LBS1.2.3 mutants are also expressed in the LAMP-1 positive organelles of the SHSY5Y cells (Figure 1(d)). Note that the expression of LBS mutants rose well above the endogenous ATP13A2 protein levels (FLUC), since the endogenous expression of ATP13A2 in FLUC is only weakly detectable (Figure 1(d)) [14]. These results show that the LBS mutants are expressed in the late endo-/lysosomes to levels well above the endogenous ATP13A2, which was confirmed by immunoblotting (Figure 1(b), compared to ATP13A2 levels in FLUC cell line which fall below the detection limit [14]), and LBS mutants). According to the immunoblot analysis, the protein levels in the LBS mutant cell lines were at least 10-fold lower than WT. This difference in protein expression between WT and LBS mutants was repeatedly observed when several WT and LBS1–3 clones were evaluated using various viral vector dilutions. In addition, the inhibition of the proteasome by MG-132 only partially enhanced the expression levels of the mutated proteins suggesting that protein instability may not be a major issue (Figure 1(b)).

Next, we confirmed that overexpression of ATP13A2 WT protects, whereas KD of ATP13A2 sensitizes cells to rotenone (Figure 2(a)), in line with our previous findings [14]. Moreover, pharmacological inhibition of the PIKfyve lipid kinase by YM-201636 or of the PLD activity by FIPI prevented the cellular protection in ATP13A2 overexpression cell lines but had no significant effect on the ATP13A2 KD cells, suggesting a direct and activating effect of both lipids on ATP13A2 (Figure 2(a)). Notably, the inhibitors exerted no significant toxic effect in the absence of rotenone (Figure 2(a)).

To further assess the role of ATP13A2 at the level of the mitochondria, we compared the sensitivity of our cell lines to rotenone and MPP+, another complex I inhibitor (Figures 2(b)–2(e)). Data demonstrated that, as for rotenone, ATP13A2 WT protects and KD sensitized cells to MPP+

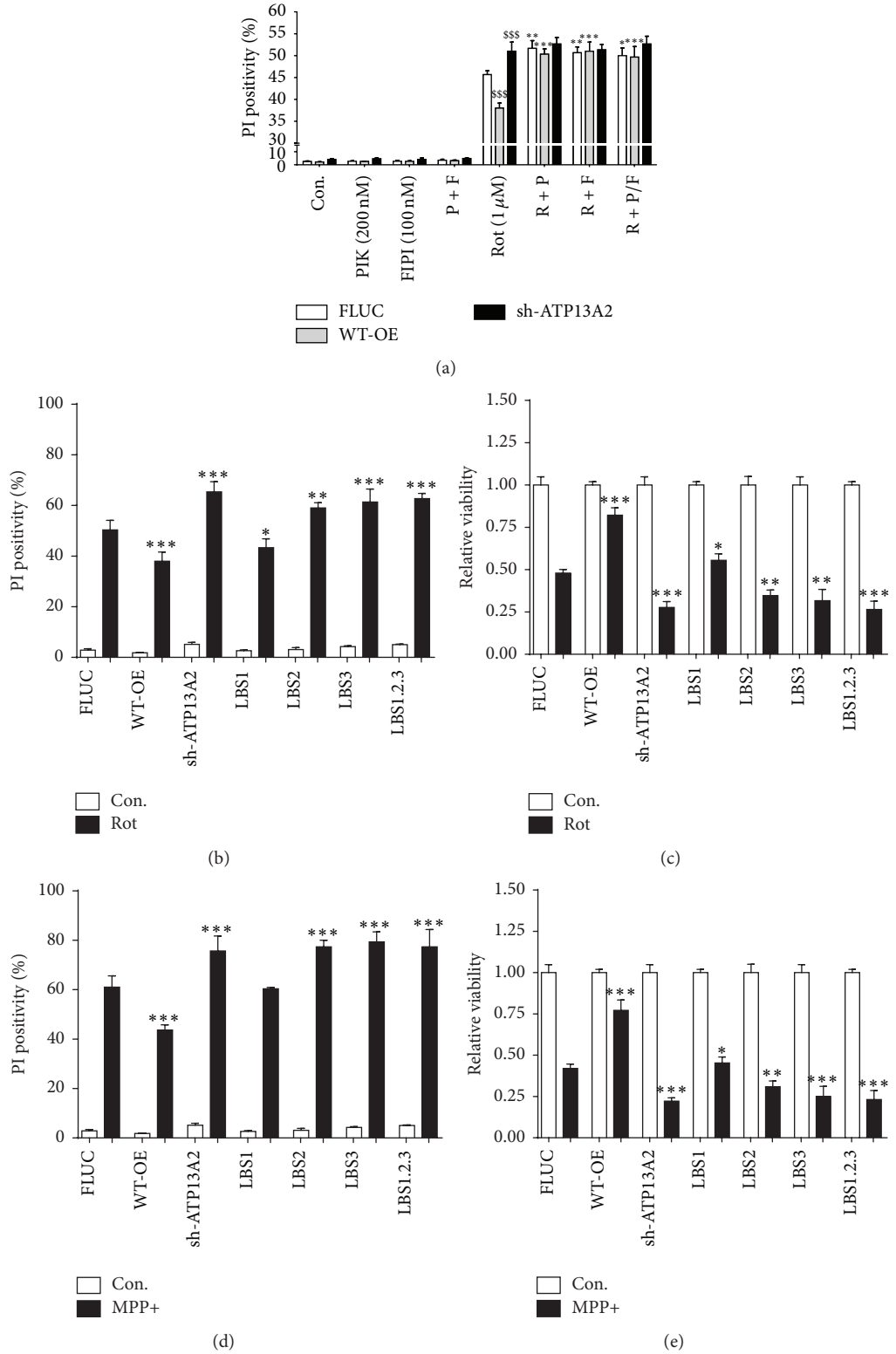

Figure 2: LBS mutations prevent ATP13A2-mediated cytoprotection. (a) ATP13A2's protective response at 24 h exposure to rotenone (Rot, 1 μM) and the effect of pharmacological inhibition of PIKfyve lipid kinase with YM-201636 (PIK or P, 200 nM) and inhibition of PLD with FIPI (F, 100 nM) on cell death were assessed via a propidium iodide (PI) based assay. \$ statistical differences between FLUC and WT-OE/sh-ATP13A2, $*$ statistical differences within cell line following treatment with inhibitor (1 mark, $P < 0.05$; 2 marks, $P < 0.01$; 3 marks, $P < 0.001$) (ANOVA with Bonferroni post hoc test). ((b)–(e)) Stable cell lines were exposed to 1 μM rotenone ((b)-(c)) or 50 μM MPP+ ((d)-(e)) for 24 h and cell death was assessed by PI stained flow cytometry, whereas cell viability was assayed by the MUH protocol. Data are the mean of 3 independent experiments ± SD. $*$ statistical differences between FLUC and WT-OE/sh-ATP13A2/LBS1/LBS2/LBS3/LBS1.2.3 (1 mark, $P < 0.05$; 2 marks, $P < 0.01$; 3 marks, $P < 0.001$) (ANOVA with Bonferroni post hoc test).

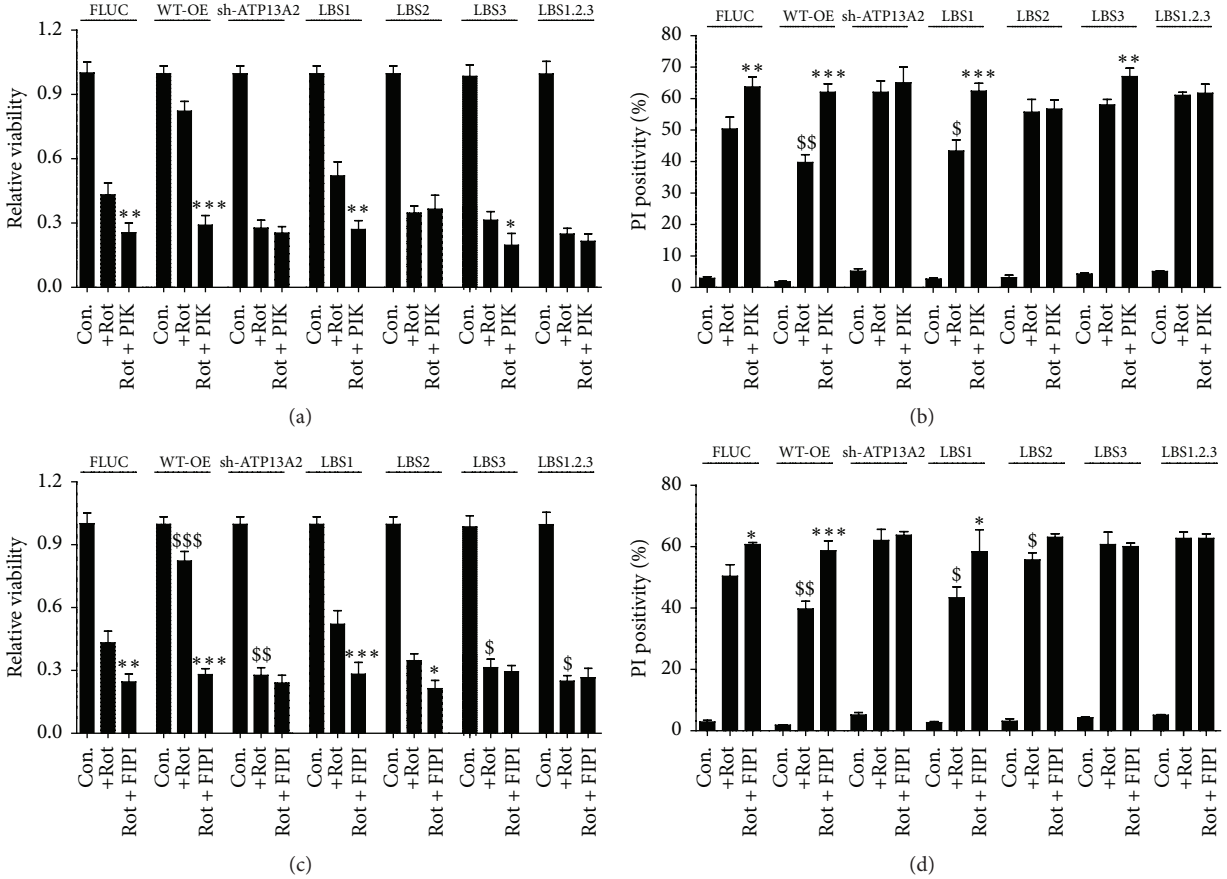

Figure 3: PA and PI(3,5)P2 mediated protection to mitochondrial stress occurs via ATP13A2. SHSY5Y cells stably expressing FLUC, shRNA for KD of ATP13A2, ATP13A2, and full length mutants of ATP13A2 with mutated putative lipid binding sites (LBS1–3) were exposed to rotenone (Rot, 1 μM) in the presence or absence of 1 h pretreatment with the pharmacological inhibitors of PIKfyve lipid kinase (((a) and (b)) YM-201636, 200 nM) or phospholipase D (((c) and (d)) FIPI, 100 nM). Following 24 h exposure to rotenone, cells were assessed for cell viability ((a) and (c)) or death ((b) and (d)) by, respectively, MUH and propidium iodide (PI) assays. Data are the mean of 3 independent experiments ± SD. \$ statistical differences between FLUC and WT-OE/sh-ATP13A2/LBS1/LBS2/LBS3/LBS1.2.3, ∗ statistical differences within cell line following treatment with inhibitor (1 mark, $P < 0.05$; 2 marks, $P < 0.01$; 3 marks, $P < 0.001$) (ANOVA with Bonferroni post hoc test).

toxicity (Figures 2(d) and 2(e)). Interestingly, in comparison with WT, overexpression of the LBS2, LBS3, and LBS1.2.3 mutants failed to protect against either rotenone- or MPP+-induced mitochondrial stress, in line with a critical role of the LBS2/3 sites in the ATP13A2-mediated cellular protection (Figures 2(b)–2(e)). Moreover, compared to FLUC, LBS1 demonstrated a slightly weaker than WT, but significant protective effect against rotenone or MPP+ in the cell viability assay. In contrast to rotenone, the protection of LBS1 to MPP+ was not significant in the cell death assay (Figures 2(b) and 2(c)). The failure of the LBS2-3 mutants to protect against either rotenone or MPP+ is not merely related to their lower expression levels, since, for either cell death induction (Figures 2(b) and 2(d)) or inhibition of cell viability (Figures 2(c) and 2(e)) a significant sensitization to either agent was observed in the LBS2, LBS3, and LBS1.2.3 cell lines to the level of ATP13A2 KD, whereas in LBS1 such a sensitization was not observed. Finally, we tested whether mutagenesis of the key lipid interacting sites influenced the autophosphorylation properties of ATP13A2 (Figure 1(c)). No autophosphorylation signal was observed in the LBS2/3/1.2.3 cells, whereas in LBS1 cells a faint, but reproducible autophosphorylation signal was detected.

Altogether, these data pieces point to an inhibitory effect of the LBS2-3 mutations on the functionality of ATP13A2 in conditions of mitochondrial stress.

3.2. PA and PI(3,5)P2 Mediated Protection against Mitochondrial Stress Occurs Specifically via ATP13A2. We investigated whether pharmacological inhibition of PA and PI(3,5)P2 production may influence the response of the LBS cell lines to rotenone-induced mitochondrial stress (Figure 3). In the LBS1 and LBS3 cell lines, inhibition of PIKfyve by YM-201636 significantly increased rotenone-induced toxicity (Figures 3(a) and 3(b)). In contrast, YM-201636 exposure was unable to incite further stress in either the LBS2 or LBS1.2.3 cell lines. In the case of PA, inhibition of PLD by FIPI was unable to potentiate the rotenone-elicited toxicity in LBS3 and

LBS1.2.3 (Figures 3(c) and 3(d)). Yet, for the LBS1 cells, FIPI significantly potentiated rotenone-induced toxicity (Figures 3(c) and 3(d)), whereas, in the case of LBS2, a significant effect of FIPI was only observed in the cell viability assay (Figure 3(c)). These observations correlate well with previous observations that PI(3,5)P2 predominantly interacts with LBS2 and PA with LBS3 [14]. Altogether, these results are in line with an inhibitory effect of the N-terminus on ATP13A2 activity, which is reversed by specific and direct binding of PA and PI(3,5)P2 to the LBS sites in conditions of rotenone-induced mitochondrial stress.

3.3. PA and PI(3,5)P2 Provide ATP13A2-Mediated Protection to PD-Related Metal Toxicity. The protective effect of ATP13A2 on various metal ions (Mn^{2+} [16, 17], Zn^{2+} [18], and Fe^{3+} [19]) depends on the catalytic activity of ATP13A2, since a catalytic dead mutant is unable to provide cellular protection. Here, we tested whether the activation of ATP13A2 during metal toxicity also depends on PA and/or PI(3,5)P2. In the stable SHSY5Y cell lines we observed that similar to rotenone and MPP+ toxicity ATP13A2 overexpression protected against, whereas KD sensitized cells to metal toxicity following exposure to Zn^{2+}, Mn^{2+}, and Fe^{3+} (Figures 4(a) and 4(b)), in line with previous reports [14, 16, 17, 19, 23].

Next, we addressed whether the ATP13A2-mediated protection to metal toxicity also depends on PA and PI(3,5)P2 interaction. Similar to that in conditions of rotenone (Figure 2(a)), inhibition of PI(3,5)P2 formation significantly blunted the protective effect following metal exposure observed in both FLUC and WT overexpression cells, but not in the KD cell line (Figures 4(c)–4(e)). Like the data obtained for YM-201636, FIPI also blunted the protective effect of ATP13A2 on the metal ions Zn^{2+}, Mn^{2+}, and Fe^{3+} (Figures 4(c)–4(e)). However, unlike YM-201636, FIPI increased the sensitivity of FLUC and WT overexpression cells up to the level of the ATP13A2 KD cells, whereas coexposure of YM-201636 and FIPI did not further potentiate cell death. Finally, in ATP13A2 KD cells YM-201636 or FIPI did not exert an effect on metal ion toxicity, whereas in all cell lines treatment with either YM-201636 or FIPI alone did not induce cell death (Figures 4(c)–4(e)).

In addition, the LBS1–3 cells were exposed to the metal ions Zn^{2+} or Mn^{2+}, and cytotoxicity and cell death were assessed (Figure 5). Compared to ATP13A2 WT and FLUC, LBS3 and LBS1.2.3 showed a significant loss in the protective effect of ATP13A2 towards either Zn^{2+} or Mn^{2+} (Figures 5(a)–5(d)). Although LBS2 demonstrated a significant, albeit small, reduction in the protective capacity based on the cell death assay, no significant difference was found for LBS2 in the cell survival assay, suggesting that the sensitization to heavy metal toxicity was most consistent and significant in the LBS3 cell line.

4. Discussion

4.1. Inhibition of ATP13A2 Activity by the N-Terminus Is Reversed by PA and PI(3,5)P2 Interaction. The catalytic activity of ATP13A2 provides cellular protection against various PD-related insults like mitochondrial stress [14, 23] and metal exposure (Mn^{2+} [16, 17], Zn^{2+} [18], and Fe^{3+} [19]). Here we established that these various cytoprotective effects depend on the same activation mechanism of ATP13A2 involving an N-terminal lipid switch. Our results show that both PI(3,5)P2 and PA are required to exert ATP13A2-mediated cellular protection, which is explained by a specific and direct interaction of PI(3,5)P2 and PA at the ATP13A2 N-terminal lipid binding sites LBS2 and LBS3, respectively.

Whereas LBS1.2.3 mutants do not respond to either PIKfyve or PLD inhibition, pharmacological inhibition of PIKfyve or PLD had no impact on rotenone toxicity in, respectively, the LBS2 and LBS3 cell lines, strongly indicating that PI(3,5)P2 binds to LBS2 and PA to LBS3. This is in agreement with previous results of protein lipid overlays with purified N-terminal fragments of ATP13A2 [14]. In addition, our data demonstrate that both lipids need to bind together to allow ATP13A2 activation, since pharmacological inhibition of only PA or PI(3,5)P2 formation is sufficient to abolish the cellular protection of ATP13A2 WT to rotenone. No autophosphorylation signal was detected for the LBS2/3/1.2.3 mutants, whereas a weak signal was detected for LBS1, suggesting that the LBS2/3/1.2.3 mutants might exhibit loss of autophosphorylation activity. However, we cannot fully exclude that the autophosphorylation signal might have fallen below the detection level of the assay due to the lower expression levels of the mutants. But since we previously demonstrated that application of PA and PI(3,5)P2 stimulates the autophosphorylation reaction [14] and we now show that the protective effects of ATP13A2 depend on lipid interactions on LBS2/3, it is reasonable to speculate that the functional LBS2-3 sites are required for the catalytic autophosphorylation reaction.

Together, our observations highlight the critical dependence of ATP13A2 activation on the lipid interactions at the membrane-associated N-terminal domain and suggest that the N-terminus might be an autoinhibitory domain preventing ATP13A2 activity. Autoinhibition of P-type ATPases by N- or C-terminal regions is frequently observed, for example, in the human plasma membrane Ca^{2+}-ATPase PMCA [24] and the proton pump in plants [25].

