MOLECULAR LINKS BETWEEN MITOCHONDRIAL DAMAGE AND PARKINSON'S DISEASE AND RELATED DISORDERS

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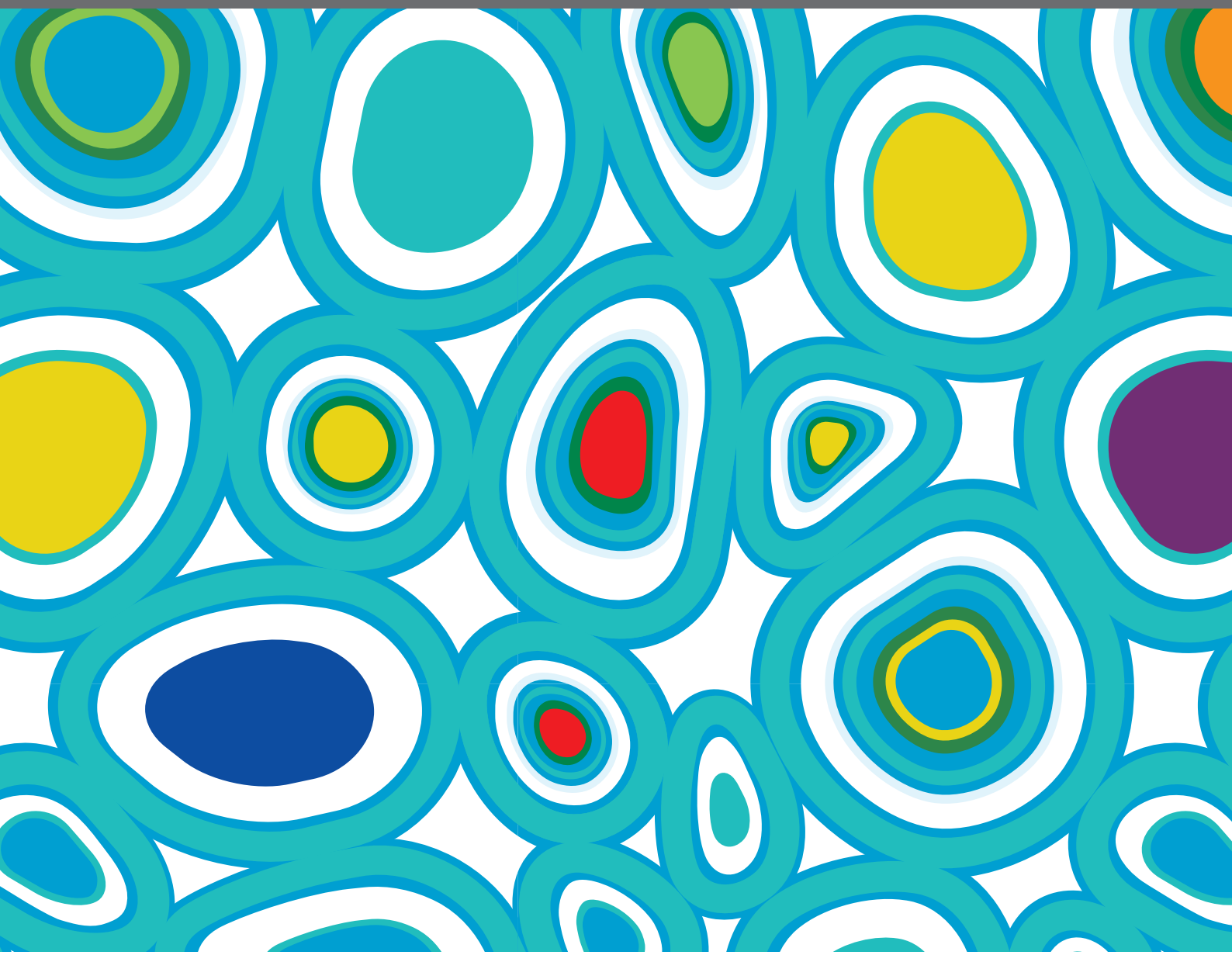

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MOLECULAR LINKS BETWEEN MITOCHONDRIAL DAMAGE AND PARKINSON'S DISEASE AND RELATED DISORDERS

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EDITORIAL
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Editorial: Molecular Links Between Mitochondrial Damage and Parkinson's Disease and Related Disorders

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Editorial on the Research Topic

Molecular Links Between Mitochondrial Damage and Parkinson's Disease and Related Disorders

Mitochondria are organelles that play a variety of roles, including energy production, regulation of intracellular Ca^{2+}, lipid and iron metabolism, redox regulation, inflammation, and cell death. The involvement of mitochondria in the etiology of Parkinson's disease has been long suspected; however, the reason for the selective degeneration of dopaminergic neurons remains a mystery. This Research Topic was envisioned to provide insights that could aid in uncovering this mystery and consists of seven excellent reviews, two unique perspectives, and two original papers that pioneer new fields of study. These studies demonstrate that mitochondrial dysfunction is more complex than previously thought and its association with other organelles as well as the association between neurons and glia must be elucidated.

Jung et al. provide a concise overview of recent studies on mitochondrial Ca^{2+} regulation during neural activity and mechanisms of brain aging and neurodegeneration caused by functional abnormalities in molecules involved in mitochondrial Ca^{2+} regulation. In particular, Table 1 provides an excellent summary of studies that involve genetically encoded calcium indicators and will be of great help to researchers who will be conducting studies in this field.

A review by Lin et al. indicates the importance of communication between mitochondria and other organelles, such as the endoplasmic reticulum, the Golgi apparatus, peroxisomes, and lysosomes. At organelle contacts, Ca^{2+}, lipids, proteins, and other substances are exchanged. This area deserves further attention as VPS13, the protein family responsible for neurodegenerative diseases, including Parkinson's disease, functions at the level of these organelles (Ugur et al., 2020). This review describes findings and genetic evidence that supports the theory of organelle communication disorders leading to neurodegeneration.

Nicoletti et al. present an extensive list of studies reporting the effects of Parkinson's disease-related gene mutations on the mitochondria. In addition, they mention the pathogenic roles of mitochondria in atypical parkinsonism, progressive supranuclear palsy (in which tau aggregates accumulate in both neurons and astrocytes), and Huntington's disease (in which neurotoxic protein aggregation is caused by N-terminal polyglutamine elongation of Huntingtin protein). Moreover, Li et al. briefly summarize the effects of 11 monogenic Parkinson's disease genes (*SNCA*,

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PRKN, PINK1, DJ-1, LRRK2, ATP13A2, PLA2G6, FBXO6, VPS35, CHCHD2, and VPS13C) on mitochondrial function and α-synuclein aggregation. However, further discussion is needed to decipher whether mitochondrial abnormalities are the cause or the consequence of these diseases.