Surprisingly, the LBS2/3/1.2.3 but not LBS1 cell lines displayed an increased sensitivity to either rotenone or MPP+ in comparison to FLUC control cells and reached similar toxicity levels as observed for KD cells. This result may point to a dominant negative effect of the LBS2/3/1.2.3 mutants suppressing the protective effect of the endogenous ATP13A2. Of interest, this dominant negative effect was not observed with LBS1 (Figure 2) or the catalytic dead mutant D508N [14], which both contain intact LBS2/3 sites. The LBS2/3 mutated N-terminus might therefore inhibit the endogenous ATP13A2 by direct interaction or by irreversibly trapping regulatory proteins and/or lipids required for ATP13A2 activation. Alternatively, the endogenous WT ATP13A2 levels might be reduced upon expression of the LBS2/3/1.2.3 mutants, which might also explain why the LBS mutant cell lines display an increased sensitivity to mitochondrial stress as compared to control cells. These possibilities will be addressed in future experiments.

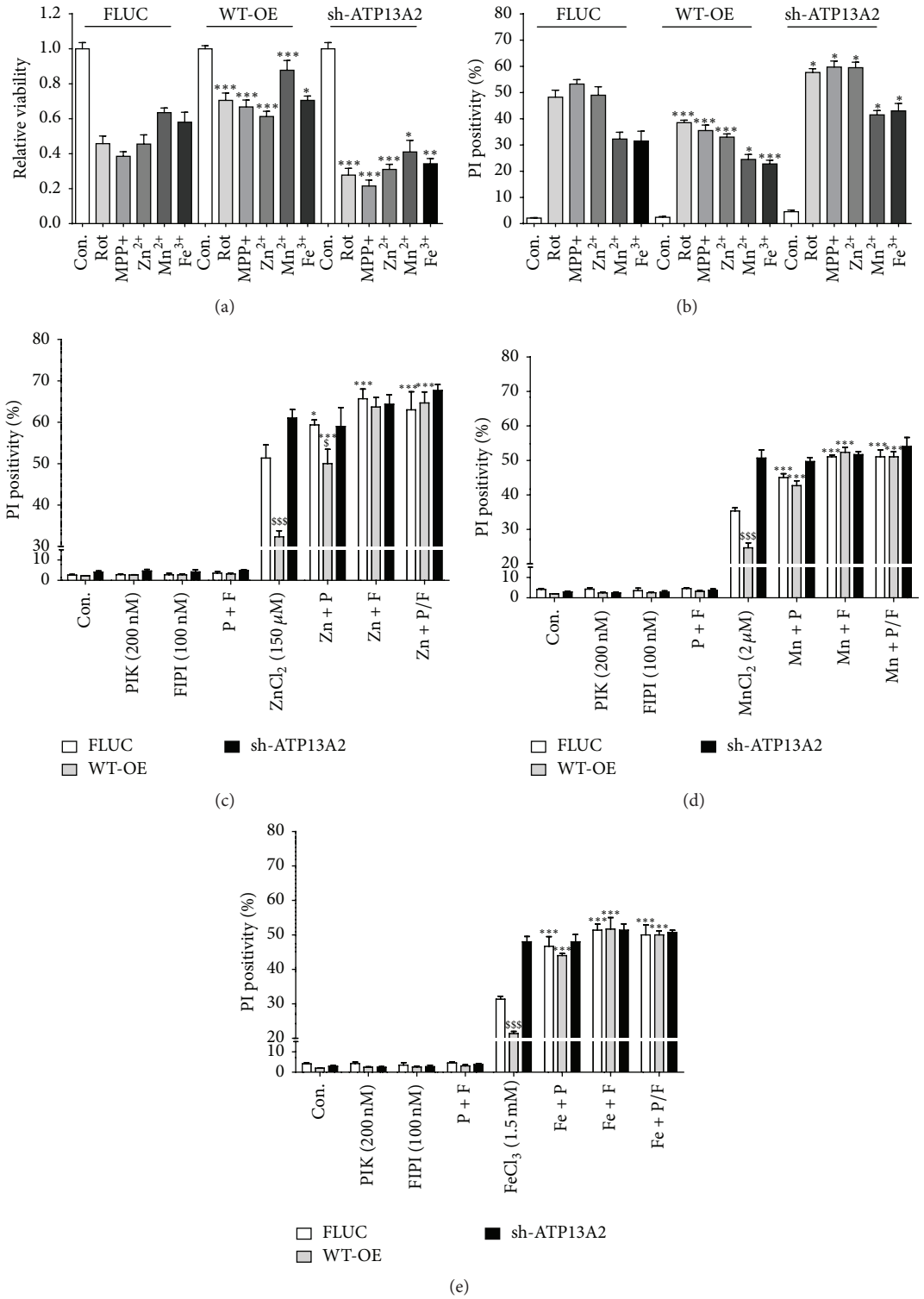

Figure 4: PA and PI(3,5)P2 provide ATP13A2-mediated protection to PD-related metal toxicity. ((a)-(b)) The SHSY5Y cell models stably overexpressing FLUC, WT-OE, or sh-ATP13A2 were exposed for 24 h to rotenone (Rot, 1 μM, positive control) and MPP+ (50 μM), as well as the heavy metals Zn^{2+} (150 μM), Mn^{2+} (2 μM), and Fe^{3+} (1.5 mM). Cell viability was evaluated by the MUH assay (a), whereas cell death was assessed by propidium iodide (PI) stained flow cytometry (b). To test whether the protective role of ATP13A2 depends on the signaling lipids PI(3,5)P2 and PA, the SHSY5Y cell lines were pretreated for 1 h with the PIKfyve inhibitor (200 nM; P or PIK) and/or the phospholipase D inhibitor (100 nM; F or FIPI) prior to the addition of the heavy metals Zn^{2+} (c), Mn^{2+} (d), and Fe^{3+} (e). The level of cellular protection was evaluated by PI stained flow cytometry. Data are the mean of 3 independent experiments ± SD. \$ statistical differences between FLUC and WT-OE, ∗ statistical differences within cell line following treatment with inhibitor (1 mark, $P < 0.05$; 2 marks, $P < 0.01$; 3 marks, $P < 0.001$) (ANOVA with Bonferroni post hoc test).

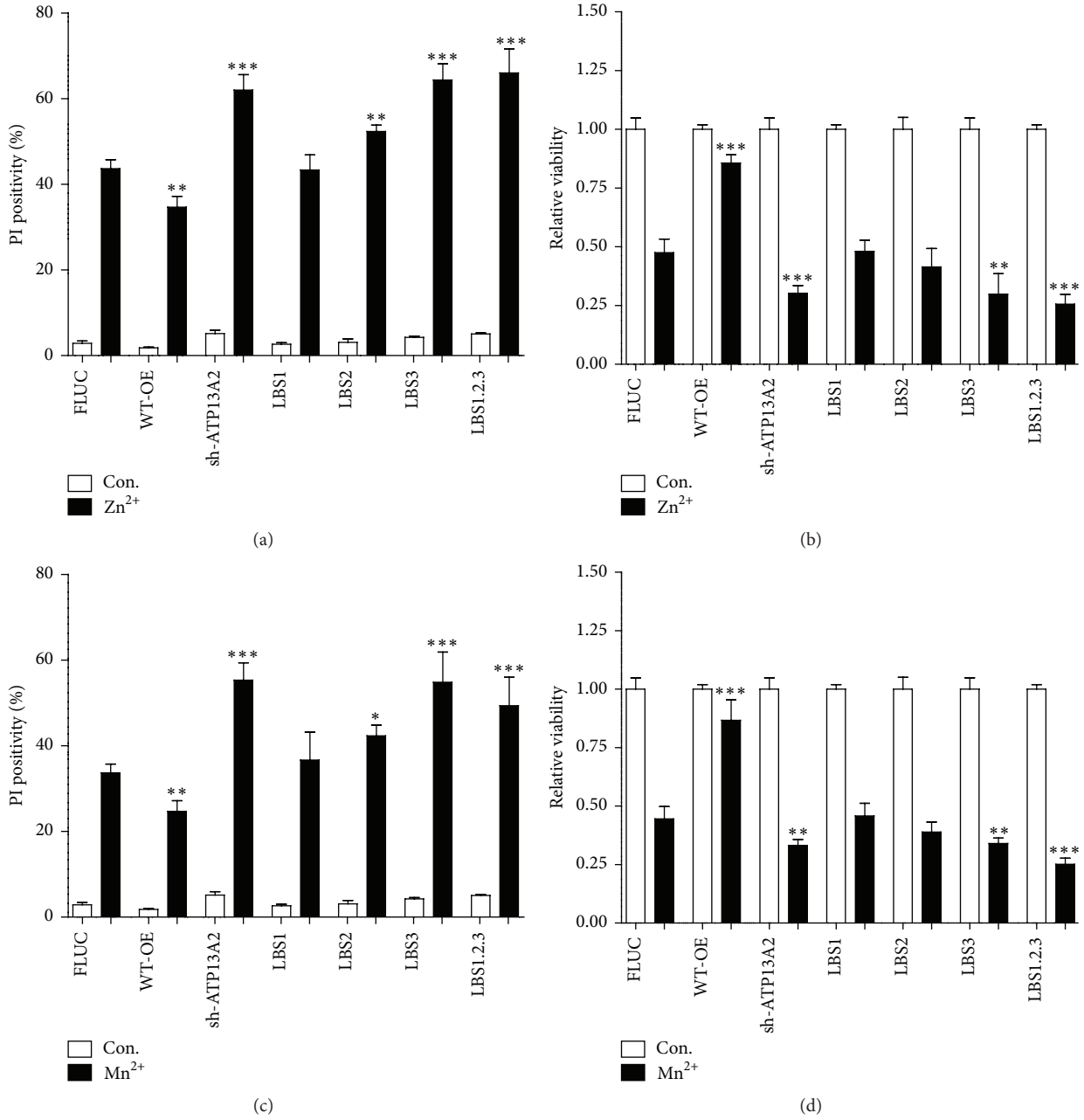

Figure 5: The capacity of ATP13A2 to interact with PA/PI(3,5)P2 is essential to protect against heavy metal-induced cytotoxicity. SHSY5Y cell lines stably overexpressing FLUC, ATP13A2 shRNA, ATP13A2 WT, and LBS1–3 mutants were exposed to toxic heavy metal concentrations of Zn^{2+} (150 μM ((a) and (b))) or Mn^{2+} (2 μM ((c) and (d))). Cell death induction was assessed by propidium iodide (PI) based flow cytometry ((a) and (c)) and cell viability by MUH assay ((b) and (d)). Data are the mean of 3 independent experiments ± SD. ∗ statistical differences between FLUC and WT-OE/sh-ATP13A2/LBS1/LBS2/LBS3/LBS1.2.3 (1 mark, $P < 0.05$; 2 marks, $P < 0.01$; 3 marks, $P < 0.001$) (ANOVA with Bonferroni post hoc test).

4.2. Lysosomal and Mitochondrial Dysfunctions Are Tightly Connected in PD. So far the exact cellular function of ATP13A2 remains obscure, since the transported substrate of ATP13A2 is not yet identified. However, a role of ATP13A2 in the clearance of mitochondria and proteins is gradually emerging. ATP13A2 is involved in the biogenesis and release of exosomes mediating α-synuclein clearance [18], whereas a role of ATP13A2 in autophagy-dependent pathways has also been proposed, although mechanistic details are lacking [21, 22, 26]. Indeed, ATP13A2 KD in SHSY5Y cells reduces autophagic flux [22], whereas lysosomal mediated clearance of autophagosomes is impaired in patient-derived $ATP13A2^{-/-}$ fibroblasts [20]. A decreased autophagic flux associated with ATP13A2 deficiency may critically affect mitochondrial quality control, which may explain mitochondrial fragmentation and elevated ROS production [22].

Of interest, the dependency of ATP13A2 activation on PA and PI(3,5)P2 further connects ATP13A2 to autophagy-mediated clearance pathways [14, 23]. Indeed, PLD1 regulates α-synuclein clearance via autophagy [27], whereas PI(3,5)P2 regulates endo-/lysosome morphology and is also implicated in autophagy [28, 29]. By stimulating ATP13A2 activity both lipids might regulate autophagy and promote mitochondrial quality control and overall cellular health. ATP13A2 activity might be specifically required in conditions of mitochondrial stress and damage, which can be induced by various insults, such as the complex I inhibitors rotenone/MPP+ or also by toxic concentrations of Zn^{2+}, Mn^{2+}, or Fe^{3+} [30, 31]. Indeed, loss of ATP13A2 in olfactory neurosphere cultures results in Zn^{2+} dyshomeostasis, which on its term contributes to mitochondrial dysfunction [18].

PD studies highlight the importance of mitochondrial maintenance and clearance by PINK1/parkin mediated mitophagy, a macroautophagy pathway involving the encapsulation of defective mitochondria in autophagosomes [32–34]. But how lysosomes accept the mitochondria for subsequent degradation via mitophagy pathways or alternative routes such as mitochondrial derived vesicles [35] and what the role of ATP13A2 is herein remain poorly understood. Nevertheless, lysosomal and mitochondrial dysfunctions may be tightly connected in PD. Besides ATP13A2, also mutations in the lysosomal protein glucocerebrosidase (GBA) are genetic risk factors for PD leading to impaired lysosomal sphingolipid degradation, mitochondrial fragmentation, and elevated ROS levels [36]. In addition, LRRK2 affects lysosomal functionality and regulates mitochondrial dynamics [37, 38].

In conclusion, PA and PI(3,5)P2 are required for ATP13A2-mediated protection to rotenone/MPP+-induced mitochondrial stress and toxic Mn^{2+}/Zn^{2+}/Fe^{3+} concentrations, suggesting a general lipid-dependent ATP13A2 activation mechanism that relieves the N-terminal autoinhibition. Thus, targeting the N-terminal lipid binding sites of ATP13A2 might offer a therapeutic modality to activate ATP13A2 and reduce cellular toxicity of various PD insults.

Disclosure

Shaun Martin and Sarah van Veen Joint are the first authors. Tine Holemans and Sarah van Veen are research fellows of the Flanders Research Foundation FWO.

Competing Interests

The authors declared that there are no competing interests.

Acknowledgments

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Research Article

Altered Mitochondrial Respiration and Other Features of Mitochondrial Function in *Parkin*-Mutant Fibroblasts from Parkinson's Disease Patients

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Mutations in the *parkin* gene are the most common cause of early-onset Parkinson's disease (PD). Parkin, an E3 ubiquitin ligase, is involved in respiratory chain function, mitophagy, and mitochondrial dynamics. Human cellular models with *parkin* null mutations are particularly valuable for investigating the mitochondrial functions of parkin. However, published results reporting on patient-derived *parkin*-mutant fibroblasts have been inconsistent. This study aimed to functionally compare *parkin*-mutant fibroblasts from PD patients with wild-type control fibroblasts using a variety of assays to gain a better understanding of the role of mitochondrial dysfunction in PD. To this end, dermal fibroblasts were obtained from three PD patients with homozygous whole exon deletions in *parkin* and three unaffected controls. Assays of mitochondrial respiration, mitochondrial network integrity, mitochondrial membrane potential, and cell growth were performed as informative markers of mitochondrial function. Surprisingly, it was found that mitochondrial respiratory rates were markedly higher in the *parkin*-mutant fibroblasts compared to control fibroblasts ($p = 0.0093$), while exhibiting more fragmented mitochondrial networks ($p = 0.0304$). Moreover, cell growth of the *parkin*-mutant fibroblasts was significantly higher than that of controls ($p = 0.0001$). These unanticipated findings are suggestive of a compensatory mechanism to preserve mitochondrial function and quality control in the absence of parkin in fibroblasts, which warrants further investigation.

1. Introduction

Parkinson's disease (PD) is a progressive and debilitating neurodegenerative disorder, characterized by a distinct motor phenotype and the selective loss of dopaminergic neurons in the substantia nigra. While the etiology of PD is not fully understood, it is thought to involve a combination of different genetic, cellular, and environmental factors that independently or concurrently contribute to neurodegeneration. To date, several PD-causing genes have been identified, and investigations of their function have provided novel insights into the pathobiology of this disease [1].

Recently, particular attention has been drawn to *parkin*, mutations in which are the most common genetic cause of early-onset PD. Over 200 different pathogenic *parkin* mutations have been reported to date, including missense and nonsense mutations, small insertions/deletions (indels), and

large whole exon deletions and duplications, across various ethnic groups [2, 3]. Parkin is a RING-between-RING- (RBR-) type E3 ligase that ubiquitinates protein substrates and targets such substrates for degradation via the ubiquitin proteasome system (UPS). Therefore, the loss of functional parkin may result in the deleterious dysregulation of its substrates and may lead to cellular dysfunction and neuronal cell death [4].

Parkin's enzymatic activity has also been implicated in the maintenance of mitochondrial health, and mitochondrial dysfunction is commonly reported in animal models of parkin deficiency [5]. For example, *parkin*-knockout *Drosophila* demonstrate prominent mitochondrial abnormalities, muscle degeneration, and dopaminergic degeneration [6–8]. While *parkin*-knockout mice exhibit milder neurological deficits, they consistently show mitochondrial impairment and oxidative damage [9, 10]. Recent studies have elegantly demonstrated the pivotal role of parkin in promoting the sequestration and autophagic degradation of damaged mitochondria (mitophagy) [11–14]. Upon mitochondrial depolarization, parkin is recruited to the outer mitochondrial membrane (OMM) through direct phosphorylation by PINK1, where it ubiquitinates several OMM proteins [15–18]. This widespread ubiquitination of OMM proteins results in the recruitment of the autophagy machinery and the autophagic clearance of damaged mitochondria, promoting cell survival [19].

In addition to its important role in mitophagy, parkin is also involved in the regulation of mitochondrial fission and fusion, continuous processes that orchestrate a dynamic cellular network of mitochondria. These processes fine-tune the mitochondrial network in response to changes in the metabolic environment, in order to maintain favorable mitochondrial function during metabolic perturbations [20]. Moreover, parkin promotes mitochondrial biogenesis via its UPS-dependent regulation of the PARIS-PGC-1α pathway [21]. Interestingly, parkin also directs the localized translation of mitochondrial respiratory chain component mRNA at the OMM [22]. Hence, it is evident that parkin plays important roles in the promotion and coordination of various aspects of mitochondrial health, including degradation of damaged mitochondria, mitochondrial dynamics, and mitochondrial biogenesis. It is hypothesized that dysregulation of the careful balance between these processes may significantly compromise mitochondrial health [23]. However, the exact role of mitochondrial function in the pathogenesis of PD remains largely unclear.

Notably valuable in the investigation of PD-associated mitochondrial dysfunction are patient-derived primary cell models of PD [24]. *Parkin*-mutant dermal fibroblasts in particular are a useful and easily accessible tool to study mitochondrial phenotypes in an *ex vivo* setting. However, previous studies of fibroblasts from patients with *parkin* mutations have been inconsistent [25–29]. We have previously reported subtle mitochondrial abnormalities in dermal fibroblasts obtained from three South African early-onset PD patients carrying homozygous loss-of-function *parkin* mutations [28]. The present study serves to follow-up our previous report with a more comprehensive analysis of mitochondrial respiration, and with the inclusion of three age- and gender-matched control individuals.

2. Materials and Methods

2.1. Study Participants and Tissue Culture. This study gained ethical approval from the Health Research Ethics Committee of Stellenbosch University, Cape Town, South Africa (Protocol number 2002/C059). Written informed consent was obtained from all participants.

Dermal fibroblasts were previously obtained from three South African PD patients with homozygous *parkin* mutations, namely, patient 1 (P1) and a pair of affected siblings patients 2 and 3 (P2 and P3) [28]. All three patients underwent a standardized examination by a movement disorder specialist (JC) and met the UK Parkinson's Disease Society Brain Bank diagnostic criteria for PD diagnosis [30]. P1 presented with mild dyskinesia, resting tremor, and dystonia of the left leg and responded well to levodopa therapy. Both P2 and sibling P3 presented with typical PD features as well as dystonia, while P3 exhibited greater disease severity. Each patient's mutation status (P1, homozygous *parkin* exon 3-4 deletion; P2 and P3, homozygous *parkin* exon 4 deletion) was confirmed by means of multiplex ligation-dependent probe amplification (MLPA) analysis and cDNA sequencing, as previously reported [31, 32].