The involvement of astrocytes in the pathogenesis of Parkinson's disease is of recent interest. Bantle et al. summarize Research Topics related to astrocyte mitochondria, such as metabolism of glutamate, Ca^{2+}, and lipids as well as inflammation. Mitochondrial transfer between astrocytes and neurons is a newly discovered phenomenon and a topic of peculiar interest, including its therapeutic potential (Hayakawa et al., 2016). Similarly, Jeon et al. discuss a different perspective on glial mitochondrial function and α-synuclein toxicity. Their review discusses the potential modulation of α-synuclein propagation to neurons and oligodendrocytes by glial mitochondrial dysfunction and neuroinflammation.

Mitochondrial dysfunction is hallmark of cancer, leading to an increase in glycolytic metabolism, which is known as the Warburg effect. Lu and Ho have proposed a similar scenario that occurs in brain tumors where tumor-associated macrophages and microglia play an active role in cancer immunity. Mitochondrial dysfunction induces mtDNA release, which in turn activates glia-associated macrophages and microglia to elicit anti-tumor response through the STING signaling (Mathur et al., 2017). They indicate that Parkinson's disease-associated PINK1-Parkin signaling is a potential therapeutic target as it negatively regulates the STING pathway through the removal of damaged mitochondria (Sliter et al., 2018). Furthermore, PINK1-Parkin signaling is also believed to be involved in mitochondrial quality control *via* mitochondrial motility arrest and mitophagy (Imai, 2020). In contrast, ubiquitin-specific proteases, which cleave polyubiquitin chains, possess the potential to suppress the progression of mitophagy (Bingol et al., 2014; Niu et al., 2020). Among them, USP14 is involved in both the ubiquitin-proteasome system (Lee et al., 2010; Kim and Goldberg, 2017) and mitophagy (Chakraborty et al., 2018). The study by Banerjee et al. on the expression of USP14 and Prohibitin 2, an inner mitochondrial membrane LC3 receptor (Wei et al., 2017), in a variety of brain regions of both young and adult rats discusses the potential of USP14 as a therapeutic target.

Odnokoz et al. report that the inhibition of mitochondrial thiol-dependent peroxidases, i.e., peroxiredoxins (Prxs), in a *Drosophila* model leads to a significant reduction in *Drosophila* lifespan. Although the physiological functions of Prxs remain to be elucidated, it has been suggested that Prxs may play a role in suppressing premature aging. The elucidation of its effects on neurodegenerative models is warranted in the future.

Liu et al. reveal that the combination of NADPH, which is required for the production of reduced glutathione, and NADPH oxidase (NOX) inhibitors effectively suppresses kainic acid-induced neurotoxicity. As NOX produces reactive oxygen species with NADPH (Bedard and Krause, 2007), inhibition of NOX in turn inhibits the production of reactive oxygen species. Although there are still several issues to be addressed before NADPH can be used clinically, this study provides a possible therapeutic approach.

Prasuhn et al. discuss, in detail, mitochondria as a prospective therapeutic target. In particular, brain imaging analysis at the prodromal stage and inexpensive exercise therapy seem to be realistic methods for early detection and control of mitochondrial diseases, respectively. Moreover, they point out that clinical phenotypes of hereditary mitochondrial diseases do not usually have attributes similar to those of Parkinson's disease or related disorders. Therefore, focusing solely on the well-known functions of mitochondria may hinder in the elucidation of the real causes of neurodegeneration.

The wide variety of reviews and original publications listed here, which address the multifaceted roles of mitochondria and their communication with other organelles within and beyond the cell, confirm that mitochondrial studies remain a research focus in the field of neurodegenerative diseases. Furthermore, the studies listed here will serve as a valuable introduction to this field.

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YI wrote the manuscript. YI, KK, ZW, and SS corrected the manuscript. All authors contributed to the article and approved the submitted version.

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USP14 as a Therapeutic Target Against Neurodegeneration: A Rat Brain Perspective

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In the recent past, many of the deubiquitinases (DUB) were found to modulate mitochondrial clearance or mitophagy and thus they are currently projected as therapeutic targets against neurodegeneration. Among these DUBs, USP14 stands at a distinctive juncture, since it can influence both proteasome complex activity and autophagy process. USP14 interference can enhance mitochondrial clearance and thus can protect Parkinsonian phenotypes in *Drosophila* model. However, in higher animal models of neurodegenerative disorders, evaluation of the protective role of USP14 is yet to be done. In this perspective, we pointed out a few of the major considerations that should be classified before designing experiments to evaluate the therapeutic potential of this DUB in rodent models of neurodegeneration. These are mainly: level of USP14 in the concerned brain region and how the level alters in the model system. Because USP14 mediated mitophagy is Prohibitin2 dependent, the anticipated impact of this protein in this aspect is also discussed. To illustrate our view, we show that USP14 levels increases in adult rat brain substantia nigra (SN) and cerebellum compared to the young ones. We also depict that rotenone treatment can immediately lead to increased SN specific USP14 levels. Our perception thus portrays USP14 as a therapeutic target, especially for addressing SN specific neurodegeneration in adult rat brain, but may vary with the disease model.

Keywords: USP14, Prohibitin2, mitophagy, neurodegeneration, Parkinson's disease, substantia nigra, rotenone, 3-nitropropionic acid

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INTRODUCTION

Dysfunctional mitochondria can lead to Cytochrome c release in cytosol and thus multiple levels of adjustments are required to maintain a healthy mitochondrial population in a normal cell. While doing so, the damaged ones are degraded by a process called mitochondrial autophagy or mitophagy. Other than autophagic machineries, the process also depends on ubiquitin proteasome system (UPS) to some extent (Tanaka et al., 2010; Chan et al., 2011; Yoshii et al., 2011). The current understanding of this dependency suggests that UPS degrades outer mitochondrial membrane (OMM) proteins and thus exposes inner mitochondrial membrane (IMM) LC3 receptor – Prohibitin2 (PHB2) (Wei et al., 2017). This facilitates the process of engulfment by autophagic isolation membranes. During mitophagy, UPS degrades the OMM proteins which are targeted by

a few E3 ubiquitin ligases; among them, Parkin is the most studied one. Activation of Parkin and recruitment onto depolarised mitochondria is facilitated by PINK1 (a kinase) and this pathway has been studied with great details (Narendra et al., 2008, 2010; Ziviani et al., 2010; Chakraborty et al., 2017). Mutations in PINK1 and Parkin have been directly linked to familial form of Parkinson's disease (PD) (Kitada et al., 1998; Silvestri et al., 2005). Though most of the cases are sporadic, anomaly in the maintenance of a healthy mitochondrial population is equivocally accepted in both genetic and sporadic forms of the disorder (Parker et al., 1989; Mizuno et al., 1998). In this disorder, dopaminergic neurons at substantia nigra (SN) region of the brain progressively degenerate. The manifestation at the periphery includes tremor, akinesia and rigidity. Cure of the ailment involves dopamine supplementation but comes with severe side effects and the current therapies do not halt or slow down neurodegeneration. However, huge efforts are made to delineate the cause for the region specificity in PD and strategies are designed to make the hit points "druggable." Among these, recent discoveries indicate that deubiquitinase enzymes (DUBs) might have a higher impact on the development and progression of neurodegeneration. Here, we discuss how DUBs are important for PD and a few key aspects that should be considered before evaluating USP14 as a mediator of mitophagy in PD animal models.

DEUBIQUITINASES IN PARKINSON'S DISEASE: A BRIEF DESCRIPTION

As the name suggests, these enzymes cleave ubiquitin chains from the substrate. There are more than one hundred DUBs, involved in numerous pathways and they have some sort of substrate specificity, which makes them targets for drug development (Komander et al., 2009). As far as PD is concerned, a few DUBs have been implicated in the progression of the disease, both in cellular and animal models. The idea in principle suggests that where efficient Parkin mediated ubiquitination is compromised, inhibiting DUBs might linger the remaining signal which originates from the other routes of mitophagy (SIAH, Mul1, Gp78, etc.). In general, these DUBs influence the disease scenario by antagonizing Parkin activity or by modulating UPS and autophagy. In this regard, USP15 and USP30 were found to antagonize Parkin activity by competing for the common substrates on OMM (Bingol et al., 2014; Cornelissen et al., 2014). Downregulation of both of these DUBs delivered protective effects against PD progression in *Drosophila* model. USP8 on the other hand regulates Parkin activation and downregulation of which is also known to be protective in *Drosophila* model of PD (Durcan et al., 2014; Von Stockum et al., 2019). Another DUB- Ataxin 3 has huge potential to be a drug target as it can directly interact with Parkin, but the impact on PD is yet to be documented (Durcan et al., 2012). Mutation of UCH L-1 in this respect is the only DUB directly linked with familial PD (Kabuta et al., 2008). It can inhibit autophagy by interacting with LAMP-2A. USP24 is another modulator of autophagy which may also influence PD progression as it can regulate dopaminergic neurite outgrowth, but the potential as a disease modulator is not evaluated yet (Li et al., 2006; Haugarvoll et al., 2009).

Among the DUBs, USP14 is unique as it can influence autophagy (Xu et al., 2016) and UPS activity (Lee et al., 2010; Kim and Goldberg, 2017) independently, both are the prerequisites for mitophagy. Recently, it was demonstrated that USP14 inhibition enhances mitophagy and protects against the disease progression in *Drosophila* model (Chakraborty et al., 2018). So the current understanding depicts that higher levels of USP14 may influence UPS activity, autophagy, and in turn mitophagy levels. All of these processes are affected during many of the age-related neurodegenerative disorders, including PD. Verification of the protective ability of USP14 inhibition in higher animal models of neurodegeneration is yet to be done. The lone report which supports this potential of USP14 inhibition is done in cerebral ischemia/reperfusion-induced neuronal damage model (Min et al., 2017). None the less, this study indicates that the inhibitor of USP14 – IU1 might be blood brain barrier permeable. In this perspective, we highlighted a few of the aspects that should be considered for better interpretation of data, as far as USP14 vs. neurodegeneration is concerned.

USP14 VS. NEURODEGENERATION: CONSIDERATIONS FOR EXTRAPOLATION

Inhibitors of USP14 are available. A popularly used inhibitor – IU1 increases UPS activity, enhances Tau degradation in primary cultured neurons and increases mitochondrial elimination in neuronal cell lines (Lee et al., 2010; Boselli et al., 2017; Chakraborty et al., 2018). For evaluating the potential of USP14 inhibitor in rodent models of PD, here we discuss some of the key factors that might influence the outcome of the experiments.

First of all, level of USP14 in a brain region or subset of neurons may vary during aging. If this hypothesis is true, the amount of USP14 inhibition required for neuroprotection might change with the age of the model. In other words, if a neurodegenerative disease model can be generated using animals from different age groups, level of USP14 should be monitored in that particular age group to adjust the dose of the inhibitor. To elaborate our opinions, first we selected rats from two age groups (Sprague-Dawley, young −1 month and adult- 6 to7 months old) and compared USP14 levels at different brain regions. We found that USP14 is increased in adult rat brain SN and cerebellum (**Figures 1E,G**), while there were no significant alterations in cortex, striatum, VTA and hippocampus (**Figures 1A–D,F**). Accessibility and distribution of USP14 inhibitors among these brain regions are yet to be characterized. Obviously the level of the protein and the accessibility of the inhibitor in that region should equally complement each other. Most commonly, while developing PD models with rotenone, adult rat is preferred as the young ones often show mild behavioral or neuroanatomical changes in response to acute neurotoxic insults (Cannon et al., 2009). Our results are indicative that changes in USP14 levels might make a brain region more vulnerable than the others (**Figure 1E**).