Three age- and gender-matched control individuals were also used, namely, Ct1, Ct2, and Ct3. The three controls had no history of neurological disease and were confirmed to be wild-type with regard to the *parkin* gene by means of cDNA sequencing. Relevant genotypic and phenotypic details of the three PD patients and three controls are summarized in Table 1.

Dermal fibroblasts were obtained from P1, P2, P3, and Ct1 by means of skin punch biopsies taken from the inner upper arm. Ct2 and Ct3 fibroblast cell lines were purchased from Sciencell Laboratories (USA) and were selected to be age- and gender-matched to patient fibroblasts. Fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Switzerland) with 4.5 g/L glucose supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, and 1% (v/v) penicillin/streptomycin, in a 5% CO_2 humidified incubator at 37°C. All experiments were performed on fibroblasts with comparable passage numbers, ranging from 6 to 12, in order to avoid possible effects of cellular senescence.

2.2. Mitochondrial Respiration Analysis. Measurements of mitochondrial respiration are strong indicators of the functional bioenergetic capacity of mitochondria, and of overall cellular health. The Seahorse Extracellular Flux Analyzer uses a plate-based approach and fluorescence detectors to accurately and simultaneously measure cellular oxygen consumption rates (OCR) of multiple samples in real time [33]. Moreover, the Seahorse Analyzer allows for the sequential addition of pharmacological reagents to probe the function of individual components of the mitochondrial respiratory chain in a single experiment. This can be expressed as various informative parameters of mitochondrial function [34].

Table 1: Genotypic and demographic characteristics of the six dermal fibroblast donors used in this study.

Identifier	Lab ID	*Parkin* mutation	Gender	AAO (years)	AAR* (years)	PD duration (years)
PD patients						
P1	53.44	Deletion exon 3-4 hom	Female	27	39	12
P2	P2	Deletion exon 4 hom	Female	27	54	27
P3	P3	Deletion exon 4 hom	Female	27	52	25
Controls						
Ct1	WT2	n/a	Female	n/a	62	n/a
Ct2	WT3	n/a	Female	n/a	56	n/a
Ct3	WT4	n/a	Female	n/a	44	n/a

*The age of the donor at the time of skin punch biopsy. AAO, age at onset; AAR, age at recruitment; hom, homozygous; n/a, not applicable; PD, Parkinson's disease.

Mitochondrial respiration assays were performed using a Seahorse XF96 Cell Mito Stress Test Kit (Seahorse Biosciences, USA), in accordance with manufacturer's instructions. Briefly, fibroblasts were seeded at an optimized density of 22 000 cells per well in a 96-well Seahorse cell culture plate and incubated overnight. Each fibroblasts cell line was seeded in eight replicate wells ($N = 8$). After 24 h, the Seahorse XF^{e}96 Extracellular Flux Analyzer (Seahorse Biosciences, USA) along with XF^e Wave software (Seahorse Biosciences, USA) was used to measure the OCR of each well. A period of 1 h before the measurements was initiated, the culture media in each well was replaced with 175 μL of Seahorse assay media supplemented with 1 mM pyruvate, and the plate was further incubated for 1 h at 37°C without CO_2. Thereafter, successive OCR measurements was performed for each well, consisting of three basal OCR measurements, three OCR measurements following the automated injection of 1 μM oligomycin, three OCR measurements following the injection of 1 μM carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP), and finally three OCR measurements following the dual injection of 1 μM rotenone and 1 μM antimycin A. Subsequently, relative DNA content in each well of the plate was measured using a CyQUANT® cell proliferation assay kit (Life Technologies, USA).

The Seahorse assays were analyzed using XF^e Wave software, according to manufacturer's instructions. All OCR measurements were normalized to cell number and used to calculate various mitochondrial parameters, including basal mitochondrial OCR, OCR due to the proton leak across the inner mitochondrial membrane, OCR due to ATP synthesis, ATP coupling efficiency, and maximum OCR and spare respiratory capacity, following established methods [34]. The minimum OCR after rotenone and antimycin A injection was interpreted as the OCR due to nonmitochondrial respiration, and this rate was subtracted from all other measurements in order to isolate mitochondrial OCR.

2.3. Mitochondrial Network Analysis. Mitochondrial morphology of fibroblast cells was assessed by means of live-cell fluorescence microscopy, where staining with the Mitotracker® Red dye was used to visualize the mitochondrial network. Cells were seeded at a density of 3000 cells per well in a Nunc® Lab-Tek® 8-well chamber slide (Thermo Scientific, USA), which was then incubated overnight. Subsequently, cells were stained with a 100 nM solution of Mitotracker Red CMXRos (Life Technologies, USA) and imaged inside a live-cell environmental chamber of an Olympus IX-81 motorized inverted fluorescence microscope (Olympus Biosystems GmbH, Japan) equipped with a F-view-II cooled CCD camera (Soft Imaging Systems, Germany). Fluorescence was excited through a 572 nm excitation filter, and fluorescence emission collected at 599 nm using a UBG triple-bypass emission filter cube and an Olympus Plan AP N 60X/1.42 oil-immersion objective. All images were acquired as Z-stacks, with 7–12 image frames per stack and increments of 0.26–0.3 μm between frames, using Cell^R imaging software (Olympus Biosystems GmbH, Tokyo, Japan).

Following image acquisition, micrographs were deconvoluted in order to remove out-of-focus fluorescent signal. Cells were individually analyzed using ImageJ Software version 1.47 (http://imagej.nih.gov/ij/) with an average of 40 cells analyzed per sample. Raw images were binarized and optimized by manual contrast adjustment. The individual morphological characteristics of the mitochondria within a given cell, such as area, perimeter, and major and minor axes, were measured and used to calculate aspect ratio (ratio between the major and minor axes of the ellipse equivalent to the mitochondrion) and form factor ($\text{perimeter}^2/(4\pi \times \text{area})$) [25]. Aspect ratio is consistent with mitochondrial length, whereas form factor is a quantification of the degree of branching of the mitochondrial network.

It should be noted that while the mitochondrial respiratory and network analyses was performed on all three patient-derived fibroblast cell lines P1, P2, and P3, due to microbial contamination of the stocks of P1's fibroblasts these cells had to be discarded; therefore only P2 and P3 were available for the assays of $\Delta\psi_m$ and cell growth.

2.4. Mitochondrial Membrane Potential Analysis. In the present study, mitochondrial membrane potential ($\Delta\psi_m$) was assessed with the tetraethyl benzimidazolyl carbocyanine iodide (JC-1) cationic dye and flow cytometric analysis. JC-1 exhibits potential-dependent accumulation in mitochondria, resulting in a fluorescence emission shift from 525 nm (green)

to 590 nm (red). Therefore, loss of $\Delta\psi_m$ is detectable by the decrease in the red : green fluorescence emission ratio [35].

Cultured fibroblasts were incubated with 0.5 μg/mL JC-1 (Life Technologies, USA) in the dark for 1 h. The stained cells were collected, rinsed, and resuspended in prewarmed sterile phosphate-buffered saline (PBS). JC-1 dye equilibration was allowed for 10 min at room temperature, after which the stained cell suspensions were immediately analyzed on a BD FACSCalibur flow cytometer (Becton Dickinson, USA) using BD CellQuest PRO software (Becton Dickinson, USA). The JC-1 fluorophore was excited with a 488 nm argon-ion laser after which red and green emission were separately detected in the FL1 and FL2 channels, respectively, using standard PMT detectors. Debris and aggregates were gated out by establishing a population of interest based on forward scatter/side scatter (FSC/SSC) properties. Compensation between FL1 and FL2 was carefully adjusted in reference to a CCCP-treated positive control sample, according to the manufacturer's instructions. A total of 10 000 events were collected per sample in each of three separate experiments ($N = 3$).

2.5. Cell Growth Assays. Cell growth rate is considered to be one of the most sensitive and reliable indicators of overall cellular health [36]. The present study investigated cell growth of fibroblasts by means of CyQUANT assays, which measures cellular DNA content via fluorescent dye binding. As DNA content is tightly regulated, CyQUANT assays can be used as accurate measurements of cell number. Fibroblasts were seeded in quadruplicate into a 96-well plate at a density of 5000 cells per well and left to adhere overnight. Culture media was then replaced and supplemented with either 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP, to induce cellular stress) or 0.1% (v/v) ethanol (vehicle control), and the plate incubated further for 24 h. Cell growth assays were performed using a CyQUANT NF Cell Proliferation Kit (Life Technologies, USA), according to manufacturer's instructions. Briefly, adherent cells in the 96-well plate were gently rinsed once with prewarmed sterile PBS and a volume of 100 μL of 1x dye binding solution was added to each well. The plate was then incubated in the dark for 1 h. Subsequent fluorescence intensity was measured in a Synergy HT luminometer (BioTek, USA) with excitation at 480 nm and emission detection at 530 nm.

2.6. Statistical Analysis. Linear mixed-effects modeling was used to compare grouped patient-derived and control fibroblasts, with groups as fixed effects. The freely available program R, a language and environment for graphics and statistical computing (https://www.r-project.org/), and R packages *nlme* and *effects* were used [37]. Adjustments were made for the effect that the different observations on a specific cell line will be correlated. Separate experimental runs were modeled as random effects. Where appropriate, a 2^2 factorial design was used to model effects of pharmacological treatment on outcomes. Outcome distributions were graphically depicted as boxplots with indicated medians. Where appropriate, notched boxplots were used to indicate the 95% confidence intervals of the medians. For analysis of mitochondrial network morphology, all outcome distributions were transformed (taking the natural logarithm) in order to approach normality, as the untransformed distributions of form factor and aspect ratio were positively skewed. Results were not adjusted for multiple testing because it has been suggested that corrections, such as Bonferroni, are too conservative when several associations are tested in the same group of individuals [38]. All p values were derived from the results of the specific models, where p values of < 0.05 were considered to be of nominal statistical significance.

3. Results

3.1. Mitochondrial Respiratory Rates Are Elevated in Parkin-Mutant Fibroblasts. In order to compare the bioenergetic status of the three *parkin*-mutant and three wild-type control fibroblasts, mitochondrial respiration analyses were performed. All OCR readings were normalized to cell number. The overall respiratory responses of all patient-derived and control fibroblasts are illustrated in Figure 1(a), from which several important respiratory parameters can be assessed (Figure 1(b)).

A comparison of these parameters in grouped *parkin*-mutant and wild-type control fibroblasts is provided in Figure 2. Patient-derived fibroblasts had a markedly higher mitochondrial respiration than control fibroblasts under basal conditions ($p = 0.0093$; Figure 2(a)). This mitochondrial respiration is composed of two components: the oxygen consumption devoted to ATP synthesis and the oxygen consumption due to the natural proton leak across the inner mitochondrial membrane. The addition of the ATP synthase inhibitor oligomycin allowed for these contributory components to be isolated. While *parkin*-mutant fibroblasts demonstrated higher proton leak ($p = 0.0375$; Figure 2(b)), the elevation in ATP-linked respiration was more pronounced in these cells ($p = 0.0060$; Figure 2(c)). A comparison of the ATP-coupling efficiency demonstrated similar coupling efficiencies in *parkin*-mutant and control cells ($p = 0.5541$; Figure 2(d)), suggesting that the lack of parkin did not significantly impair respiratory efficiency in the patient fibroblasts.

The addition of the accelerator FCCP allowed for an estimation of the maximum, uncontrolled OCR. FCCP is an ionophore which directly transports protons across the inner mitochondrial membrane instead of via the ATP synthase proton channel. Hence, addition of FCCP collapses $\Delta\psi_m$, leading to a rapid consumption of oxygen without the generation of ATP. The maximal respiratory rate is determined by several factors, including the functional capacity of the electron transport chain. It was found that the patient-derived cells had a markedly higher maximum respiratory rate than control fibroblasts ($p = 0.0081$; Figure 2(e)), whereas spare respiratory capacity was comparable between these two groups ($p = 0.1145$; Figure 2(f)).

3.2. Parkin-Mutant Fibroblasts Demonstrate More Fragmented Mitochondrial Networks. As parkin is involved in the

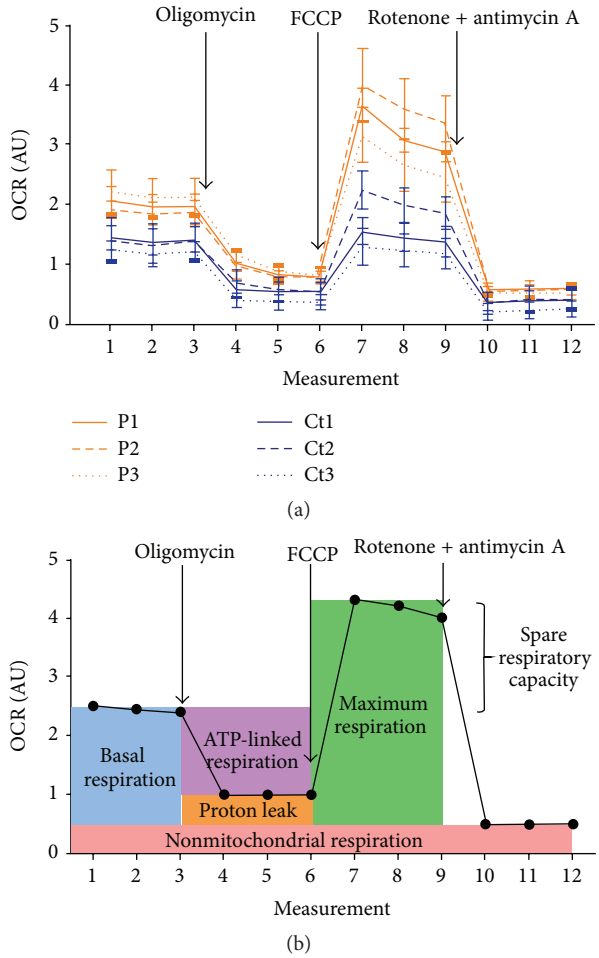

Figure 1: Respiratory flux profiles of patient-derived and control fibroblasts, as determined by a Seahorse Extracellular Flux Analyzer with twelve consecutive measurements of oxygen consumption rate (OCR). Addition of ATP synthase inhibitor oligomycin, electron transport chain uncoupler FCCP and complex I and III inhibitors rotenone and antimycin A are indicated. (a) Respiratory flux profiles of patient-derived and control fibroblasts. Results are expressed as mean ± SEM. (b) Illutrative respiratory flux profile indicating various parameters of respiratory control. These include: OCR due to non-mitochondrial respiration (rotenone/antimycin A response); basal mitochondrial OCR (basal measurement minus rotenone/antimycin A response); ATP-linked OCR (basal measurement minus oligomycin response); OCR due to proton leak (oligomycin response minus rotenone/antimycin A response); ATP coupling efficiency (basal mitochondrial OCR divided by ATP-linked OCR); maximum OCR (FCCP response minus rotenone/antimycin A response) and spare respiratory capacity (maximum OCR divided by basal mitochondrial OCR). AU, arbitrary units; Ct, control; OCR, oxygen consumption rate; P, patient; SEM, standard error of the mean.

regulation of mitochondrial dynamics, mitochondrial morphology was assessed in all *parkin*-mutant and control fibroblasts by means of live-cell microscopy and image analysis. Approximately 40 cells were analyzed from each fibroblast cell line, and representative images of *parkin*-mutant and control fibroblasts are shown in Figure 3(a). Each image was assessed with regard to form factor (degree of mitochondrial branching) and aspect ratio (degree of mitochondrial elongation). The form factor of patient-derived fibroblasts was significantly lower than that of control cells ($p = 0.0304$; Figure 3(b)), which is consistent with a more fragmented mitochondrial network. No significant differences were observed between the aspect ratios of patient-derived and control fibroblasts ($p = 0.1638$; Figure 3(c)).

3.3. Mitochondrial Membrane Potential ($\Delta\psi_m$) Was Similar in Parkin-Mutant and Control Fibroblasts. Given that $\Delta\psi_m$ is a central parameter of mitochondrial integrity, it was decided to assess $\Delta\psi_m$ in the fibroblast cell lines. The fibroblasts were stained with the JC-1 potentiometric dye, and the green and red fluorescent emissions of each cell population were simultaneously measured by means of flow cytometry. Differences in $\Delta\psi_m$ were detected by dissimilarities in red : green florescent emission ratios. The obtained red : green florescent emission ratios of the patient-derived and control fibroblasts are graphically illustrated in Figure 4. No significant differences in $\Delta\psi_m$ were observed for *parkin*-mutant and control fibroblasts ($p = 0.1533$).

3.4. Parkin-Mutant Fibroblasts Have Increased Cellular Growth While Being More Susceptible to Mitochondrial Insult. CyQUANT assays of cell growth were performed to determine whether the overall state of cellular health differed between *parkin*-mutant and control fibroblasts. As illustrated in Figure 5, cell growth was significantly higher in patient-derived fibroblasts than controls under basal conditions ($p = 0.0001$). Fibroblast cell growth was also assessed under conditions of cellular stress, as any differences between patient-derived and control cells may not be readily apparent under basal conditions. Here, the fibroblasts were treated with CCCP to induce mitochondrial impairment and subsequent parkin recruitment to the damaged mitochondria. It was found that cell growth was similar between patient and control fibroblasts even after CCCP treatment ($p = 0.0922$). However, a comparison of the effect of CCCP treatment within each fibroblast group (i.e., with and without cellular stress) demonstrated that the growth of patient-derived fibroblasts was significantly more blunted by CCCP compared to the growth of control fibroblasts ($p = 0.0013$). This is indicative of a heightened sensitivity to CCCP of *parkin*-mutant fibroblasts in comparison to control fibroblasts.