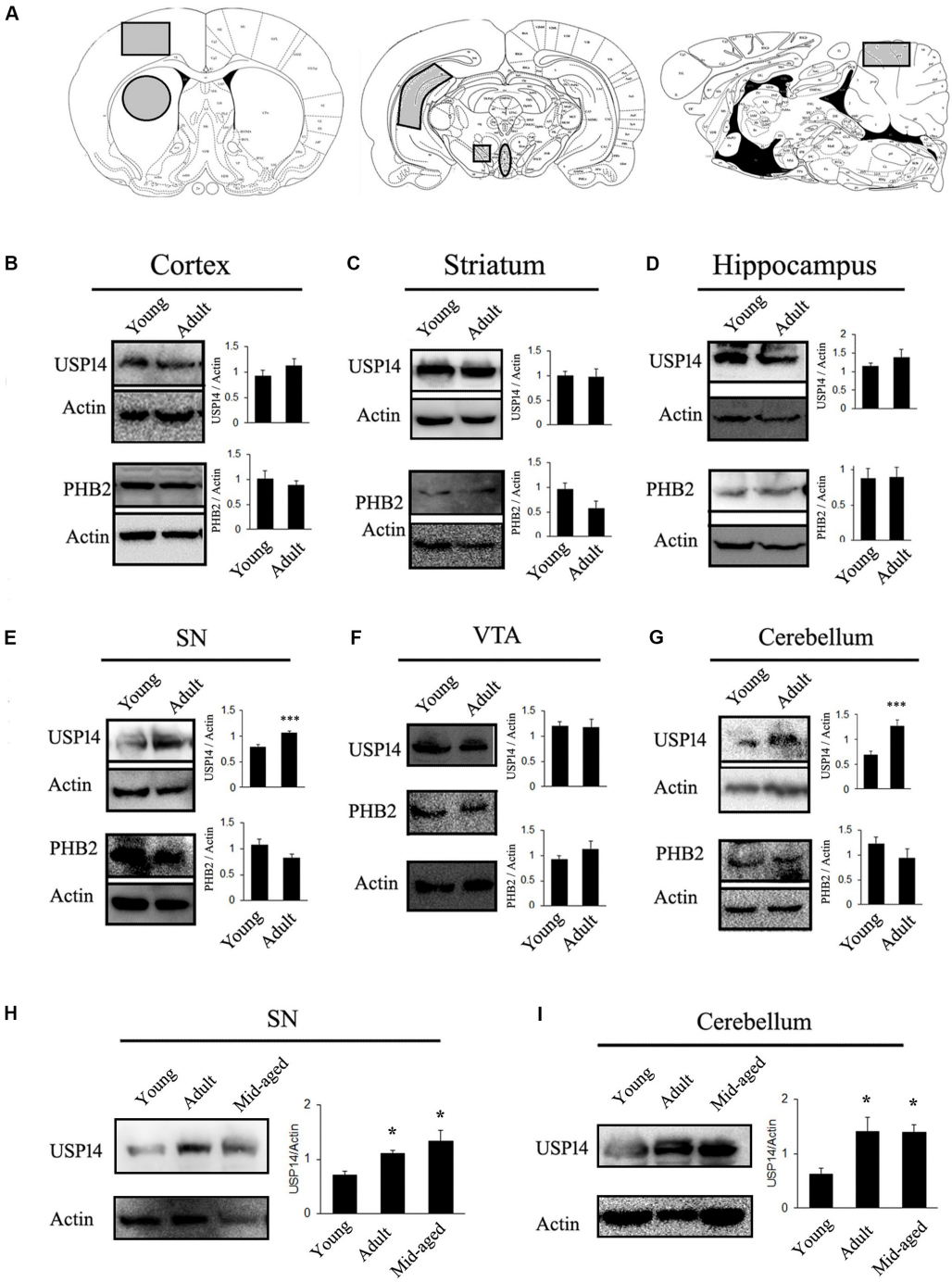

FIGURE 1 | USP14 increases with age in substantia nigra and cerebellum. **(A)** Different brain regions showing the area that were taken for the study (marked as gray). **(B–G)** Immunoblot analysis of USP14, PHB2 and Actin from the mentioned area of brain from young (1 month) and adult (6–7 months) animals. **(H,I)** Immunoblot analysis of USP14 from the mentioned areas of young (1 month), adult (6–7 months) and mid-aged (10–12 months) rats. Bar graphs represent mean ± SEM. $N = 8$ for **(B–G)** and 3 for **(H,I)**. We employed student's t test for **(B,G)** and one way ANOVA followed by Newman Keul's multiple comparison test for statistical significance for **(H,I)**. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

However, further independent investigations are required to press upon this point and decipher whether or not other UPS related DUBs compensate this effect. We further wanted to quantify if this increase in USP14 is maintained in older rats or it reverses back. So we included another age group (10–12 months) to further investigate. We found that the increase in USP14 was maintained in mid-aged rat brain SN and cerebellum (**Figures 1H,I**).

Secondly, if evaluation of USP14 as a therapeutic agent is connected with mitophagy, it should be kept in mind that mitophagy, in general, may depend to a certain extent on PHB2 (Wei et al., 2017) and USP14 mediated mitophagy is PHB2 dependent (Chakraborty et al., 2018). However, PHB2 levels should not be considered directly as an indicator of neuron's ability to thrust for mitophagy. PHB2 mediated mitophagy is mostly UPS dependent and there are different pathways that can still drive mitophagy in response to diverse cellular stimuli. Though many of the functions of PHB1 and 2 are well documented (Merkwirth et al., 2008), their brain region-specific distribution is not fully classified yet. Also, the correlation between USP14 and PHB2 level is yet to be deciphered. However, it can be envisioned that if a group of neurons express a very low amount of PHB2, USP14 inhibition might not be able to enhance mitophagy significantly and may lead to unpredictable changes at the cellular level. We found that PHB2 levels do not alter due to age in the mentioned areas of rat brain (**Figure 1**).

The third major point that should be considered is the process of the model generation. Some models of neurodegenerative disorders show protein aggregate formation at some point in time and those aggregates may block UPS functionality (Bence et al., 2001). Unless there is formation of unoccupied proteasome complex, USP14 blockade might not be able to execute the desired effect. So, the time point from when the inhibitor is administered is something vital to consider, preferably before the formation of the protein aggregates. Many of the animal models of neurodegeneration are generated by the use of mitochondrial electron transport chain (ETC) complex inhibitors, like rotenone (Sherer et al., 2003; Cannon et al., 2009), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Burns et al., 1984; Heikkila et al., 1984) and 3-nitropropionic acid (3-NP) (Beal et al., 1993; Chakraborty et al., 2014a). How USP14 is modulated by these toxins should also be taken under consideration. To elaborate the hypothesis that different mitochondrial toxins may alter USP14 differently, even before the initiation of neurodegeneration, we selected two of the commonly used mitochondrial toxins, namely rotenone (Sherer et al., 2003; Cannon et al., 2009) and 3-NP (Beal et al., 1993; Pandey et al., 2008; Chakraborty et al., 2014a). One of the reasons to choose these two toxins was their ability to block mitochondrial ETC complex I (by rotenone) and II (by 3NP) ubiquitously. Rotenone is widely used to generate neurodegeneration in dopaminergic cells of SN (Sherer et al., 2003; Cannon et al., 2009) and 3-NP is used mostly for striatal lesions (Beal et al., 1993; Benchoua et al., 2008; Chakraborty et al., 2014a). Though 3-NP is also known to induce mild neuronal loss at SN (Fernagut et al., 2002), these toxins have different mechanisms to generate area-specific neurodegeneration. Along with SN, two major brain regions were selected: striatum and cerebellum. We also selected ventral tegmental area (VTA) because of its proximity and similarity with SN in terms of dopaminergic neuronal population.

Previously it was found that 20 mg/kg dose of 3-NP starts showing neuronal lesions from 4th day of treatment, however, the initial behavioral symptom starts appearing from 3rd day onwards (Pandey et al., 2008; Chakraborty et al., 2014a,b). To monitor the effect of complex II inhibition on USP14 at the very early stage, before majority of the neurons are lost, we treated 3-NP for 2 days and the animals were sacrificed on the 3rd day. We did not find any change in USP14 levels after 3-NP administration in any of the mentioned brain regions (**Figures 2A–D**).

We wanted to monitor the immediate effect of rotenone treatment (1 mg/kg) on USP14 levels, as well. We found that rotenone treatment (1 mg/kg) did not show reduced dopamine levels upto 6th day of treatment, but starts showing weight loss, increased catalepsy and reduced rearing from 4th day (data not shown). So, we sacrificed the animals on 3rd day after two doses of rotenone. We found a significant increase in USP14 in SN after rotenone treatment, while no alteration in the other regions was observed (**Figures 2A–D**). As the effect of rotenone or 3-NP treatment on PHB1-PHB2 complex is not characterized yet, we also measured PHB1 along with PHB2 in these brain regions after these toxin treatments. Only striatum showed increased levels of PHB2, along with PHB1 after rotenone administration (**Figure 2B**).

From these two examples of mitochondrial ETC complex inhibitors, it is clear that different neurotoxins might offer differential alterations of USP14 (and PHB2) in different brain regions. It is always advisable to determine the level of USP14 in the respective brain regions of the animal model, before evaluating the prospects of USP14 inhibition. From these experiments it is not clear though whether the increase in USP14 is neuron-specific or not. Semi-quantitative immunofluorescence based co-localisation study is advised to delineate this point a step further. From these experiments, it is also not clear whether or not the increase in USP14 is accompanied by increased proteasome complex, along with other associated DUBs (USP14, UCH37, and RPN11) which are important for maintaining free ubiquitin pool. Proteasome mediated degradation of protein depends on the functioning of these three DUBs. While USP14 and UCH37 antagonize degradation, RPN11 seems to promote substrate degradation (Lee et al., 2010; De Poot et al., 2017). If a substrate related to mitophagy is shared between these DUBs, how they interplay and decide the fate of the protein requires further independent study. However, if USP14 mediated enhanced proteasome activity and subsequently mitophagy is to be investigated, a simpler way can be followed. The effect of the inhibitor on proteasome activity in specific areas can be monitored by using synthetic flourogenic substrates. In this regard, it should also be kept in mind that many of these substrates enter the core of proteasome complex quite freely and thus may minimize the quantitative differences between the groups.

To simplify our point of view, we refer to **Figure 2E**. Here, we presented a few of the considerations that should be determined beforehand. We also represented a few anticipated outcomes concerning mitophagy after USP14 inhibition, based on two situations: (+) means high and (−) mean low, prior to inhibitor administration. The current understanding suggests that if age and area associated USP14 levels is already at a low level, USP14 inhibition might not be able to enhance mitophagy further. The same can be expected if PHB2 level is low. So an unaltered or higher level of USP14 and PHB2 is expected for inhibitor mediated enhanced mitophagy. If a particular model of neurodegenerative disorder downregulates USP14 or negatively