4. Discussion

A substantial body of evidence supports parkin's involvement in mitochondrial function. However, many of these studies rely on artificially overexpressed or recombinantly tagged parkin, which may introduce experimental artifacts [39, 40]. It is therefore pivotal to investigate parkin-associated mitochondrial effects in appropriate cellular models where parkin is expressed at endogenous levels. Patient-derived dermal fibroblasts are particularly suitable for this, as their use creates an *ex vivo* model system with the defined *parkin*

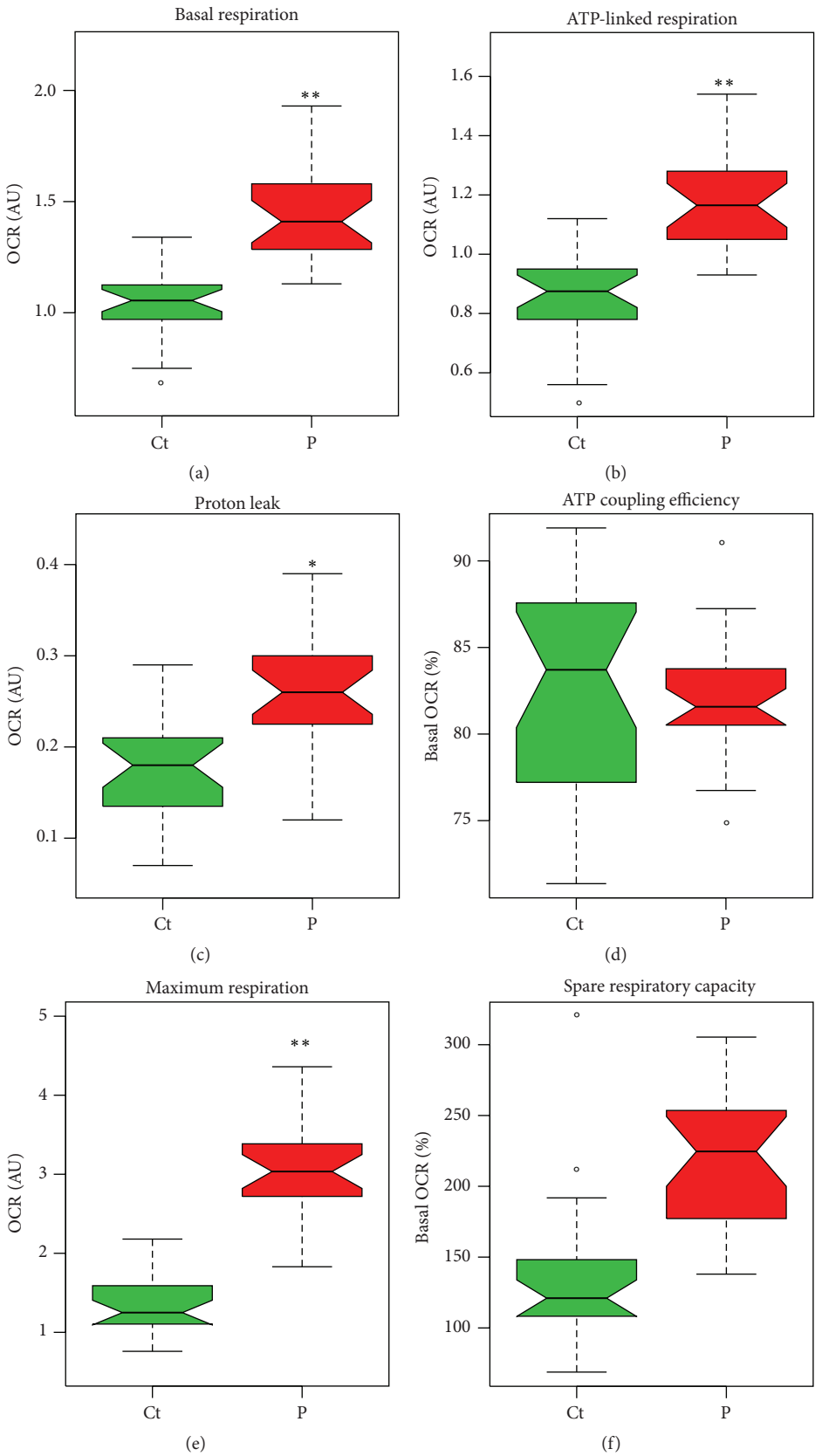

Figure 2: Parameters of respiratory control in patient-derived and control fibroblasts. Boxplots depict grouped patients (P) and control (Ct) values. (a) Basal mitochondrial OCR. (b) ATP-linked OCR. (c) OCR due to proton leak. (d) ATP coupling efficiency (percentage OCR due to ATP synthesis). (e) Maximum OCR. (f) Percentage spare respiratory capacity. $^{*}P < 0.05$; $^{**}P < 0.01$; ∘, outlier; AU, arbitrary units; OCR, oxygen consumption rate.

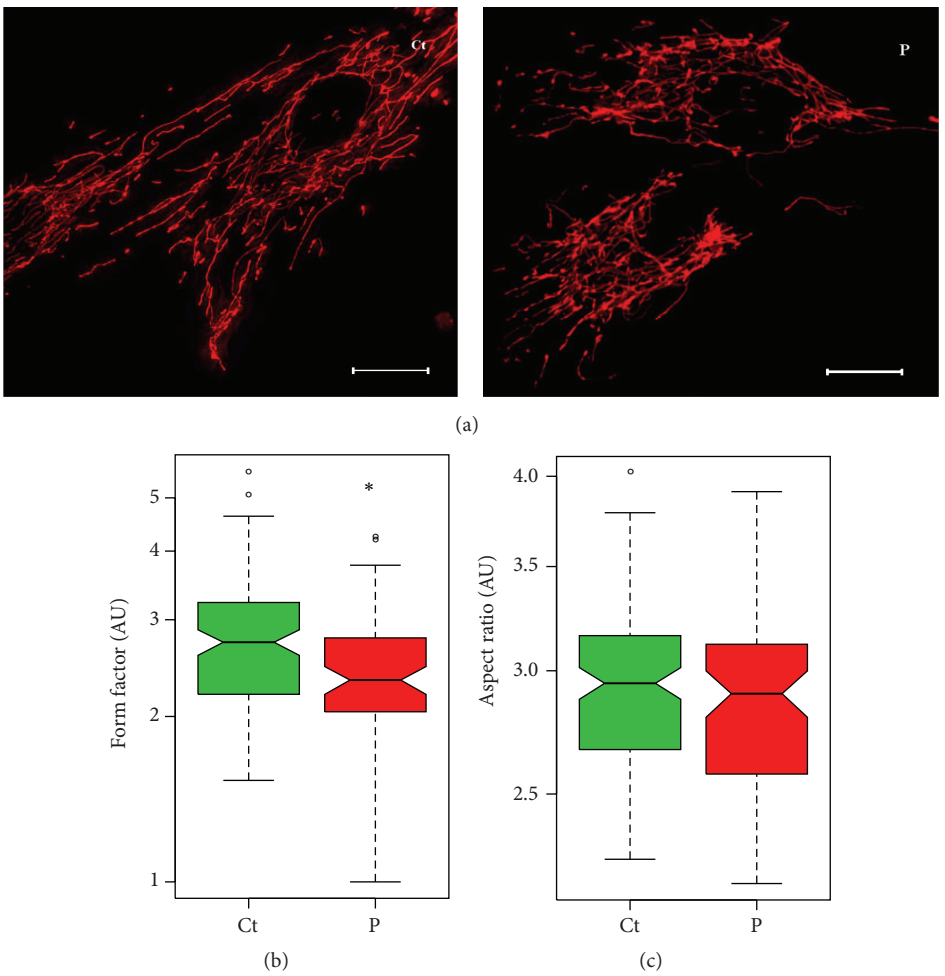

Figure 3: Mitochondrial network analysis of patient-derived and control fibroblasts. Mitotracker Red and live-cell microscopy were used to visualize the mitochondrial network. (a) Representative images of control fibroblasts (left) and patient fibroblasts (right). Scale bars = 20 μm. All images were assessed in regard to the degree of mitochondrial branching (form factor) and degree of mitochondrial elongation (aspect ratio). The distribution of these parameters in grouped patient-derived (P) and grouped control (Ct) fibroblasts are represented on logarithmic scale in boxplots, $N = 40$. (b) Comparison of form factor, which was significantly lower in patient cells than in control cells ($p = 0.0304$). (c) Comparison of aspect ratio, which was similar in patient and control cells ($p = 0.1638$). $^{*}p < 0.05$; ∘, outlier; AU, arbitrary units; N; cells analyzed.

mutations and age-accumulated cellular damage of patients, while being minimally invasive to donor individuals [24]. The present study functionally compared *parkin*-mutant fibroblasts from South African PD patients with wild-type control fibroblasts using a variety of assays of mitochondrial health and function.

Surprisingly, it was found that the rate of mitochondrial respiration was increased in *parkin*-mutant fibroblasts in comparison to control fibroblasts. In particular, the patient cells demonstrated markedly increased basal respiration, elevated ATP-coupled respiration, and a higher maximal respiratory rate. This is indicative of increased electron flow through the respiratory chain, which is coupled to elevated oxidative phosphorylation. The unanticipated increment in mitochondrial respiration is in contrast to numerous studies which have reported decreased respiratory activity in fibroblasts from PD patients with *parkin* mutations. For example, Mortiboys et al. [25] described significant impairment of mitochondrial complex I activity in *parkin*-mutant fibroblasts, which was linked to a loss of $\Delta\psi_m$ and decreased cellular ATP content. Similarly, Pacelli et al. [27] reported that both the basal and maximal respiratory rate were significantly decreased in fibroblasts with *parkin* mutations. Further investigation of the specific respiratory complexes contributing to the decline in respiratory flux demonstrated that activity of complexes I, III, and IV was significantly reduced in patient fibroblasts [27].

It is interesting to compare these results to a recent report by Zanellati et al. [29], who used a similar experimental approach to the present study to investigate mitochondrial respiration in *parkin*-mutant fibroblasts from four PD patients. The authors likewise observed increased basal and maximal respiration in patient cells; however, despite this increment, they reported significantly lower ATP-coupled

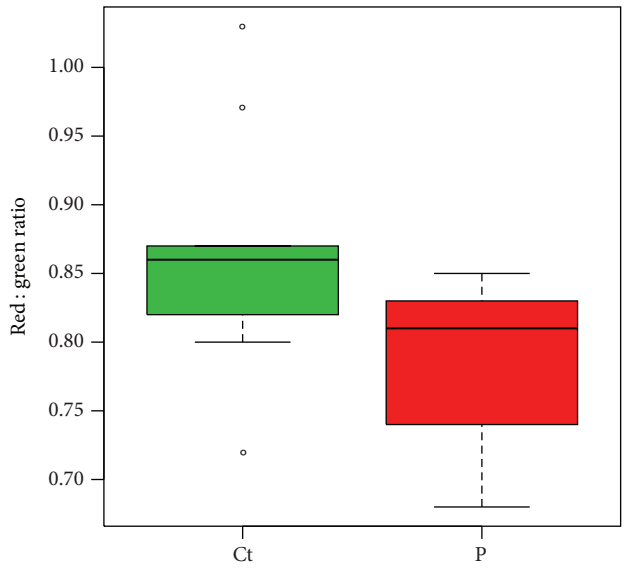

Figure 4: Relative $\Delta\psi_m$ of patient-derived and control fibroblasts. Relative $\Delta\psi_m$ was determined by JC-1 red : green fluorescent emission ratios for each fibroblast cell line in three experimental runs. Fibroblasts from P1 were not available; results pertain to a comparison of P2 and P3 *parkin*-mutant fibroblast with the three controls. Similar $\Delta\psi_m$ was seen for patient-derived (P) and control (Ct) fibroblasts ($p = 0.3285$). ∘, outlier; $\Delta\psi_m$, mitochondrial membrane potential.

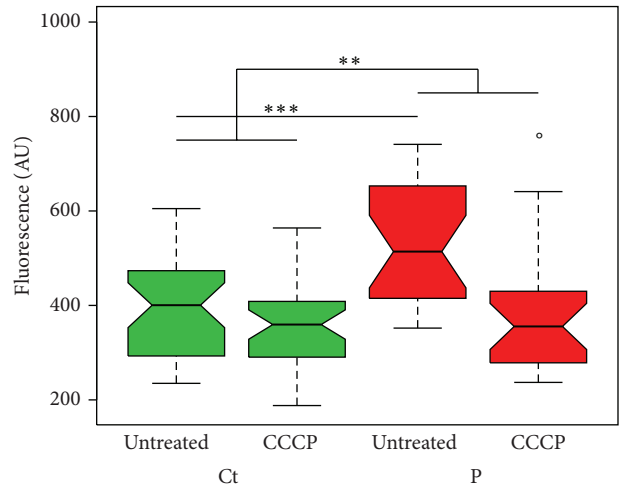

Figure 5: Cell growth in patient-derived and control fibroblasts under basal (untreated) and CCCP-stressed conditions, as assessed by a CyQUANT assay. Boxplots depict grouped patients (P) and control (Ct) measurements for three experimental runs. Patient cells demonstrated higher cell growth under basal conditions ($p = 0.0001$). A comparison of the magnitude of the effect of CCCP treatment within each fibroblast group (i.e., with and without cellular stress) demonstrated that the growth of patient-derived fibroblasts was significantly more suppressed by CCCP than the growth of control fibroblasts ($p = 0.0013$). Fibroblasts from P1 were not available; results pertain to a comparison of P2 and P3 *parkin*-mutant fibroblast with the three controls. $^{**}p < 0.01$; $^{***}p < 0.001$; ∘, outlier; AU, arbitrary units.

respiration in mutants compared to controls. These findings were associated with accordingly reduced cellular ATP levels and impairment of $\Delta\psi_m$, suggesting that *parkin*-mutant fibroblasts had uncoupled mitochondria. In contrast, the present study revealed a significantly increased ATP-coupled respiration in *parkin*-mutants, without any deficit in ATP-coupling efficiency or impairment of $\Delta\psi_m$. The paradoxically improved ATP-coupled respiration in the absence of parkin seen in the present study likely reflects a compensatory response in these *parkin*-mutant fibroblasts.

The present study found marked differences between the mitochondrial network morphology of patient and control fibroblasts. Whereas the degree of mitochondrial elongation (aspect ratio) was comparable between patient-derived and control fibroblasts, the amount of mitochondrial branching (form factor) was significantly decreased in patients. This

decrease in form factor is consistent with increased fragmentation of the mitochondrial network [41]. We have previously reported that mitochondrial networks were unaffected in P1, P2, and P3 fibroblasts [28]. However, this follow-up study used a larger control sample size and was therefore more powered to detect differences in network parameters.

In contrast to the results obtained here, Mortiboys et al. [25] found that fibroblasts with *parkin* mutations had a marked increase in mitochondrial branching, as quantified by the form factor, suggestive of increased mitochondrial fusion in the absence of parkin. Two other studies indicated that *parkin*-mutant and wild-type control fibroblasts demonstrated comparable form factors under basal conditions [26, 42]. However, both studies found that treatment with mitochondrial stressors (paraquat and valinomycin, resp.) decreased the form factor and induced mitochondrial network fragmentation in *parkin*-mutant and control fibroblasts; these decreases were only statistically significant in the fibroblasts with *parkin* mutations. These findings are supported by the results of Pacelli et al. (2011), who observed a noticeably more fragmented mitochondrial network in *parkin*-mutant fibroblasts even under basal conditions; however, this difference was not quantified in terms of form factor. The results obtained here support these findings, but not the contrasting findings of Mortiboys et al. [25].

Moreover, in the present study it was found that cell growth was significantly higher in the *parkin*-mutant fibroblasts under basal conditions, which is in contrast to the published literature. For example, Mortiboys et al. [25] reported that cell growth was similar in fibroblasts from controls and patients with *parkin* mutations, whereas Pacelli et al. [27] reported that *parkin*-mutant fibroblasts displayed significantly lower growth than control fibroblasts. Both of these studies reported cell growth under basal, unstressed conditions. It is conceivable that the increased cell proliferation in the absence of parkin observed in the present study is a result of a metabolic shift in response to parkin deficiency, which is known to promote cell proliferation in various cancers with *parkin* mutations [43]. Furthermore, while significant efforts were made to only use fibroblast cell lines at low and comparable passage numbers, we cannot exclude the possibility that some of the cell lines may have undergone spontaneous transformation, which is known to affect cell growth rates.

It is speculated that the significant increases in basal, ATP-linked, and maximal respiratory rates of *parkin*-mutant fibroblasts, as well as elevated growth rates of these cells, may be due to a compensatory effect. In fact, several compensatory responses to parkin deficiency have been described in the literature. Pacelli et al. [27] reported that the defective ATP production in *parkin*-mutant fibroblasts was compensated by an upregulation of PGC1-α protein expression, suggestive of a compensatory increase in mitochondrial biogenesis [44]. However, the expressions of several PGC1-α target genes directly involved in mitochondrial biogenesis (including *NRF1*, *TFAM,* and *COX II*) were unchanged or even decreased in patient-derived fibroblasts. Pacelli et al. postulated that an unknown posttranslational modification of PGC1-α modulated its function in *parkin*-mutant fibroblasts, preventing a compensatory increase in mitochondrial biogenesis. It is interesting to speculate that the unique genetic backgrounds of the fibroblasts in the current study may allow for the PGC1-α-mediated increase in mitochondrial biogenesis. This deserves further study as it may, in part, explain the conflicting results obtained here and by Pacelli et al.

Other studies have also implied a compensatory increase in mitochondrial biogenesis in parkin deficient fibroblasts. Grünewald et al. [26] investigated citrate synthase activity as an index of total mitochondrial mass and found that such activity was significantly higher in *parkin*-mutant fibroblasts than wild-type controls. Indeed, Grünewald et al. did not observe any impairments of mitochondrial complexes I–IV under basal conditions. Hence, increased citrate synthase activity and elevated mitochondrial biogenesis in general may explain the milder phenotype of *parkin*-mutant fibroblasts observed by Grünewald et al. While markers of mitochondrial biogenesis were not assessed in the present study, increased biogenesis may underlie the compensatory increase in mitochondrial respiration seen here. It is noted that a possible compensatory elevation of mitochondrial biogenesis in the parkin-deficient fibroblasts would be paradoxical: parkin is involved in the promotion of mitochondrial biogenesis; hence, these processes are expected to be decreased in the absence of parkin [45]. However, future investigation of the exact nature and mechanism of the respiratory compensation observed in *parkin*-mutant fibroblasts may reveal a more complex and nuanced view of parkin and its interaction with mitochondria.

These compensatory responses are likely dependent on cell- and tissue-specific metabolic capacity and adaptations [46]. Hence, the observations made here on patient-derived fibroblasts should not be extrapolated to possible effects in a neuronal environment, as neurons may be more restricted in their compensatory repertoire than dermal fibroblasts. Furthermore, many of the described functional roles of parkin are cell-type specific which will result in different functional effects of parkin deficiency in fibroblasts and neurons [47]. Ideally, the observations made in this study should be verified in a neuronal model, such as induced pluripotent stem cell- (iPSC-) derived neurons with *parkin* mutations.

We recognize several limitations of this study. The findings are limited by the small sample size of three patient-derived fibroblasts cell lines, of which only two were available for $\Delta\psi_m$ and cell growth assays. Furthermore, two of the patients recruited for this study were siblings. The small sample size reflects the scarcity of *parkin*-mutant fibroblast models, which is also apparent in the small sample sizes (two to six) of previous studies on *parkin*-mutant fibroblasts [25–29]. It is recommended that the findings reported here be verified in a larger group of patients and controls. We also recommend stringent quality control measures when performing functional assays in fibroblasts, considering that cell growth, respiration, and oxidative stress can be greatly influenced by cellular senescence, spontaneous transformation, or undetected mycobacterial infections.

The functional effects of parkin deficiency observed in this exploratory study were assessed in fibroblasts cultured under basal, unstressed conditions. Given parkin's important role in the cellular stress response, mitochondrial impairments in $\Delta\psi_m$ in *parkin*-mutant fibroblasts may only be readily observable under highly oxidative conditions, where the cells are more reliant on mitochondria for ATP production. Future studies should aim to compare the results obtained here to fibroblasts cultured under more stressed or oxidative conditions, particularly in regard to the mitochondrial respiration and $\Delta\psi_m$ analyses.