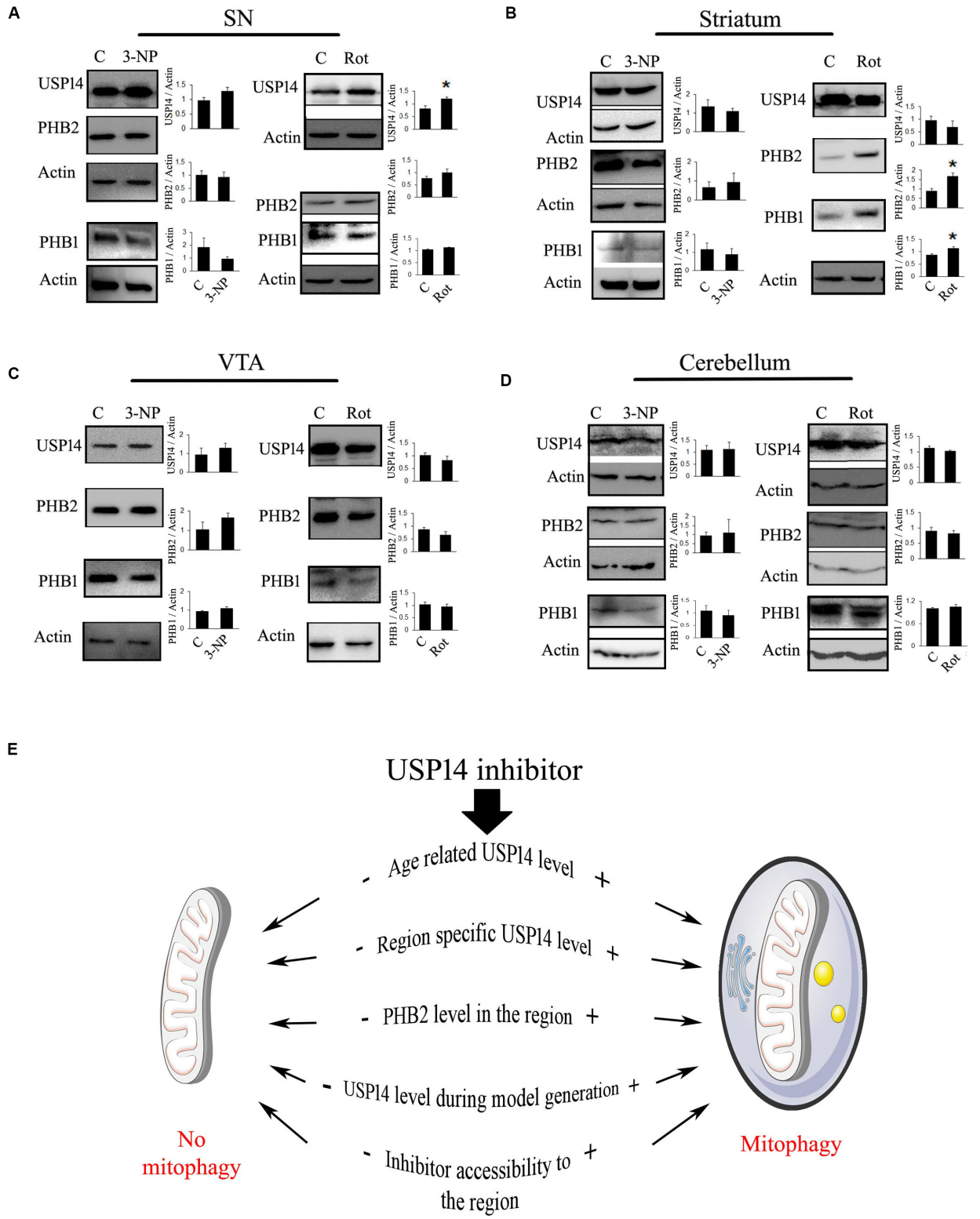

FIGURE 2 | Blockade of mitochondrial electron transport chain complex I increase USP14 levels in substantia nigra. **(A–D)** 6–7 month old animals were treated with rotenone (1 mg / Kg, 2 days) or 3-NP (20 mg /Kg, 2 days) were sacrificed and brain regions were isolated. Immunoblot analysis was done for the mentioned areas and proteins. Bar graphs represent mean ± SEM from 3 to 4 animal brain. Student's *t* test, $^{*}p \leq 0.05$. **(E)** Different possibilities to consider for USP14 inhibition mediated mitophagy. (+) and (–) depict increased or decreased levels of the event, respectively, before USP14 inhibitor administration and that might decide the outcome after. The list includes some of the possibilities and might not be exhaustive.

impacts the accessibility of the inhibitor to the area, that should also negatively impact the outcome after the administration of the inhibitor.

DISCUSSION

Aging and autophagy are quite inversely related. This phenomenon contributes to many of the age-associated neuronal complicacies (Simonsen et al., 2008; Pyo et al., 2013; Carnio et al., 2014; Hansen et al., 2018). However, goal of the current perspective is not to highlight age-related autophagy or proteasome functioning. Instead, we wanted to point out a few of the important aspects for evaluating USP14 as a therapeutic target, which might act as an upstream effector of mitophagy in rat brain. This perspective highlights mostly one aspect: there could be age and disorder specific alterations in USP14 protein levels which might impact the outcome. As in this case, an increase in USP14 is found mostly in SN, it can be advocated that it might be a key target for therapy development, as far as PD related anomaly in mitophagy is concerned.

Though USP14 is more pronounced for its therapeutic potential in cancer (Shinji et al., 2006; Liao et al., 2018; Han et al., 2019), its role in neurodegenerative disorders is also known (Boselli et al., 2017; Min et al., 2017; Chakraborty et al., 2018). It is well accepted that the influence of USP14 on proteasome complex activity is inversely correlated (Lee et al., 2010; Kim and Goldberg, 2017). Because in many of the neurodegenerative disorders proteasome complex is known to be inhibited and one of the causative factors for protein aggregate formation (Keller et al., 2000; Mcnaught et al., 2001, 2002; Diaz-Hernandez et al., 2006; Thibaudeau et al., 2018) inhibitors of USP14 has been proposed as curative agents (Lee et al., 2010; Boselli et al., 2017). Whether or not the available inhibitor – IU1 or its derivative is toxic for neuronal cells, is a matter of debate as reports indicate both the ways for cultured neurons (Boselli et al., 2017; Kiprowska et al., 2017). Boselli et al. (2017) did not find any toxicity with IU1-47 (a more potent inhibitor of USP14 than IU1) and demonstrated that it can enhance Tau degradation. Kiprowska et al. (2017), however, showed that IU1 decreases neuronal survivability and inhibits ETC complex I in isolated mitochondrial fraction. It should be noted that culture conditions for cortical neurons are important in this regard as it can influence the neuronal toxicity of IU1 or its derivatives.

How USP14 can influence autophagy is yet to be fully characterized. Though the study by Xu et al. (2016) suggests that USP14 inhibition leads to stabilization of K63 ubiquitination of Beclin1 and thus enhances autophagy (Xu et al., 2016) whether long term inhibition will also lead to the same or not, might be a matter of discussion. Long term enhancement of proteasome functioning might induce degradation of some of the protein members of autophagy machinery and thus might have a negative feedback loop. This feedback loop between autophagy and UPS is unavoidable and the duration of USP14 inhibition may decide the outcome. Precautions should be taken to utilize this window period and avoid such paradoxes. In our opinion determining and utilization of the therapeutic window period is vital for exploring the protective effects of USP14 inhibition. Here, we would like to highlight that USP14 is required for maintaining the free ubiquitin pool and it is vital for normal neuronal functioning and plasticity (Anderson et al., 2005; Vaden et al., 2015b). USP14 deficient mice exhibit post-natal lethality, motor incoordination and defects in neuromuscular junction, which can be rescued by overexpression of the protein (Crimmins et al., 2006; Chen et al., 2009; Vaden et al., 2015b). USP14 inhibition in amygdala also results in impairment of long term potentiation against fear conditioning (Jarome et al., 2013). So, chronic USP14 inhibition might lead to unavoidable damage to peripheral and central nervous system in rodents. If USP14 mediated mitophagy is to be targeted in rodent models of neurodegenerative disorders, we propose intermittent USP14 inhibition for exploring the therapeutic aspects. In our view, this will enhance the chances of mitochondrial rejuvenation with minimal toxic effects on neurophysiology. The dose and duration of the inhibition have to be standardized in a disease-specific manner. It has to be mentioned here, Vaden et al. (2015a) demonstrated that acute, intermittent administration of IU1 does not affect structure and arborization of neuronal endplates as such, but reduce miniature endplate current frequency and enhance AChR-γ expression in gastrocnemius muscle. The study further demonstrated that USP14 deficiency mediated complicacies in neuromuscular junction is executed via c-Jun N- terminal kinase (JNK) signaling. JNK inhibition rescued motor dysfunction and abnormalities in synaptic structures caused by USP14 deficiency. So incorporating JNK inhibitors in the treatment paradigm might minimize the anticipated impairments caused by USP14 inhibition and extend therapeutic window period in rodent models of neurodegeneration.