In conclusion, our results do not support the findings of impairment of mitochondrial respiration in *parkin*-mutant fibroblasts, while concurring with previous reports of altered mitochondrial dynamics in these cells. These preliminary findings suggest a compensatory response in the patient fibroblasts used in this study. Future studies should aim at investigating the molecular mechanism of the mitochondrial compensation in the absence of parkin; proteomic analyses of *parkin*-mutant fibroblasts may be particularly suitable to identify dysregulated biological processes. Insights derived from these studies may have important implications for therapeutic strategies aimed at preserving mitochondrial function in PD patients.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

A Feed-Forward Circuit of Endogenous *PGC-1α* and *Estrogen Related Receptor α* Regulates the Neuronal Electron Transport Chain

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Peroxisome proliferator-activated receptor γ coactivator 1α (*PGC-1α*) is a central regulator of cellular and mitochondrial metabolism. Cellular bioenergetics are critically important in "energy-guzzling" neurons, but the components and wiring of the transcriptional circuit through which *PGC-1α* regulates the neuronal electron transport chain have not been established. This information may be vital for restoring neuronal bioenergetics gene expression that is compromised during incipient Parkinson's neuropathology and in aging-dependent brain diseases. Here we delineate a neuronal transcriptional circuit controlled by endogenous *PGC-1α*. We show that a feed-forward circuit of endogenous neuronal *PGC-1α* and the orphan nuclear estrogen-related receptor α (*ERRα*) activates the nuclear-encoded mitochondrial electron transport chain. *PGC-1α* not only *trans*-activated expression of *ERRα*, but also coactivated *ERRα* target genes in complexes I, II, IV, and V of the neuronal electron transport chain via association with evolutionary conserved *ERRα* promoter binding motifs. Chemical activation of this transcriptional program induced transcription of the neuronal electron transport chain. These data highlight a neuronal transcriptional circuit regulated by *PGC-1α* that can be therapeutically targeted for Parkinson's and other neurodegenerative diseases.

1. Introduction

PGC-1α is a central regulator of cellular and mitochondrial metabolism in metabolically highly active nonneuronal cell types—brown fat cells, cardiomyocytes, and muscle cells [1]. *PGC-1α* dysfunction is linked to diseased states of these cell types such as diabetes [2], cardiomyopathy [3], and sarcopenia [4]. *PGC-1α* orchestrates a remodeling of cells to increase "clean energy" production [5]. It quantitatively and qualitatively increases energy production as well as the detoxifying enzymes necessary to remove the reactive oxygen species that are the byproduct of increased ATP production [1]. *PGC-1α* induces mitochondrial biogenesis in response to a number of physiological clues such as exercise, cold, and fasting [1]. It remodels individual organelles by increasing levels of electron transport chain (ETC) complexes as well as ATP synthase within isolated mitochondria [4, 6].

The brain is the most energy-demanding organ [7], but the components and wiring of the transcriptional circuits through which *PGC-1α* regulates energy production in brain have not been dissected. This is in contrast to other cell types and organs for which considerable progress has been made in elucidating *PGC-1α* function [1, 4, 6, 8–13]. This information may be vital for restoring the neuronal bioenergetics that are compromised in several brain diseases, including Parkinson's (PD) [14], Huntington's (HD) [15, 16], and amyotrophic lateral sclerosis (ALS) [17].

We previously meta-analyzed laser-captured human dopamine neuron and substantia nigra transcriptomes of hundreds of individuals with Parkinson's and controls,

followed by two-stage replication [14]. We found ten gene sets (i.e., groups of transcripts that encode the same biological pathway) with previously unknown associations with PD [14]. These gene sets pinpointed defects in mitochondrial electron transport, glucose utilization, and glucose sensing and indicated that these systems changes may occur already at earliest, subclinical stages of Lewy body neuropathology. Genes controlling cellular bioenergetics that are expressed in response to *PGC-1α* were underexpressed in dopaminergic neurons laser-captured from substantia nigra of motor PD patients [14]. Mechanistically, transduction with *PGC-1α* blocked mutant *α*-synuclein and rotenone toxicity in rat primary mesencephalic cultures [14]. Other laboratories showed that *PGC-1α* potently modulates dopaminergic neurodegeneration in two mouse models of PD [18–20]. The findings in sporadic PD are supported in a *PARK2*-linked, autosomal recessive variant of PD [19], where repression of *PGC-1α* by the parkin substrate PARIS contributes to neurodegeneration [19].

Here we set out to clarify a specific, open question: the transcriptional circuit through which endogenous *PGC-1α* regulates the neuronal electron transport chain in neuronal cells and brain. Our data indicate that endogenous *PGC-1α* and estrogen-related receptor *α* (*ERRα*) coactivate the nuclear-encoded electron transport chain in neuronal cells through a feed-forward loop. This transcriptional network can now be further defined and therapeutically exploited as chemical activation induced a pervasive increase in endogenous neuronal electron transport chain gene expression.

2. Materials and Methods

2.1. Mouse Brains. Snap-frozen whole brain tissue from *PGC-1α* KO mice, originally characterized by Dr. Bruce Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School), were obtained from Jackson Laboratory (stock number 008597). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee.

2.2. Cell Culture. SK-N-MC neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS. All cells were cultured in the presence of 100 U/mL penicillin and 100 μg/mL of streptomycin sulfate in 5% CO_2 at 37°C.

2.3. Transfections and Adenoviral Transductions. Low passage SK-N-MC cells were plated at 8×10^5 cells/well in a 6-well plate the day before transfection in media lacking antibiotics. Routinely, cells were transfected with a total of 1 to 5 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For adenoviral transductions, SK-N-MC cultures were transduced with adenovirus encoding *PGC-1α* or LacZ (50 MOI) for 24 hours as described elsewhere [12]. Cells were harvested after 48 hours of treatment.

2.4. RNA Isolation and Quantitative Real-Time PCR. RNA was extracted from SK-N-MC cells or snap-frozen brain tissue samples by TRIzol (GIBCO/BRL) extraction similar to what we describe in [12]. RNA quality was determined by spectrophotometry and by visual inspection of electropherograms using the RNA 6000 NanoChip Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). For quantitative gene expression analysis in human biospecimens, TaqMan Assay-on-demand primers and probes (Applied Biosystems) were used. Amplification products were analyzed for specificity by agarose gel electrophoresis. To detect *PGC-1α* mRNA, we have used TaqMan probe Hs01016719_m1, which does not differentiate between various *PGC-1α* isoforms. The comparative threshold cycle method was used for analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *RPL13* ribosomal RNA were used as RNA loading controls. Equal amplification efficiencies were confirmed for target and reference genes.

2.5. siRNA Transfection. Low passage SK-N-MC cells were seeded into 6-well dishes at 40% confluency. The required amount of target siRNA (Invitrogen) and 9 μL of Lipofectamine RNAi MAX (Invitrogen) were each diluted into a final volume of 250 μL in Opti-MEM (GIBCO), then combined, gently mixed, and incubated at room temperature for 25 min. 500 μL of this transfection solution was overlaid onto cells at a final concentration of 80 nM siRNA. Transfection of SK-N-MC cells with RNAi Negative Control (Dharmacon, with no significant homology to any known gene sequences from mouse, rat, or human) served as a negative control. After 48 hr incubation at 37°C in the presence of 5% CO_2, cells were lysed by TRIzol reagent, and total RNA was isolated by chloroform/isopropanol precipitation. To detect *PGC-1α* protein levels by Western blot analysis we used a rabbit polyclonal antibody (H300, Santa Cruz, CA, USA).

2.6. Quantitative Chromatin Immunoprecipitation Analysis. Chromatin immunoprecipitation assays (ChIPs) were performed in asynchronously growing SK-N-MC cells transfected with the myc-*PGC-1α* construct or the empty vector. Cross-linking was carried out with 1% formaldehyde for 10 min at room temperature. Cross-linking was subsequently quenched by adding glycine to a final concentration of 250 mM for 10 min. Cells were collected and washed twice with PBS and then resuspended in 2.5 mL of lysis buffer (150 mM NaCl, 50 mMTris-HCl pH 8.0, 1% NP-40, 25 μM MG-132, and 1x Complete® Protease inhibitor cocktail). After 10 min on ice, cells were sonicated to obtain DNA fragments of ~500 bp as determined by agarose gel electrophoresis with ethidium bromide staining. Protein-DNA complexes were isolated by centrifugation at 15,000 rpm for 20 min. Supernatants with protein-DNA complexes were incubated for 16 hrs with rabbit polyclonal antibody directed against *PGC-1α*. Normal rabbit IgG was used as a control. Antibody-protein-DNA complexes were further incubated with 100 μL of magnetic DYNA beads (Invitrogen) to isolate antibody bound fractions of chromatin. Immunocomplexes were washed with the following buffers: low salt (20 mM

Tris-Cl, pH 8.1, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 1x complete protease inhibitor), high salt (20 mM Tris-Cl, pH 8.1, 500 mM NaCl, 1% Triton X-100, and 2 mM EDTA), LiCl (10 mM Tris-Cl, pH 8.1, 250 mM LiCl, 1% deoxycholate, 1% NP-40, and 1 mM EDTA), and twice in TE (10 mM Tris-Cl, pH 8.1, and 1 mM EDTA). Protein-DNA complexes were eluted in 1% SDS and 100 mM $NaHCO_3$. Cross-links of pulldown fractions and inputs (2% of total IP fraction) were reversed by overnight incubation in elution buffer and 0.2 M NaCl. DNA was then extracted, purified, precipitated, and resuspended in TE for qPCR. Immunoprecipitated DNA was analyzed by real-time PCR as previously described. The primer sequences are available in supplement. The dissociation curves showed that PCRs yielded single products. Samples from three or more independent immunoprecipitation assays were analyzed.

2.7. Statistical Analysis. Values were expressed as mean ± standard error of the mean (SEM). Differences between groups were examined for statistical significance using one-way ANOVA or two-tailed Student's *t*-tests, using GraphPad Prism 5 software. A P value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Endogenous PGC-1α Regulates Nuclear-Encoded Electron Transport Chain Genes in Neuronal Cells and in Brain. To determine whether endogenous *PGC-1α* systematically regulates the expression of the endogenous, neuronal electron transport chain, we silenced native *PGC-1α* using small interfering RNA (siRNA) in dopaminergic SK-N-MC neuroblastoma cells. Transfection with 100 nM *PGC-1α* siRNA reliably knocked down *PGC-1α* mRNA abundance by 80% compared to cells transfected with negative control siRNA (Supplementary Figure S1, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2405176). Similar results were obtained when *UBC* instead of the ribosomal gene *RPL13* was used to control for RNA loading. Silencing of endogenous *PGC-1α* repressed the relative abundance of 14 of 18 nuclear-encoded electron transport chain genes (ETC) analyzed chosen to representing complexes I, II, III, IV, and V of the electron transport chain with P values below 0.05 (Figure 1(a)). Importantly, similar results were observed in brain of *PGC-1α* null mice [21]. Expression of 5 out of 6 ETC subunits probed was significantly decreased in *PGC-1α* knockout mice (Figure 1(b)) compared to age- and sex-matched wild-type littermates ($N = 3$) with P values below 0.05.

Conversely, we previously showed that transduction with adenovirus carrying *PGC-1α* (but not transduction with the control LacZ gene) *trans*-activated the expression of endogenous genes encoding nuclear subunits of complexes I, II, IV, and V of the mitochondrial respiratory chain in primary rat midbrain cultures [14]. We independently confirm this here in an additional cell line, SK-N-MC cells (Figure 1(c)). In the catecholaminergic SK-N-MC cells, 15 of 18 ETC genes analyzed were overexpressed in response to transduction with *PGC-1α* (Figure 1(c)). Collectively, these data show that endogenous *PGC-1α* regulates electron transport chain gene expression in neuronal cells and in brain.

3.2. The Orphan Nuclear Estrogen-Related Receptor α (ERRα) Is an Early Target of Endogenous, Neuronal PGC-1α. ERRα was identified on the basis of its sequence similarity to classical, hormone-regulated steroid receptors [23]. It recognizes similar DNA motifs as the estrogen receptors but does not bind naturally secreted estrogens in animals [24]. However, *PGC-1α* is a peptide ligand for *ERRα* in nonneuronal cells [13]. There, *PGC-1α* induces the expression of *ERRα* and potently converts *ERRα* from a factor with little or no transcriptional activity to a potent regulator of gene expression via interaction with leucine-rich motifs in the *PGC-1α* peptide [13]. To determine whether *PGC-1α* similarly exerts its regulatory control on the *neuronal* electron transport chain genes in coordination with endogenous *ERRα*, we silenced endogenous *PGC-1α* in SK-N-MC cells. Knockdown of *PGC-1α* dramatically repressed endogenous *ERRα* expression (Figure 2(a)) by more than 90% and also repressed the late target gene nuclear respiratory factor-1 (*NRF1)* by more than 50% (Figure 2(c)) compared to controls transfected with scrambled siRNAs. To further delineate the underlying transcriptional program, we then silenced endogenous *ERRα* (Supplementary Figure S2). Silencing *ERRα* not only repressed the *NRF1* gene expression (Figure 2(d)) but also recapitulated the reduction in electron transport chain gene expression observed in response to *PGC-1α*-silencing (with the exception of *COX7A2* expression) (Figure 2(b)). This is consistent with previous studies in nonneuronal cells, that is, murine myoblasts [8] and human osteosarcoma cells [13].

Collectively, these data suggest that in neuronal cells, endogenous *PGC-1α* is a potent transcriptional coactivator of the early target gene *ERRα* and that both endogenous *PGC-1α* and *ERRα* activity modulate the late target gene *NRF1* and the expression of most components of the human neuronal electron transport chain.

3.3. PGC-1α Physically Associates with Evolutionary Conserved ERRα Binding Motifs in the Promoters of Neuronal Electron Transport Chain Genes That Are Dysregulated in Parkinson's Disease. Transcriptional coregulators like *PGC-1α* exert their function through transcriptional complexes that occupy the promoters of distinct target genes. Transcription factors direct these complexes (including the transcriptional coregulator) to specific target sequences. The transcription factor *ERRα* occupies a nine-nucleotide extended half-site sequence with the consensus TNAAGGTCA, referred to as *ERRα* response element (ERRE) [25, 26]. These *ERRα* binding motifs are evolutionary conserved and enriched in electron transport chain genes (Figure 3(a) and Supplementary Figure S3) [8, 27]. In order to evaluate whether *PGC-1α* regulation of ETC genes is the result of an interaction of its transcriptional complex with these evolutionarily conserved *ERRα* binding motifs, we performed quantitative chromatin immunoprecipitation (ChIP) analyses in SK-N-MC neuroblastoma cells overexpressing *PGC-1α* protein.

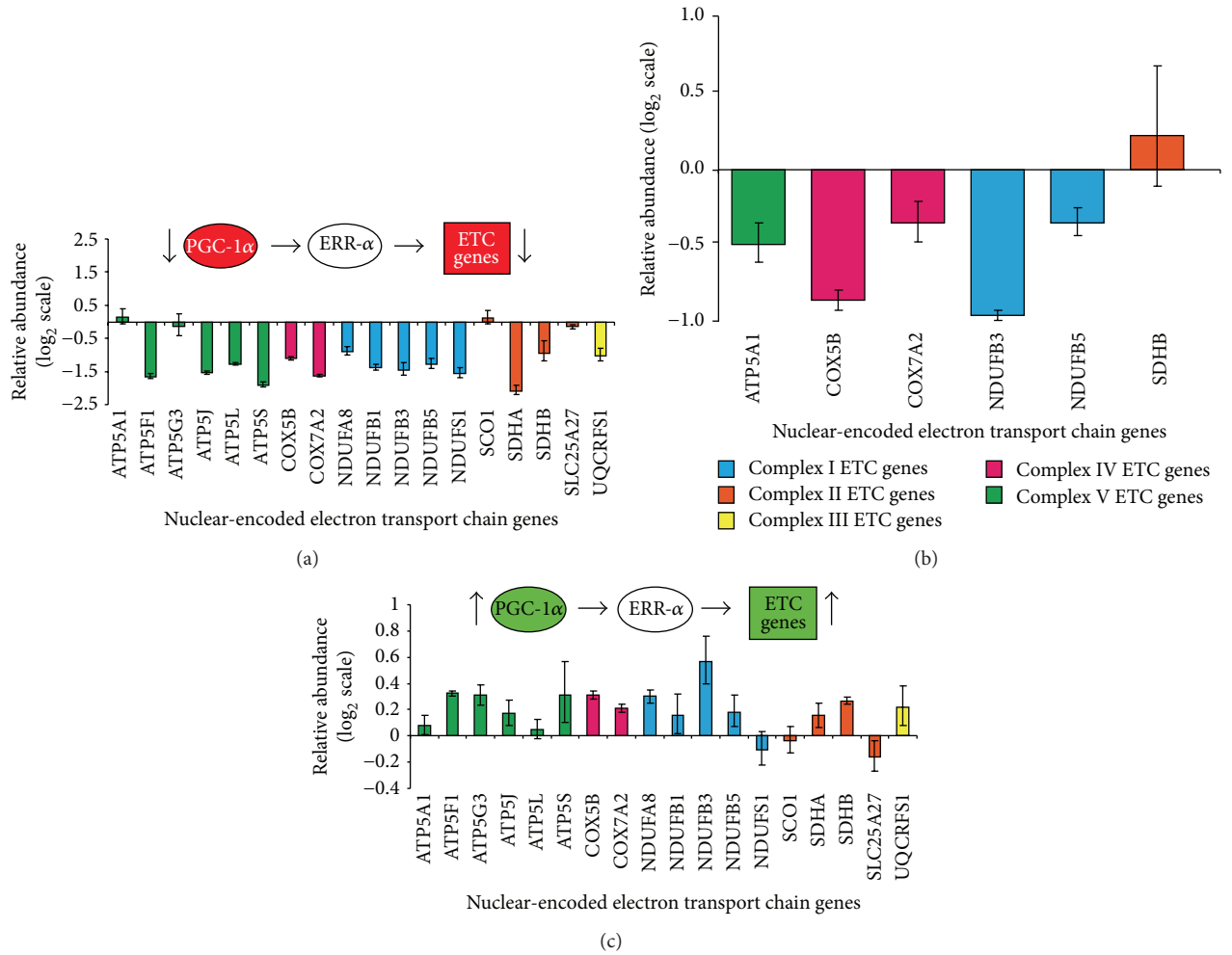

Figure 1: Endogenous *PGC-1α* regulates the nuclear-encoded electron transport chain genes in neuronal cells and in mice. (a) Silencing of endogenous *PGC-1α* repressed the expression of nuclear-encoded electron transport chain genes representing complexes I, II, III, IV, and V of the electron transport chain in SK-N-MC cells by quantitative PCR analysis (note $\log_2$ scale). Model circuit and observed effects in circuit components are shown (top). Subunits of complexes I to V of the electron transport chain are color-coded in panels (a)–(c) in accordance with the color legend shown in (b). Means ± SEM are shown ($N = 3$ for each treatment). The ribosomal gene *RPL13* was used to control for input RNA. (b) Expression of representative electron transport chain genes was similarly reduced in brain of *PGC-1α* null mice [21] compared to age- and sex-matched wild-type littermates ($N = 3$). The ribosomal gene *RPL13* was used to control for input RNA. (c) Transduction with adenovirus carrying *PGC-1α trans*-activated the expression of endogenous genes encoding nuclear subunits of complexes I, II, IV, and V of the mitochondrial respiratory chain in SK-N-MC cells compared controls transduced with the LacZ gene. Model circuit and observed effects in circuit components are shown (top).

We evaluated *PGC-1α* cooccupancy of conserved *ERRα* in the promoters of electron transport chain genes that are underexpressed in laser-captured nigral dopamine neurons of patients with symptomatic PD neuropathology as well as in individuals with incipient, subclinical PD neuropathology [14]. One gene representative for each of complexes I, II, IV, and V of the electron transport chain was investigated. *ATP5A1* (complex V), *COX5B* (complex IV), *NDUFB5* (complex I), and *SDHB* (complex II) were evaluated. Promoter fragments were specifically enriched in the IP fraction of *PGC-1α* compared to IgG control indicating *PGC-1α* occupancy of the conserved ERRE motifs (Figure 3(b)). *UCP-2*, a known transcriptional target of *PGC-1α* [22], was used as positive control. No *PGC-1α* occupancy was seen in intergenic regions lacking a predicted *ERRα* binding site that were included as negative controls (Figure 3(b)).