How USP14 increases immediately in response to ETC complex I blockade is not clear. Dopamine itself might not be a factor for this enhancement, as VTA did not show any alterations after rotenone treatment. Whether or not this effect persists in long term treatment is a matter of further study. However, the implications of such site-specific increase could be vast. First of all, this might lead to a decrease in proteasome activity and in turn can lead to early accumulation of protein oligomers, which are known to act as the "seeds" for further protein aggregation (Gregori et al., 1995; Lindersson et al., 2004; Diaz-Hernandez et al., 2006). Secondly, this increase in USP14 might also lead to reduced levels of mitophagy. The most important point here we state is the increase in USP14 level is SN specific. Investigations are warranted to determine whether or not this is the initiation point for decreased UPS activity and reduced mitophagy in PD.

MATERIALS AND METHODS

Materials

All the chemicals are analytical grade and purchased from Sigma Aldrich Chemicals Pvt Ltd. or Sisco Research Laboratories Pvt. Ltd. (SRL, India) unless otherwise specified. Reagents were prepared and stored according to the manufacturer's guidelines.

Animal Treatment

Animal experimentations were carried out as per national guidelines on the "Care and Use of Animals in Scientific Research," formed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Environment and Forests, Govt. of India. The protocol was accepted by animal ethics committee of CSIR-Indian Institute of Chemical Biology, Kolkata, India. Sprague Dawley male rats (1 month, 6–7 and 10–12 months old) were kept in the animal house ($22 \pm 2°C$, $60 \pm 5\%$ humidity, with 12 h light-dark cycle). Food and water were provided *ad libitum*. 3-NP was made freshly before each injection in saline (0.85% NaCl) and the pH was adjusted to 7.4 by 5.0 N NaOH. Rats were treated with 20 mg/kg (i.p.) once daily, for 2 days. Rotenone was dissolved in 30:70 DMSO and mineral oil and 1 mg/kg (i.p.) dose was administered for 2 days.

Immunoblotting

In brief, isolated brain regions were homogenized in ice cold radio immunoprecipitation buffer (RIPA, 50 mM Tris HCl containing 1 mM EDTA, 150 mM NaCl, 1% Nonidet p-40, 0.25% sodium deoxycholate; pH 7.5) supplemented with protease inhibitors (Invitrogen). The lysate was kept on ice for 30 min and centrifuged at $10{,}000 \times g$ for 10 min at 4°C and the supernatant was collected. 25–40 μg protein was separated by 10% polyacrylamide SDS gels and transferred to PVDF membrane. Blocking and antibody dilution was done in 5% or 2.5% skimmed milk, respectively. The following antibodies were used: anti-Actin (1:2,000; Santa Cruz Biotechnology), anti-PHB2 (1:3,000; Sigma), anti-PHB1 (1:1000; Abcam) and Anti USP14 (1: 3000, Abcam). Appropriate HRP tagged secondary antibodies (rabbit or mouse, 1:2000) were procured from Bangalore Genei Private Limited (India).

Protein bands were detected using chemiluminescence substrate (Sigma) and images were captured in a chemidoc instrument (Biorad). Band intensities were measured by ImageJ and were normalized by respective Actin band intensity.

Statistics

We used two tailed student's *t*-test and one way ANOVA followed by Newman Keul's multiple comparison test for statistical significance. In all cases, results are provided as mean ± S.E.M., and $p \leq 0.05$ was considered as significant.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal ethics committee and experimentations were carried out in accordance with national guidelines on the "Care and Use of Animals in Scientific Research," formed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Environment and Forests, Govt. of India. The protocol was accepted by animal ethics Committee of CSIR-Indian Institute of Chemical Biology, Kolkata, India.

AUTHOR CONTRIBUTIONS

JC, CB, and MR performed the experiments with young, adult, and mid-aged rats, as well as with the rotenone treatment. RM performed the experiments with 3-NP treatment. JC designed the experiments and analyzed the data. CB, MR, and JC wrote the perspective.

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REVIEW
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The Role of Glial Mitochondria in α-Synuclein Toxicity

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The abnormal accumulation of alpha-synuclein (α-syn) aggregates in neurons and glial cells is widely known to be associated with many neurodegenerative diseases, including Parkinson's disease (PD), Dementia with Lewy bodies (DLB), and Multiple system atrophy (MSA). Mitochondrial dysfunction in neurons and glia is known as a key feature of α-syn toxicity. Studies aimed at understanding α-syn-induced toxicity and its role in neurodegenerative diseases have primarily focused on neurons. However, a growing body of evidence demonstrates that glial cells such as microglia and astrocytes have been implicated in the initial pathogenesis and the progression of α-Synucleinopathy. Glial cells are important for supporting neuronal survival, synaptic functions, and local immunity. Furthermore, recent studies highlight the role of mitochondrial metabolism in the normal function of glial cells. In this work, we review the complex relationship between glial mitochondria and α-syn-mediated neurodegeneration, which may provide novel insights into the roles of glial cells in α-syn-associated neurodegenerative diseases.

Keywords: alpha-synuclein, mitochondrial dysfuncion, neurodegenerative diseases, non-cell autonomous, glia, neuron

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INTRODUCTION

Alpha synuclein (α-syn), first identified in 1988 by Maroteaux et al. (1988), is a small protein that consists of 140 amino acids and encodes the human *SNCA* gene. α-syn is expressed in the central nervous system (CNS), and is specifically localized in synapses and nuclei (Jakes et al., 1994). The function of α-syn is not clearly defined, but several studies have shown that α-syn regulates synaptic plasticity and vesicle trafficking (Kahle et al., 2000; Fortin et al., 2004; Lee et al., 2008) and interacts with synaptic vesicles to physiologically regulate vesicle recycling (Maroteaux et al., 1988; Huang et al., 2019). Originally, α-syn was thought to be a natively unfolded monomeric protein, but recent studies have indicated that α-syn forms an α-helix-rich tetramer (Bartels et al., 2011; Wang et al., 2011, 2014; Dettmer et al., 2013, 2015; Gurry et al., 2013; Burre et al., 2014). The biological function of α-syn is exerted through the N-terminal, non-amyloid-beta component (NAC) and C-terminal domains. The N-terminus, which contains the KTKEGV motif, maintains tetramerization of α-syn, and mutations in this motif can induce neurotoxicity (Dettmer et al., 2015). NAC, first identified in Alzheimer's disease patients, is a highly hydrophobic domain and forms a β-sheet structure for α-syn aggregation (Ueda et al., 1993). The C-terminus of α-syn is a proline-rich region. α-syn can interact with other proteins through this domain (Kim et al., 2002). Unfolded or misfolded α-syn forms fibrillar aggregates and aggregated α-syn generates insoluble inclusions in the affected neurons and glial cells of α-Synucleinopathy brains.