These results indicate that *PGC-1α* not only *trans*-activates expression of the transcription factor *ERRα* but also coactivates its target genes in the neuronal electron transport chain via occupancy of conserved *ERRα* binding motifs in their promoters.

3.4. The Endogenous PGC-1α and ERRα-Regulated Feed-Forward Circuit Can Be Targeted through Systems Pharmacology. Pioglitazone, a thiazolidinedione approved for the treatment of diabetes, is a synthetic ligand for Peroxisome proliferator-activated receptor γ (*PPARγ*) and to a lesser extent *PPARα* [28]. *PPARγ trans*-activates *PGC-1α* thereby

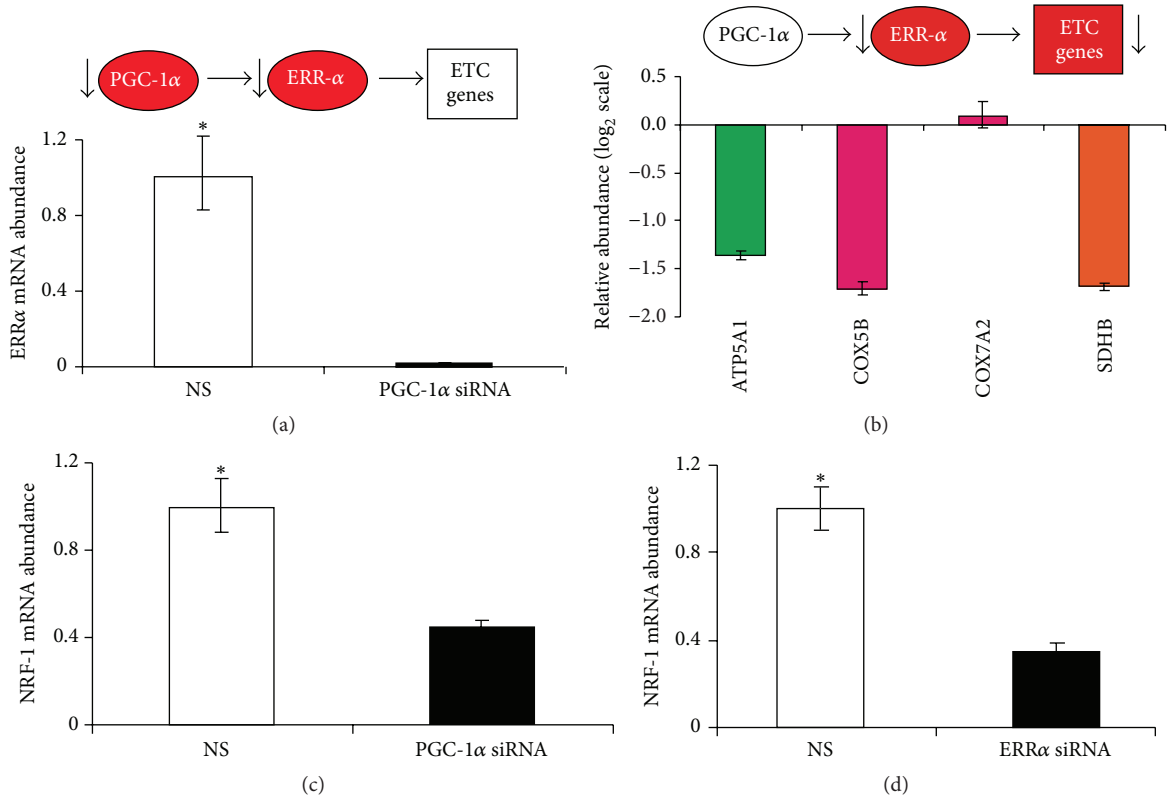

FIGURE 2: The orphan nuclear estrogen-related receptor α (*ERRα*) is an early target of endogenous, neuronal *PGC-1α*. (a) Silencing of neuronal *PGC-1α* repressed the expression of endogenous *ERRα* by more than 90%, respectively, compared to controls transfected with scrambled siRNAs (NS). Model circuit and observed effects in circuit components are shown. (b) Silencing of *ERRα* largely recapitulated the reduction in electron transport chain gene expression observed in response to *PGC-1α*-silencing (note $\log_2$ scale). *NRF1* gene expression was downregulated by more than 50% by silencing *PGC-1α* (c) or *ERRα* (d). The ribosomal gene *RPL13* was used as control for input RNA. Mean ± SEM shown ($N = 3$ for each set). ∗ denotes P value ≤ 0.05.

activating mitochondrial biogenesis in human subcutaneous tissue [29]. Importantly, for Parkinson's disease [30], treatment with pioglitazone or with related thiazolidinediones is protective in multiple animal models of PD [31–33]. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone are linked to parkinsonism in humans and rodents. Thiazolidinediones strongly suppress MPTP-induced-loss of tyrosine hydroxylase-positive cells in the substantia nigra pars compacta [31, 32] as well as motor and olfactory dysfunctions in animal models [32]. Pioglitazone also suppressed rotenone-induced reduction in striatal dopamine levels and locomotor activity in rats [34].

To test the idea that the *PGC-1α* and *ERRα*-regulated feed-forward circuit can be exploited as a target system for therapeutics, we evaluated the endogenous transcriptional response to pioglitazone treatment in neuronal cells. A dose response curve with increasing concentrations of pioglitazone was performed (Supplementary Figure S4). At a concentration of 10 μM, 48-hour treatment with pioglitazone pervasively activated the *PGC-1α-ERRα* circuit (Figure 4). It induced a statistically significant 5-fold increase in expression of endogenous *PGC-1α*, a significant 2-3 fold increase in endogenous *ERRα*, and a correlated, significant 2–5-fold-*trans*-activation of their electron transport chain target genes (Figure 4). These data confirm that the *PGC-1α* and *ERRα*-regulated feed-forward circuit is druggable for early intervention in PD and other brain diseases.

4. Discussion

Cellular bioenergetics are particularly important in "energy-guzzling" neurons, but the role of *PGC-1α* in regulating the neuronal electron transport chain has not previously been clarified. In this study we delineate a previously unconfirmed neuronal transcriptional circuit controlled by endogenous *PGC-1α*. By combining gene silencing and gene expression with quantitative chromatin immunoprecipitation analysis in neuronal cells and mouse brain, and taken together with our previous studies in primary mesencephalic cultures [14], we show evidence for a feed-forward circuit of endogenous neuronal *PGC-1α* and *ERRα* that activates the nuclear-encoded mitochondrial electron transport chain via occupancy of evolutionary conserved *ERRα* motifs. *PGC-1α*-induced ETC gene expression has been previously linked to mitochondrial respiration [35]. In muscle cells, for example, *PGC-1α*-induced ETC gene expression results in increased mitochondrial respiration [35].

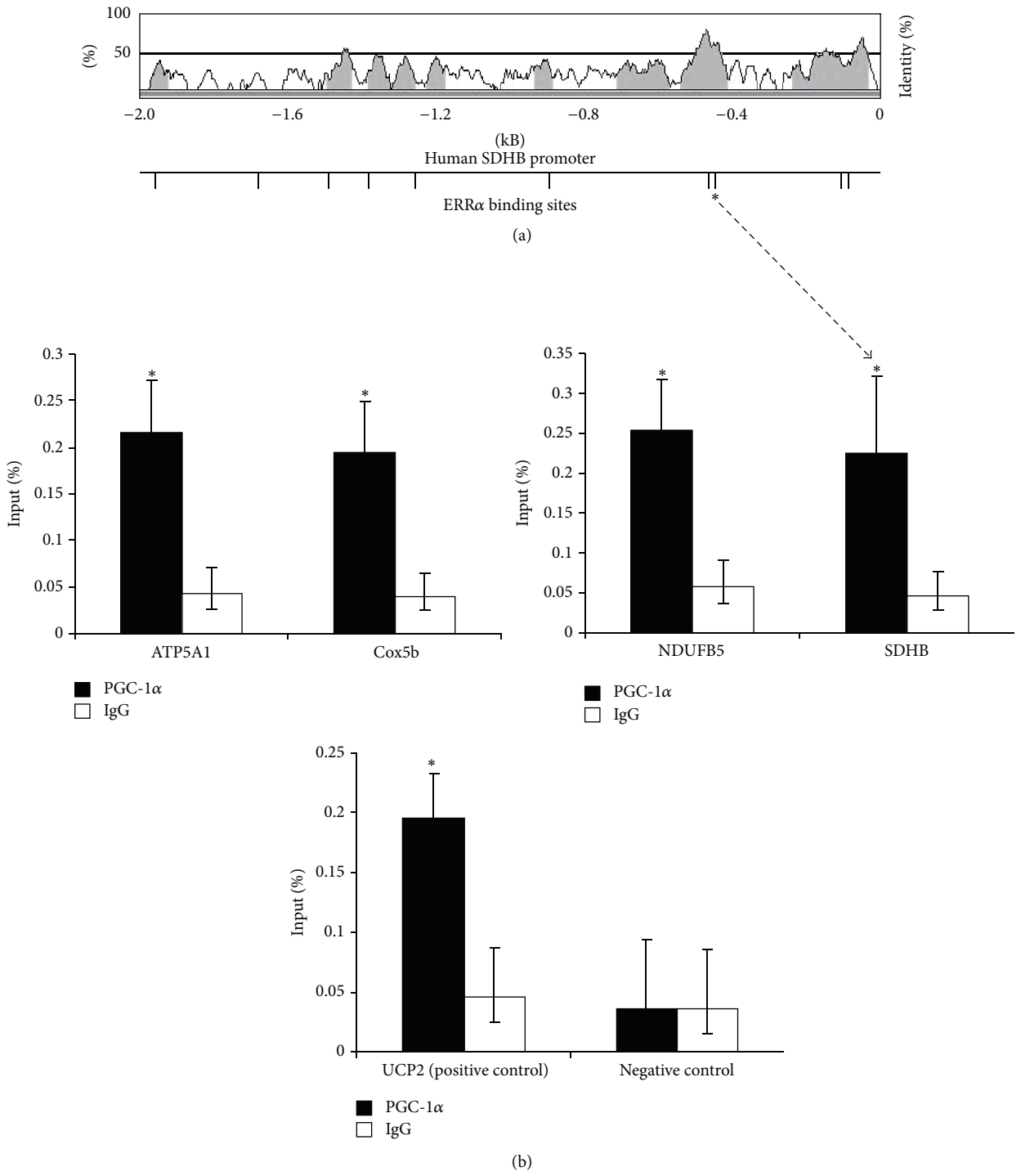

Figure 3: *PGC-1α* physically associates with evolutionary conserved *ERRα* binding motifs in the promoters of neuronal electron transport chain genes that are dysregulated in Parkinson's disease. (a) The VISTA plot of a 2-kb promoter region of the *SDHB* gene is shown with percentage identity of the human and mouse sequences. Small vertical bars indicate the location of conserved predicted ERRE binding motifs and the asterisk indicates the binding motif assayed by quantitative chromatin immunoprecipitation. (b) Quantitative chromatin immunoprecipitation (qChIP) analyses in SK-N-MC neuroblastoma cells transfected with a myc-tagged *PGC-1α* plasmid construct were performed. Promoter fragments for *ATP5A1* (complex V), *COX5B* (complex IV), *NDUFB5* (complex I), and *SDHB* (complex II) were specifically enriched in the IP fraction of *PGC-1α* compared to IgG control indicating *PGC-1α* occupancy of the conserved ERRE motifs. *UCP-2*, a known transcriptional target of *PGC-1α* [22], was used as positive control. No *PGC-1α* occupancy was seen in intergenic regions lacking a predicted *ERRα* binding site that was included as a negative control. Quantitative PCR data were normalized to genomic DNA and visualized as percent input. ∗ denotes P value ≤ 0.05.

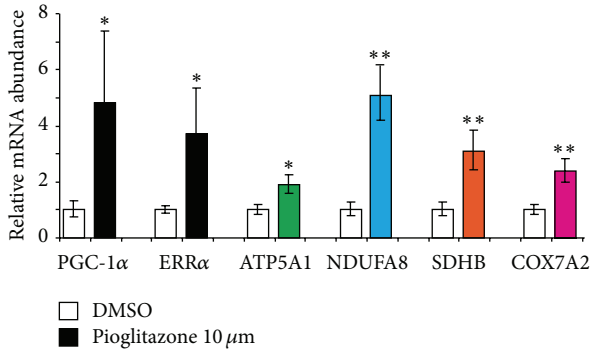

Figure 4: Endogenous *PGC-1α* is a target for drugs designed to restore electron transport chain expression. Treatment with pioglitazone (concentration of 10 μM, 48 hours of treatment) pervasively activated the *PGC-1α-ERRα* circuit. It induced a statistically significant 5-fold increase in expression of endogenous *PGC-1α*, a significant 2-3-fold increase in endogenous *ERRα*, and a resulting significant 2–5-fold-*trans*-activation of their endogenous electron transport chain target genes compared to cells treated with vehicle alone. Mean ± SEM shown ($N = 10$ for each treatment). $*$ denotes P value ≤ 0.05. $**$ denotes P value ≤ 0.005.

Mitochondrial dysfunction is impaired in common and rare neuronal diseases. Recent studies have shown that genes involved in the nuclear-encoded electron transport chain exhibit reduced expression in dopamine neurons and substantia nigra of humans with symptomatic and subclinical Parkinson's neuropathology. Systems biology analysis of human brains revealed a pervasive expression defect of *PGC-1α*-linked bioenergetics genes in laser-captured dopamine neurons of Parkinson's patients and substantia nigra of individuals with subclinical, brainstem-predominant Lewy body neuropathology [14] that likely represent preclinical PD [36]. These findings were replicated in an independent population [37]. The gene sets identified pinpointed defects in mitochondrial electron transport, glucose utilization, and glucose sensing early in the disease course [14]. Conversely, activating the *PGC-1α*-regulated program ameliorated mutant *α*-synuclein- and rotenone-induced loss of dopamine neurons in primary midbrain cultures [14]. In mouse models of PD, the *PGC-1α* transgene suppressed MPTP-induced dopaminergic neurodegeneration [18]. Conversely, deletion of *PGC-1α* dramatically enhanced MPTP-induced degeneration of nigral dopamine neurons in a mouse model of PD [20]. In mice carrying mutant *PARK2*-linked familial PD repression of *PGC-1α* by the parkin substrate PARIS contributes to neurodegeneration, while increased *PGC-1α* expression suppressed mutant parkin-induced neurodegeneration [19]. In an isogenic human induced Pluripotent Stem Cell model of Parkinson's *PGC-1α* suppressed cell loss in response to environmental toxins and mutant *α*-synuclein [38]. In short, evidence in human brain and in multiple cellular, human stem cell, genetic and toxic animal models of PD link *PGC-1α*-regulated programs to an onset mechanism of Parkinson's. Beyond Parkinson's there are clues to suggest that a *PGC-1α*-regulated transcriptional program is more generally involved in aging-related diseases such as ALS and HD [16, 39]. Mildly increased *PGC-1α* expression in skeletal muscle protects from sarcopenia during aging [4].

Is this pathway a tractable target for gene therapy? In mice, both too little and too much *PGC-1α* are detrimental. *PGC-1α* knockout leads to cardiomyopathy [12], but forced overexpression of *PGC-1α* at supraphysiologic levels induces uncontrolled mitochondrial proliferation and cardiomyopathy [12]. Analogously, adenoassociated virus-(AAV-) mediated overexpression of *PGC-1α* in the substantia nigra induces a loss of dopaminergic markers and enhances nigral vulnerability [40, 41].

Chemically restoring the activity of the endogenous *PGC-1α*-regulated circuit (i.e., reduced in Parkinson's neuropathology) back to normal may be a more advantageous strategy for early intervention in incipient PD than forced overexpression of exogenous *PGC-1α*. This could be accomplished through small molecule drugs that modulate any of the switches in the neuronal circuit we here delineated. *PGC-1α* expression can be activated through molecules acting upstream of the *PGC-1α* gene such a glitazones. For example, pioglitazone confers neuroprotection in mouse models of PD [32] and activates the entire neuronal *PGC-1α-ERRα*-regulated feed-forward circuit in neuronal cells through activation of the nuclear receptor *PPARγ*, the transcription factor of *PGC-1α*. Because *PPARγ* regulates numerous transcriptional cascades in addition to the *PGC-1α*-regulated circuit, this approach carries the risk of side effects through broad activation of unwanted programs. Moreover, initiation of treatment during earliest, preclinical disease stages might be necessary to achieve meaningful effects. In patients with clinically manifest PD (indicating advanced underlying Lewy body neuropathology and substantial loss of dopamine neurons), no efficacy was found for pioglitazone in slowing disease progression in a clinical trial [42]. However, a large, recent epidemiologic study suggested a beneficial effect for glitazones such as pioglitazone in reducing risk of PD in neurologically normal individuals with diabetes [43]. This study found an incidence rate of PD in the glitazone-exposed group of 6.4 per 10,000 patient years compared with 8.8 per 10,000 patient years in those prescribed other antidiabetic treatments [43]. *ERRα* is another switch in the circuit that could be targeted. We show that endogenous *PGC-1α* regulates neuronal *ERRα* transcription (Figure 2) and that silencing neuronal *ERRα* recapitulates the effect of *PGC-1α* knockdown on endogenous electron transport chain expression (Figure 2). *ERRα* may be both sufficient and necessary for mediating the action of *PGC-1α* on mitochondrial biogenesis as in muscle cells induction of mitochondrial biogenesis by *PGC-1α* was largely suppressed when *ERRα* was inhibited [8]. Targeting *ERRα* directly with small molecules is an attractive strategy for drug development, although the ligand-binding pocket is small [44]. Phytoestrogens activate *ERRα* [45] and a synthetic compound that inhibits *ERRα* has been reported [8, 45]. There is also precedent for a promising third strategy, targeting the *ERRα-PGC-1α* interaction with small molecules [8, 46].

These data clarify a transcriptional network regulated by neuronal *PGC-1α* that now can be therapeutically targeted for common neurodegenerative diseases. Novel chemical modulators tailored to this circuit together with a transformed

clinical trial paradigm directed at individuals with earliest, preclinical stages of neuropathology will be positioned to modify neuronal bioenergetics defects and potentially achieve substantial clinical benefits for patients with neurodegenerative disease.

Conflict of Interests

The authors have no conflict of interests to report.

Acknowledgments

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Research Article

Methyl-Arginine Profile of Brain from Aged PINK1-KO+A53T-SNCA Mice Suggests Altered Mitochondrial Biogenesis

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Hereditary Parkinson's disease can be triggered by an autosomal dominant overdose of alpha-Synuclein (SNCA) or the autosomal recessive deficiency of PINK1. We recently showed that the combination of PINK1-knockout with overexpression of A53T-SNCA in double mutant (DM) mice potentiates phenotypes and reduces survival. Now we studied brain hemispheres of DM mice at age of 18 months in a hypothesis-free approach, employing a quantitative label-free global proteomic mass spectrometry scan of posttranslational modifications focusing on methyl-arginine. The strongest effects were documented for the adhesion modulator CMAS, the mRNA decapping/deadenylation factor PATL1, and the synaptic plasticity mediator CRTC1/TORC1. In addition, an intriguing effect was observed for the splicing factor PSF/SFPQ, known to interact with the dopaminergic differentiation factor NURR1 as well as with DJ-1, the protein responsible for the autosomal recessive PARK7 variant of PD. CRTC1, PSF, and DJ-1 are modulators of PGC1alpha and of mitochondrial biogenesis. This pathway was further stressed by dysregulations of oxygen sensor EGLN3 and of nuclear TMPO. PSF and TMPO cooperate with dopaminergic differentiation factors LMX1B and NURR1. Further dysregulations concerned PRR18, TRIO, HNRNPA1, DMWD, WAVE1, ILDR2, DBNDD1, and NFM. Thus, we report selective novel endogenous stress responses in brain, which highlight early dysregulations of mitochondrial homeostasis and midbrain vulnerability.

1. Introduction

Idiopathic Parkinson's disease (PD) is the second most frequent age-associated neurodegenerative disease. It manifests itself with a movement disorder characterized by hypokinesia, rigidity, rest tremor, and postural instability. The underlying neuron loss exhibits preferential affection of the midbrain dopaminergic neurons. Within the cytoplasm of degenerating neurons, protein aggregates form and coalesce to the so-called Lewy bodies and Lewy neurites, in a process that ascends from olfactory and autonomous neurons via the midbrain to the cerebral cortex [1]. The main component of these inclusion bodies is alpha-Synuclein [2]. This protein plays a key role in the pathogenesis and the transmissibility of PD [3]. Moreover, within the past decades, so many other risk factors have been identified such that now the crucial task of understanding their interactions and shared downstream effects has to be prioritized.

In sporadic PD patients without a positive family history, genome wide investigations of genetic risk factors have identified variants at the genes alpha-Synuclein (*SNCA*) and Tau (*MAPT*) as the main contributors [4]. Alpha-Synuclein is a small lipid-membrane associated protein with chaperone features which is concentrated at presynaptic vesicles [5], but it is also found at the interface between mitochondria and the endoplasmic reticulum [6]. Tau is a microtubule-associated protein that is crucial for axonal organelle transport and growth [7].

Familial PD comprises about 10% of all PD cases [5]. Autosomal dominant forms of PD can be caused by the gain-of-function of alpha-Synuclein through various missense mutations such as A53T (responsible for the PARK1 variant

of PD) or through elevated gene dosage (PARK4 variant) [8]. Alpha-Synuclein gain-of-function leads to cumulative mitochondrial damage [9–11], while the absence of alpha-Synuclein renders neurons resistant to mitochondrial stressors [12, 13]. Autosomal recessive forms of PD have been associated very clearly with dysfunctional mitochondria and oxidative stress. A possible cause is (1) the loss-of-function of the mitochondrially targeted ubiquitin kinase PINK1 (responsible for the PARK6 variant) [14, 15], which is known for its role in mitochondrial repair by mRNA translation or fusion [16, 17] and in the autophagic degradation of mitochondria [18]. A possible cause is also (2) the loss-of-function of the PINK1-activated ubiquitin ligase PARKIN (PARK2 variant) [19, 20], which is known as a cytoplasmic regulator of trophic signals [21], but may relocalize to dysfunctional mitochondria and carry out mitophagy [22]. Yet a further cause is (3) the loss-of-function of multifunctional DJ-1 (PARK7 variant), known as an oxidation-sensitive protein that sequestrates the nuclear corepressor PSF, thus regulating the transcriptional regulation of antioxidant defense, DNA repair, and dopamine synthesis [23]. A final cause to be mentioned is (4) the loss-of-function of the lysosomal degradation enzyme Glucocerebrosidase (GBA), which influences the degradation and aggregation of alpha-Synuclein [24, 25].

Given that most PD cases have a polygenic or multifactorial origin, we have recently shown in a digenic mouse modelling approach that the combination of PINK1-KO with overexpression of A53T-SNCA in double mutant (DM) mice potentiates the phenotypes and impairs survival. Lewy-body-like pSer129-SNCA positive aggregates become detectable in the brain tissue after the age of 1 year in these DM mice, and marked mitochondrial mRNA dysregulation and DNA damage marker anomalies were documented, with the spontaneous movements being progressively reduced from the age of 3 months [26].

In view of the prime importance of posttranslational modifications in the regulation of mitophagy and PD [27], we exploited these digenic PD model brains further in several parallel characterization approaches to identify molecular events, which accompany the advent of inclusion bodies and subsequent lethality. The strongest lysine-ubiquitination target observed in brain of the aged DM mice was of course the overexpressed pathogenic alpha-Synuclein [26].

Addressing epigenetics and focusing on the lysine acetylation of proteins, we observed only sparse histone acetylation changes and tubulin acetylation changes, but documented dramatic deficits of mitochondrial acetylation levels at the mouse age of 18 months [28].

Now another hypothesis-free, quantitative label-free global proteomic mass spectrometry scan of posttranslational modifications (PTMscan®) was employed, focusing on mono-methyl-arginine, a crucial modulator of transcription factors and splicing factors [29, 30]. Thus we aimed to complement our existing knowledge about the global transcriptome profile of the DM brain with a pioneer survey of its key regulators. To our knowledge there is no publication so far on the global mono-methyl-arginine profile of brain in a neurodegenerative disorder.

Epigenetic modifications, in particular the methylation of DNA and histones have been characterized in great detail, and for the PD-susceptible midbrain dopaminergic neurons a crucial regulation of PITX3/ADH2/RA/NURR1/SIN3A/PSF through this process was described [31]. In contrast, almost nothing is known about the role of methyl-arginine modifications of other nuclear and cytoplasmic proteins, which have recently been demonstrated to exist [32]. Published reports only provide proof-of-principle that the global methyl-arginine modifications of neural cells depend on trophic cell state [33].

2. Materials and Methods

2.1. Breeding and Ageing of DM Mice with Homozygosity for Pink1$^{-/-}$ and for A53T-SNCA Overexpression. Our generation, ageing, and characterization of the DM mice were reported before [26]. In brief, the genetic background contains 129/SvEv and FVB/N in a 50 : 50 distribution on average, similar to the WT control mice that were aged F1-hybrids from a crossbreeding of 129/SvEv and FVB/N mice descended from littermates of the respective single mutant animals. The mice were kept in individually ventilated cages under 12 h light cycle with food and water *ad libitum*. Sentinel mice and regular health monitoring including blood tests for viral and parasite infections uncovered no pathology. Housing of animals was in accordance with the German Animal Welfare Act, the Council Directive of 24 November 1986 (86/609/EWG) with Annex II and the ETS123 (European Convention for the Protection of Vertebrate Animals). The mice under investigation were bred and aged at the FELASA-certified Central Animal Facility (ZFE) of the Frankfurt University Medical School. After decapitation, the organs were removed and immediately frozen in liquid nitrogen.

2.2. Global Mono-Methyl-Arginine Motif Survey by Label-Free Mass Spectrometry. Brain hemispheres from mice at age of 18 months (three DM versus three WT matched for male sex) were dissected in parallel, snap-frozen in liquid nitrogen, stored at −80°C, and shipped on dry ice for the commercial MethylScan® procedure by Cell Signaling Technology, Inc. [34, 35]. In short, tissue extracts were protease-digested and subjected to C18 solid-phase extraction. The lyophilized peptides were immunoprecipitated by protein-A/G-agarose-immobilized mono-methyl-arginine motif antibodies #8015/8711. Peptides were loaded directly onto a 10 cm × 75 μm PicoFrit capillary column packed with Magic C18 AQ reversed-phase resin. The column was developed with a 90 min linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL/min. The MS parameter settings were as follows: MS Run Time 96 min, MS1 Scan Range (300.0–1500.00), and Top 20 MS/MS (Min Signal 500, Isolation Width 2.0, Normalized Coll. Energy 35.0, Activation-Q 0.250, Activation Time 20.0, Lock Mass 371.101237, Charge State Rejection Enabled, Charge State 1+ Rejected, Dynamic Exclusion Enabled, Repeat Count 1, Repeat Duration 35.0, Exclusion List Size 500, Exclusion Duration 40.0, Exclusion Mass Width Relative to Mass,

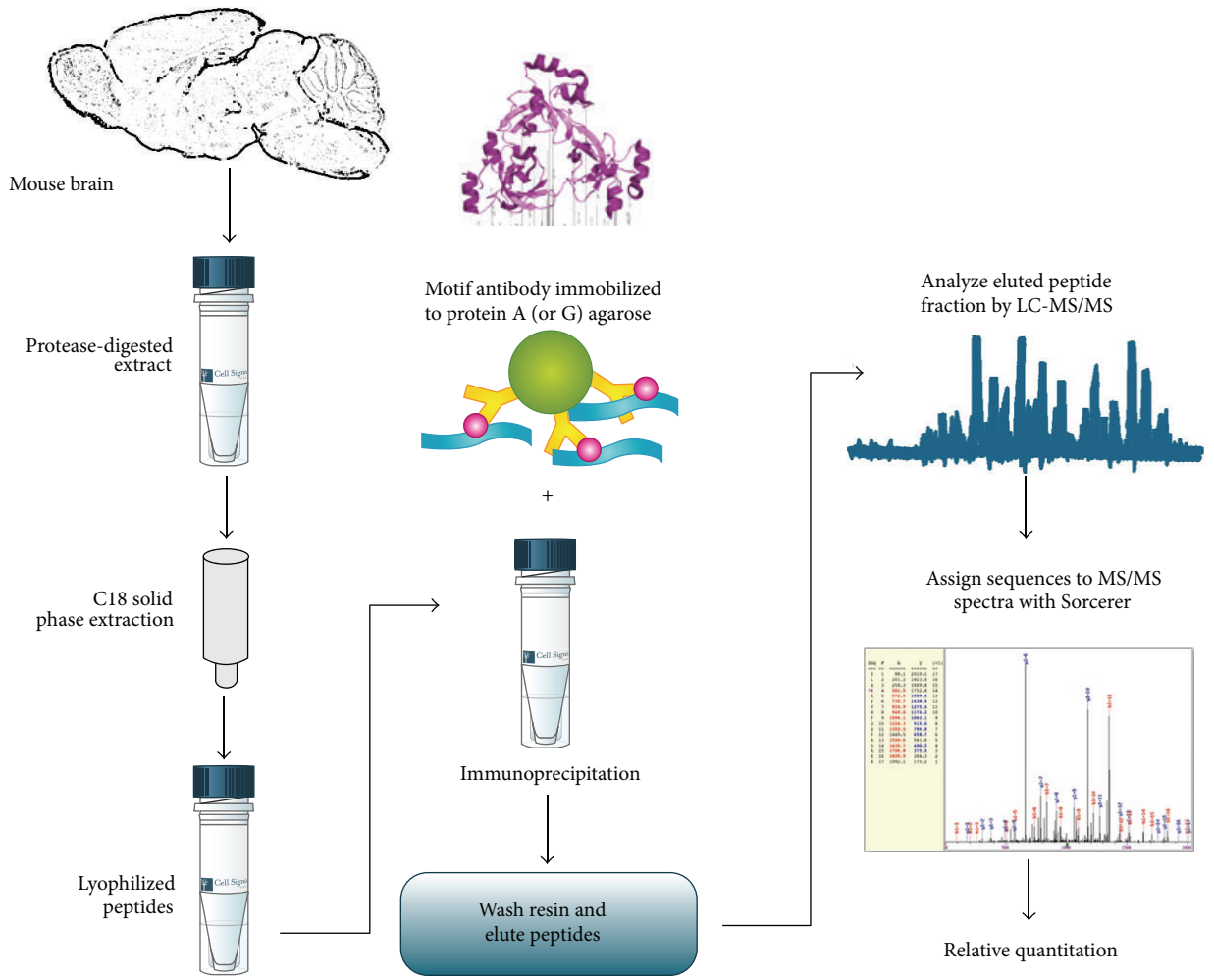

Figure 1: Workflow chart illustrating the technical approach to quantify the mono-methyl-arginine-modification of peptides throughout the global brain proteome in a quantitative and label-free manner by immunoprecipitation and mass spectrometry. The immunoprecipitation step illustrates the motif antibody (above), the agarose beads (green circle), its immunoglobulin coating (yellow), and the binding of digested peptides (blue) with mono-methyl-arginine modifications (pink). Graphic elements from internet-sites (http://www.cellsignal.com/common/content/content.jsp?id=proteomics-discovery and http://media.cellsignal.com/www/pdfs/proteomics/methylscan_workflow.pdf) were used with permission of Cell Signaling Inc.

Exclusion Mass Width 10 ppm). MS/MS spectra were evaluated using SEQUEST 3G and the Sorcerer 2 platform from Sage-N Research (v4.0, Milpitas, CA, USA) [36]. Searches were performed against the most recent update of the NCBI *Mus musculus* database with mass accuracy of ±50 ppm for precursor ions and 1 Da for product ions. The results were filtered with mass accuracy of ±5 ppm on precursor ions and presence of the intended motif (Me-R). The peptide identification with relative quantification by mass spectrometry (MS) occurred by LC-MS/MS analysis using LTQ-Orbitrap-VELOS with ESI-CID Sorcerer search.

With double injections of the 6 biological samples, 12 LC-MS/MS experiments were conducted and bioinformatically processed, using the maximum % coefficient of variation (% CV) to control replicate reproducibility. Using a 5% default false positive rate to filter the Sorcerer results, this procedure yielded a total of 2,218 redundant methylated peptide assignments to 971 nonredundant ubiquitinated peptides. The quantitative data from the three control WT mice were averaged to compare each DM mouse individually and derive the respective fold change. The original data are available from the authors upon request.

3. Results

The global brain proteome of three 18-month-old DM mice versus three matched wildtype (WT) mice was analyzed in a quantitative label-free mass spectrometry approach (see Figure 1) for the abundance of mono-methyl-arginine (Me-R) motifs (MethylScan). The original data were filtered for consistency and effect size. We excluded factors where each of the three DM mice did not show the same direction of change. We also excluded changes smaller than 1.5-fold. The remaining observations comprised only 7 upregulation

Normalized fold change								
DM all: control	DM 1: control	DM 2: control	DM 3: control	Gene name	Protein name	Site	Accession	Putative function
3.6	3.8	3.9	3.2	Patl1	PATL1	385	Q3TC46	mRNA degradation?
3.4	4.1	3.9	2.3	Crtc1	TORC1	103	Q68ED7	Synaptic plasticity, mitochondrial biogenesis
2.3	2.7	2.4	2.0	Prr18	PRR18	202	Q6PAN7	Neurite outgrowth?
2.3	1.8	3.1	1.9	Trio; Trio	TRIO; TRIO iso4	2654; 2655	Q0KL02; Q0KL02-4	Trophic signals for axons
2.2	2.5	2.1	2.1	Hnrnpa1	HNRNP A1 iso2	§232	Q5EBP8	Cytosolic mRNA transport
2.2	1.6	3.0	1.7	Dmwd	DMWD	543	Q08274	Synaptic localization
1.9	1.6	2.1	1.9	Sfpq	PSF	228, 234, 237	Q8VIJ6	Splicing factor, interactor of DJ-1, mitochondrial biogenesis
−6.6	−27.2	−16.6	−2.6	Cmas	CMAS	16	NP_034038	Cell adhesion
−2.3	−2.6	−1.9	−6.8	Tmpo; Tmpo	TMPO; TMPO iso6	85; 85	Q61029; Q61033	Midbrain dopaminergic differentiation?
−2.0	−2.0	−2.0	−1.8	Egln3	EGLN3	134	Q91UZ4	Oxygen sensor
−2.0	−2.6	−1.8	−1.7	Wasf1	WAVE1	341	Q8R5H6	Mitochondrial distribution in dendritic spines
−2.0	−1.8	−2.9	−1.5	Ildr2	ILDR2	618, 623	NP_001158000	Lipid homeostasis
−2.0	−1.8	−2.4	−1.6	Dbndd1	DBNDD1	22	NP_082422	Vesicle trafficking
−1.8	−2.0	−1.6	−1.8	Nefm	NFM	26	P08553	Axon caliber

§: published site

Figure 2: Mouse brain; trypsin digest; mono-methyl-arginine motif antibody #8015/8711. JW Goethe University Hospital (Q153802_8_25) MethylScan results.

effects and only 7 downregulation effects, which are shown in Figure 2, ordered by effect size (illustrating upregulations in red and downregulations in blue, highlighting relative effect sizes of different animals with a heat map color scale and emphasizing proteins with consistent >2-fold changes by a more intense coloring).

3.1. Upregulations in Brains from Aged DM Mice. Me-R385-PATL1 (protein PAT1 homolog 1) showed a 3.6-fold change in brains from aged DM mice.

Me-R103-CRTC1/TORC1 (CREB regulated transcription activator or transducer of regulated CAMP response element-binding protein) showed a 3.4-fold change.

Me-R202-PRR18 (proline-rich region 18) showed a 2.3-fold change.

Me-R2654-TRIO (triple functional domain Rho Guanine Nucleotide Exchange Factor) and Me-R2655-TRIO isoform 4 showed a 2.3-fold change.

Me-R232-HNRNPA1 isoform 2 (heterogeneous nuclear ribonucleoprotein A1) showed a 2.2-fold change.

Me-R543-DMWD (dystrophia myotonica WD repeat-containing protein) showed a 2.2-fold change.

Me-R228-PSF/SFPQ (polypyrimidine tract-binding protein-associated-splicing factor or splicing factor and proline- and glutamine-rich), Me-R234-PSF/SFPQ, and Me-R543-PSF/SFPQ showed a 1.9-fold change.

3.2. Downregulations in Brains from Aged DM Mice. Me-R16-CMAS (N-acetylneuraminate cytidylyltransferase) showed a −6.6-fold change.

Me-R85-TMPO (thymopoietin- or lamina-associated polypeptide 2) showed a −2.3-fold change.

Me-R134-EGLN3 (Egl nine homolog 3 or prolyl hydroxylase domain-containing protein 3, PHD3) showed a −2.0-fold change.

Me-R341-WAVE1 (Wiskott-Aldrich syndrome protein family member 1) showed a −2.0-fold change.

Me-R618-ILDR2 (immunoglobulin-like domain-containing receptor 2) and Me-R623-ILDR2 showed a −2.0-fold change.

Me-R22-DBNDD1 (dysbindin domain-containing protein 1) showed a −2.0-fold change.

Me-R26-NFM (neurofilament medium peptide) showed a −1.8-fold change.

Both the upregulation and the downregulation events clustered among proteins with nuclear localization, shuttling to cytoplasmic and cytoskeletal positions, as illustrated in Figure 3. Dysregulations were not observed for chromatin binding factors, for G protein regulators, for vesicle proteins, for translation factors, for membrane receptors/channels/transporters, and for membrane adaptors/scaffolds, which are known to undergo methyl-arginine modifications [35].

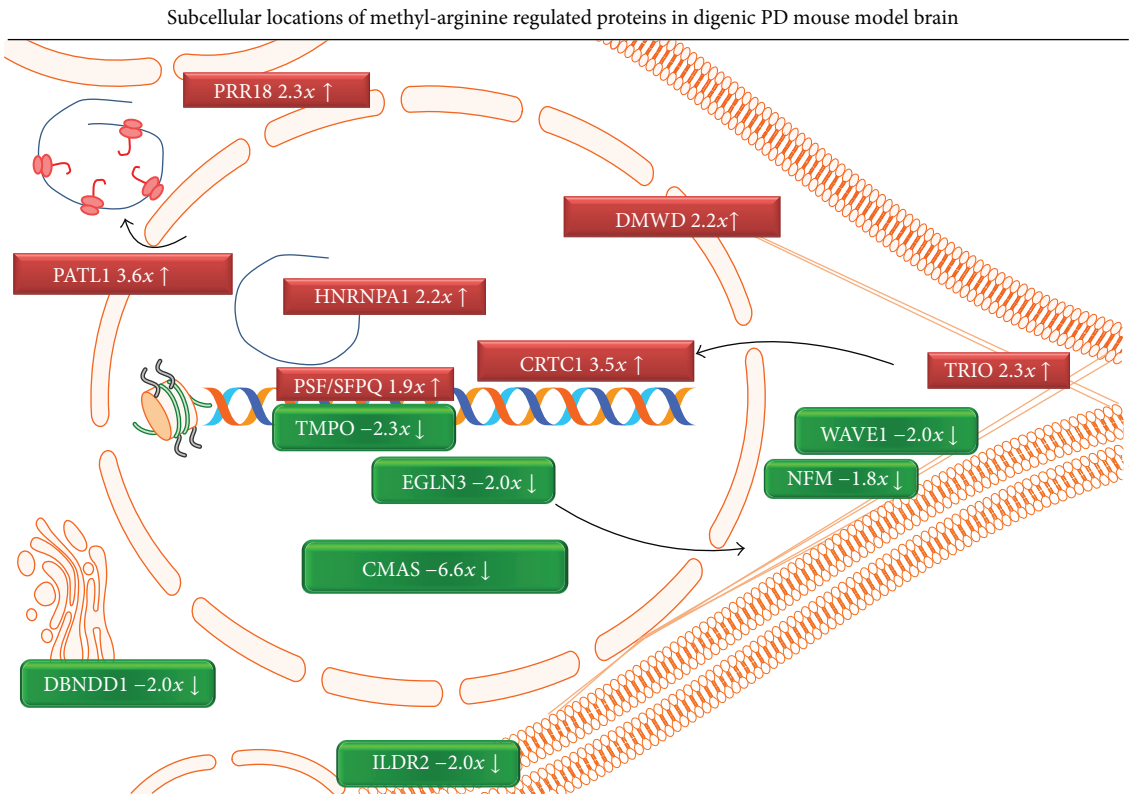

Figure 3: Subcellular localization scheme of proteins with mono-methyl-arginine that show changed abundance. The locations are shown according to GeneCards database information and published literature; diagrams from Motifolio toolkits were used for drawing. Red boxes represent upregulation events; green ellipses were used for downregulations, in sizes proportional to fold changes. Apart from the nuclear envelope around DNA and a histone in the center of the picture, the rough endoplasmic reticulum with associated translating RNA is shown in the upper left corner, the Golgi apparatus on the left side, and the smooth endoplasmic reticulum in the lower left corner; cytoskeletal elements extend from the right side of the nucleus to the cell membranes, which form a neurite towards the right side and the plasma membrane of an adjacent cell is shown in the lower right corner. Arrows indicate nuclear export and import.

4. Discussion

This study of the global mono-methyl-arginine profile in brain hemispheres by quantitative label-free mass spectrometry is the first of its kind in a neurodegenerative disorder. Although this approach might be expected to reveal epigenetic anomalies, consistent strong histone methylation changes were not observed. Of course, any global survey may produce false positive and false negative errors, but this screening yielded a surprisingly high enrichment of factors that were previously connected to neurodegeneration, PD, or dopaminergic differentiation. Furthermore, a considerable number of the identified factors are interactors in protein complexes, suggesting that they constitute particularly promising candidates for follow-up experiments. Below we comment on the relevance of each factor individually.

Regarding the upregulated events, little is known about PATL1, but it is enriched at splicing speckles and shuttles between nucleus and cytoplasm. It was observed that a viral infection may disrupt PATL1-localization at P-bodies as the sites of mRNA degradation and sequestrate PATL1 to the vicinity of lipid droplets [37]. This may be relevant given that the PINK1/PARKIN pathway was shown to mediate the cellular resistance to infections [38, 39].

CRTC1/TORC1 senses the convergence of calcium/cAMP/phosphorylation signals, relocalizes from the synapse to the nucleus in an activity-dependent manner, and triggers transcriptional responses that are key to the late phase of long-term potentiation and synaptic plasticity [39–41]. This observation is very intriguing, given that impaired synaptic function and plasticity in the nigrostriatal and corticostriatal brain projections have already been demonstrated for these mice due to their A53T-SNCA overexpression [42–52]. CRTC1 is also a potent coactivator of PGC1alpha and inducer of mitochondrial biogenesis that modulates the growth of neurites [53, 54]. CRTC1 has been implicated in several neurodegenerative diseases already. Synaptic activity induces CRTC1 dephosphorylation (Ser151), nuclear translocation, and CRTC1-dependent transcription in the hippocampus, which is deficient in Alzheimer's disease models. CRTC1 overexpression reverses amyloid-beta-induced spatial learning and memory deficits [55–57]. In models of Huntington's disease, mutant huntingtin protein interferes with the TORC1-CREB interaction to repress transcription of brain-derived neurotrophic factor [58], and also the depletion of CRTC1 contributes to Huntington's disease [59]. Moreover, CRTC1 phosphorylation is crucial for the outcome after cerebral ischemia [60]. Thus, the increased methylation at

R103-CRTC1 in our PD model confirms an important role of this DNA-binding protein in neurodegenerative processes and identifies a novel regulation mechanism.

No functional insights exist on PRR18, which has predicted localizations in the nucleus and endoplasmic reticulum, being coexpressed with neurite outgrowth regulators such as Lingo1 (leucine-rich repeat and Ig domain-containing 1) in mouse brain according to the STRING Heidelberg protein interaction database.

TRIO controls the directional extension of axons [61], modifying the signaling by FGFR and GPCR pathways and acting through AKT signaling to influence mitochondrial apoptosis [62]. Its DBL/GEF domains are thought to influence the production of membrane ruffles and the formation of stress fibers.

HNRNPA1 is involved in the packaging of pre-mRNA into hnRNP particles, the transport of poly A+ mRNA from the nucleus to the cytoplasm, and the selection of splice sites. It is coregulated together with the splice factor PSF/SFPQ (see below) by stress-induced phosphorylation signals [63]. Mutations of HNRNPA1 were reported in the motoneuron degeneration disorders Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD) [64].

Although little functional insight exists on DMWD, it is highly abundant in neurons and concentrated in synaptic connections [65]. It contains multiple WD40-repeats, which have been implicated in cytoskeleton assembly, pre-mRNA processing, and signal transduction. In the skeletal muscle degeneration disorder named myotonic dystrophy, the DMWD levels were found deficient [66].

In spite of the modest effect size, the upregulation of PSF/SFPQ methylation is intriguing because PSF/SFPQ in the nucleus interacts directly with the protein DJ-1, which is responsible for autosomal recessive juvenile Parkinson's disease [67]. DJ-1 inhibits the sumoylation of PSF/SFPQ, while elevating the expression of the dopamine homeostasis factors tyrosine hydroxylase (TH) and vesicular monoamine transporter 2 (VMAT2) [68, 69] as well as modulating the levels of PGC1alpha as a key factor of mitochondrial biogenesis [70], which is also regulated by CRTC1/TORC1 above. Nuclear PSF/SFPQ also interacts with FUS (fused in sarcoma), a protein responsible for the motoneuron degeneration disorders ALS and FTLD [71]. Nuclear PSF/SFPQ is recruited to sites of DNA damage [72]. PSF/SFPQ was found to interact directly with the internal ribosomal entry site (IRES) of the DNA repair factor TP53 (p53) [73] and with cytoplasmic PARKIN, which is responsible for the PARK2 variant of autosomal recessive juvenile Parkinson's disease [74]. The observation of its Me-R changes is also intriguing because PSF/SFPQ exists in a nuclear protein complex with LMX1B/PITX3/NR4A2 = NURR1, key factors in the development of midbrain dopaminergic neurons [75, 76], similar to TMPO below. Arginine methylation of PSF/SFPQ by the arginine N-methyltransferase PRMT1 was observed previously and shown to enhance the association with mRNA in mRNP complexes in mammalian cells [77]. The presence of PSF/SFPQ in neuronal RNA transport granules was reported, and its interaction with JNK-kinase depends on stimulation by NGF (Nerve Growth Factor) [78]. An overall role of PSF/SFPQ mRNA effects consisted, for example, in the inhibition of IGF-1-stimulated transcriptional activity and thus the trophic modulation of cells [79]. Like CRTC1, PSF/SFPQ also has been implicated in several neurodegenerative diseases already. SFPQ modulates the splicing of Tau, while Tau mediated the nuclear depletion of PSF/SFPQ in Alzheimer's and Pick's disease together with a cytoplasmic accumulation [80–83], as well as a depletion in the brain of Down syndrome cases [84]. The PSF/SFPQ transcript was upregulated in Alzheimer's disease brain [85]. PSF/SFPQ mislocalized from the neuronal nucleus to the cytoplasm in the motor neuron diseases ALS and FTLD, which were caused by TDP-43 mutations [86, 87]. Thus, also the observation of increased methylation of PSF/SFPQ at Me-R228, Me-R234, and Me-R543 in our PD model supports the relevance of this RNA-binding factor for neurodegenerative processes and describes a new regulation mechanism.

Regarding the downregulated events, the nuclear protein CMAS activates the sugar NeuNAc to the compound CMP-NeuNAc, which is needed for the addition of sialic acid to modulate cell surface glycoprotein and glycolipid interaction, thus modulating cell adhesion. No evidence existed so far that implicated this factor in neurodegenerative disorders, but it seems to be involved in Fragile-X mental retardation [88].

The nuclear protein TMPO interacts with LMX1B and NURR1, two key factors in the development of midbrain dopaminergic neurons [75], similar to PSF/SFPQ above. It has been implicated in dilated cardiomyopathy and in diabetes mellitus type 1 [89, 90], but not in neurodegenerative disorders so far.

The nucleus-cytoplasm shuttling protein EGLN3 acts as cellular oxygen sensor that hydroxylates HIF1A and HIF2A, thus regulating neuronal apoptosis [91]. It is coinduced with TP53-activation in the DNA damage response pathway [92]. Interestingly, *EGLN3* showed downregulated transcript levels in the midbrain-derived dopaminergic neuronal cell line MN9D after treatment with the Parkinsonian neurotoxin MPP+ [93]. These features are quite similar to DJ-1, which acts as an oxygen sensor, regulates HIF1A and TP53, and rescues the MPP+ toxicity of PINK1-deficient dopaminergic neurons [94–98].

WAVE1 acts downstream of Nerve Growth Factor and of the RAC1 GTPase to regulate actin filament reorganization and axonal filopodia formation via its interaction with the Arp2/3 complex [99] and controls dendritic spine morphology and neural activity-induced mitochondrial distribution in dendritic spines [100, 101]. *WAVE1* transcription is negatively regulated by the amyloid precursor protein intracellular domain, and WAVE1 protein depletion dramatically reduces amyloid beta levels and restores memory deficits in a mouse model of Alzheimer's disease [102]. WAVE1 coaggregates with hyperphosphorylated Tau and is found in neurofibrillary tangles and abnormal neurites of Alzheimer's disease brain [103].

ILDR2 localizes at tricellular tight junctions, while modulating lipid homeostasis and endoplasmic reticulum stress pathways [104, 105]. Although nothing is known yet about disease associations of ILDR2, mutations in its homologue ILDR1 were shown to be responsible for a neurosensory

degeneration disorder resulting in the autosomal recessive hearing impairment DFNB42 [106].

No functional insights exist on DBNDD1. Its homolog dysbindin-1 (DTNBP1 or BLOC1S8) is a component of the BLOC-1 complex, which targets membrane protein cargos into vesicles for delivery into nerve terminals and is thus involved in neurite extension as well as synaptic vesicle trafficking [107]. DTNBP1 regulates the cell surface presence of the dopamine receptor DRD2 and modulates prefrontal activity via the dopamine D2 pathway [108]. A disease association for DBNDD1 is not identified yet, but mutations in DTNBP1 are responsible for the Hermansky-Pudlak syndrome 7 [109], which is characterized by oculocutaneous albinism, prolonged bleeding, and pulmonary fibrosis.

NFM is important for neuronal axon caliber [110]. It is a component of Lewy bodies in Parkinson's disease brains [111] and a reduction of phospho-NFM levels was already observed in PINK1-KO mouse brains [112]. Autoantibodies against NFM are observed in the cerebrospinal fluid and blood serum of individuals with the motoneuron degeneration ALS [113].

For each of these factors, firstly the discovery that they are regulated by methylation and secondly the identity of the specific arginine, both are providing valuable insights. A downside of this novel approach, however, is the fact that no site-specific antibodies are available at present to validate these findings by a technically independent approach. This is a severe limitation of our study. Thus, we can only report in a descriptive manner that the survey supports the relevance of the proteins listed above in the early stage of Parkinsonian neurodegeneration concomitant with the appearance of Lewy-body-like pSer129-SNCA positive protein aggregates and the manifestation of motor deficits in the DM mouse line.

Even if the microscopic detection of pSer129-SNCA positive protein aggregates in the brain becomes possible only in the second year of life of our DM mice, at a submicroscopic level an insidiously progressive pathology might be ongoing much earlier. Alpha-Synuclein would adopt pathological conformations, oligomerize, undergo fibrillation, sequestrate interactor molecules into insolubility, and be compensated by degradation and extrusion efforts, before this process becomes visible in microscopes. In this light it is interesting to note that several proteins that are known to coaggregate with the disease protein in neurodegenerative conditions, did indeed show dysregulated arginine methylation in this screening, namely, NFM (coaggregating with SNCA), WAVE1 (coaggregating with Tau), and PSF/SFPQ (mislocalized from nucleus to cytoplasm by Tau and TDP-43).

It is also interesting to note that no loss of dopaminergic midbrain neurons could be substantiated in the aged DM mice; however, the dysregulated arginine methylation of PSF/SFPQ and TMPO, both of which interact with LMX1B/NURR1 in the regulation of dopaminergic midbrain neuron differentiation and regeneration, suggests that molecular anomalies in these neurons are occurring in a selective and prominent manner, while the neuronal morphology is still intact.

As an additional approach to evaluate the credibility of this survey, we questioned whether the previous transcriptome profile in the brain of aged single mutant A53T-SNCA overexpressing mice or of aged DM mice can be correlated to the dysregulated methylation of transcription factors.

In the case of aged A53T-SNCA mice, the global transcriptome was previously documented by us in the striatal region and dysregulations of a CREB regulated transcription factor named *Atf2* (cyclic AMP-responsive element-binding protein 2) and its upstream regulators *Cnr1* and *Homer1* were among the main observations [44] (see Table S2 of that reference). The same pathway is reflected in the present MethylScan by the CREB regulated transcription factor CRTC1/TORC. This pathway is crucial for trophic signaling, neurite extension, synaptic plasticity and adhesion, processes that are actively regulated by CRTC1/TORC1, TRIO, PSF/SFPQ, CMAS, WAVE1, ILDR2, NFM, and perhaps by PRR18 and DBNDD1 as MethylScan candidates, as well as by PARKIN and DJ-1 as additional causes of autosomal recessive PD [114–117].

In the case of the aged DM mice, the global transcriptome throughout brain hemispheres was previously documented by us to comprise dysregulations of the SNCA-abundance marker and cell adhesion factor *Lect1* (Leukocyte-expressed chemotaxin-1 or chondromodulin-1), of the autophagy factor *Dapk1* (death-associated protein kinase 1) and of the DNA damage marker *H2afx* (H2A histone family, member X) [26]. *Lect1* transcription upon demethylation of its core promoter region [118] occurs upon Nerve Growth Factor treatment in a TP53-dependent manner [119], in parallel to converse changes in the levels of HIF-1alpha (Hypoxia-inducible factor 1, alpha subunit) [120]. *Dapk1* transcripts are produced in dependence on its promoter methylation which is regulated by the transcription factor TP53. The *Dapk1* transcripts undergo alternative splicing [121]. *H2afx* transcript levels and protein localization depend on histone methylation and also on the DNA repair activator TP53 [122, 123]. Clearly the dependence of these three transcripts on the TP53 pathway is reflected in our MethylScan now by the PSF/SFPQ modulation of the TP53-IRES and by the TP53-effects of the PSF/SFPQ-interactor protein DJ1 [96, 124]. It was already observed in the neurodegenerative process of Huntington's disease that H2AFX, ATM, and TP53 are coactivated before the microscopic appearance of aggregates [125] and that there is a relative deficit of TP53/H2AFX dependent DNA repair [126]. Again, this pathway is also modulated by PARKIN and DJ-1 as additional causes of autosomal recessive PD [96, 97, 117, 127–133].

TP53 via PARKIN was observed to modulate glucose metabolism and the Warburg effect [132], mitochondrial length [134], and mitophagy [129, 130]. Indeed, the mitochondrial biogenesis pathway also could be affected in the brains of the aged DM mice according to the MethylScan findings, given that CRTC1/TORC1 and PSF/SFPQ in interaction with DJ-1 are known modulators of PGC1alpha, the central inducer of mitochondrial biogenesis [135]. These arginine methylations could represent a cellular compensation effort. Given that the A53T-SNCA overexpression in the DM mice

is known to exert mitochondrial toxicity but that dysfunctional mitochondria cannot be eliminated through selective mitophagy in the DM mice due to the absence of PINK1, one would expect dysfunctional mitochondria to accumulate in neurons in a similar manner as they accumulate as ragged red fibers in muscles of MERRF patients. However, such a neuronal accumulation of dysfunctional mitochondria could not be observed by microscopy or by immunoblot assessments of mitochondrial mass in the DM mice. Thus, a compensatory downregulation of mitochondrial biogenesis would appear to be a logical explanation. Furthermore, the altered methylation of the oxygen sensor EGNL3 may also represent an adaptive cellular response to the increasing mitochondrial dysfunction and oxidative stress in the brain of aged DM mice.

Of course it is interesting now to speculate how the mitochondrial dysfunction is perceived and how it elicits the compensatory efforts and downstream pathology that we have documented. It has been shown in midbrain dopaminergic neurons that neuronal activity-dependent calcium entry through L-type calcium channels triggers oxidative stress and promotes alpha-Synuclein aggregation, while the effect of calcium on oxidative stress is potentiated by the formation of alpha-Synuclein Lewy-body-like aggregates [41, 55].

Mitochondrial dysfunction induces PINK1 expression in a calcium-dependent manner [56], while PINK1 depletion compromises calcium homeostasis [3]. Both alpha-Synuclein and the PINK1 downstream effector PARKIN were shown to act at contact zones between mitochondrial membranes and endoplasmic reticulum, where calcium homeostasis and mitochondrial dynamics are controlled [1, 16, 17]. CRTC1/TORC1 depends on neuronal activity-dependent calcium in its translocation to the nucleus, where it acts to modulate mitochondrial homeostasis [18, 40, 53]. Thus, alpha-Synuclein triggered toxicity and PINK1 deficiency have convergent effects on calcium homeostasis, which may be sensed by CRTC1 and elicit compensatory efforts of mitochondrial biogenesis.

5. Conclusion

This pioneer study of the global mono-methyl-arginine profile of brain in a neurodegeneration mouse model is reporting a small number of novel posttranslational modifications with substantial fold changes. These alterations occur mostly in nuclear factors previously implicated in other neurodegenerative diseases and are clustering in the pathways of dopaminergic neuron differentiation and of mitochondrial biogenesis and antioxidant protection. Although an independent validation with other techniques is not possible and our study thus is severely limited, the data fit well with previous transcriptome findings and with functional changes of long-term-depression previously documented to be triggered by the alpha-Synuclein mutation in these mice. Particularly interesting is the increased methylation of the synaptic plasticity modulator CRTC1. We speculate (1) that the CRTC1 changes are responding to altered calcium homeostasis and represent a compensatory effort to modulate mitochondrial biogenesis and (2) that they are due to the impaired mitochondrial autophagy in these mice. Thus, this methyl-arginine profiling effort of digenic PD mouse models identifies dysregulations of CRTC1 as a potential key factor, where the effects of alpha-Synuclein on synaptic plasticity converge with the effects of PINK1 on mitochondrial quality control.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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