Accumulated α-syn is regarded as a key feature of α-Synucleinopathy. In many neurodegenerative diseases, such as Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA), show an α-Synucleinopathy phenotype. Intercellular delivery of α-syn in α-Synucleinopathy occurs through direct penetration, endocytosis, nanotube tunneling-mediated pore formation, and diffusion (Ubhi et al., 2011; Konno et al., 2012; Tosatto et al., 2012; Dieriks et al., 2017; Eguchi et al., 2017). Cell-to-cell delivery of α-syn contributes to neurodegeneration (Desplats et al., 2009; Bruck et al., 2016). Many studies have provided evidence that α-syn transmission occurs in a prion-like manner (Bernis et al., 2015; Steiner et al., 2018). However, this finding remains controversial because of the incomplete understanding of the factors that control the spread of pathogenic proteins and how they work.

Aggregated α-syn leads to many pathological features, such as mitochondrial dysfunction (Liu et al., 2009; Cali et al., 2012; Vicario et al., 2018), dysregulation of calcium homeostasis (Cali et al., 2012; Melachroinou et al., 2013), neuroinflammation, endoplasmic reticulum (ER) stress, protein quality control impairment (Melo et al., 2018; Rocha et al., 2018), Golgi fragmentation (Gosavi et al., 2002), and lysosomal dysfunction (Meredith et al., 2002). In MSA, unlike PD and DLB, aggregated α-syn mainly appears in oligodendrocyte, also called glial cytoplasmic inclusions (GCIs; Ahmed et al., 2013; Bruck et al., 2016; Mori et al., 2020). Gila cells are non-neuronal cells in brain and play a critical role in maintaining neuronal system. The majority of brain cells are glial cells, and they modulate neurogenesis and synaptogenesis (Argente-Arizon et al., 2017). Moreover, glial cells affect brain-blood barrier (BBB) development and function through interactions with neurons and endothelial cells to defend the brain from pathogens (Banerjee and Bhat, 2007; Broux et al., 2015). Glia cells are composed of astrocytes, microglia, and oligodendrocytes in the CNS (Bruck et al., 2016). A major function of astrocytes and microglia involve the immune response. Under pathological conditions, microglia and astrocytes are activated. Activated microglia and astrocytes can release pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6; Liu et al., 2011; Tjalkens et al., 2017). These cytokines lead to the production of reactive oxidative stress (ROS) and dysfunction of the BBB. Finally these factors induce neuronal cell death (Yang et al., 2007; Pan et al., 2011) and cause neurodegenerative disease. In general, protein aggregation in affected neurons is also detected in glial cells (Li and Haney, 2020). In particular, α-syn inclusions in glial cells causes a reduction in trophic support, which in turn leads to neuronal loss (Bruck et al., 2016).

Protein aggregation generally occurs in neurodegenerative diseases. In glial cell, aggregated proteins have also been observed (Li and Haney, 2020). Currently, many studies emphasize the role of mitochondria in glia (Yang et al., 2017; McAvoy and Kawamata, 2019; Yan et al., 2020). The mitochondria of glial cells regulate calcium homeostasis, ATP production and the inflammatory response (Yang et al., 2017). Mitochondrial impairment in glia can affect neuronal survival, so glial mitochondrial dysfunction has recently emerged as a major etiology of neurodegeneration (Yang et al., 2017; McAvoy and Kawamata, 2019; Yan et al., 2020).

In this review, we provide new insights into the roles of glial cells in α-syn-related neurodegenerative diseases and examine the importance of glial mitochondria in the disease progression of α-Synucleinopathy.

NEURODEGENERATIVE DISORDERS ASSOCIATED WITH α-SYNUCLEINOPATHIES

PD

Parkinson's disease is the second most common neurodegenerative disease and is defined as a α-Synucleinopathy. The main pathological features of PD are the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc; Damier et al., 1999; Kalia and Lang, 2015) and protein aggregates (called Lewy bodies and Lewy neurites) consisting mainly of α-syn proteins present in neurons (Spillantini et al., 1997; Gomez-Benito et al., 2020). Moreover, PD has clinical features such as cognitive impairment and motor dysfunction including stiffness, postural instability, and akinesia. PD is mostly known as a sporadic disease, but several gene mutations, such as α-syn, LRRK2, PINK1, DJ-1, and parkin, cause disease (Davie, 2008). Moreover, mutations in α-syn (duplications, triplications, or point mutations) facilitate aggregate formation in neurons and affect the exacerbation and progression of PD. Many pathological features of PD, such as mitochondrial dysfunction (Park et al., 2018; Wang X.L. et al., 2020), neuroinflammation (Wang et al., 2015; Pajares et al., 2020), ER stress (Colla, 2019), and impaired protein quality control (Cook and Petrucelli, 2009; Sala et al., 2016), can lead to neuronal toxicity. α-Synucleinopathy features the accumulation of aggregated α-syn in neuronal and glial cells (McCann et al., 2014). Previous studies showed that healthy neurons transplanted into the striatum of PD patient exhibited α-syn pathology (Kordower et al., 2008; Li et al., 2008). These results indicated that α-syn can propagate into other cells. Moreover, cell-to-cell transmission of aggregated α-syn accelerates Synucleinopathy. Mitochondrial dysfunction is a key feature in PD progression, which is caused by disrupted mitochondrial respiration, decreased mitochondrial membrane potential, and impaired mitophagy (Lin et al., 2019). Moreover, α-syn also influences mitochondrial function (Devi et al., 2008; Pozo Devoto and Falzone, 2017). α-Synucleinopathy induces mitochondrial dysfunction, especially in the context of calcium homeostasis (Vicario et al., 2018; Lin et al., 2019). Mitochondria regulate calcium levels through interactions with ER. However, abnormal α-syn accumulates in both the mitochondria and ER and stresses the ER to release too much calcium into mitochondria. Excessive calcium in mitochondria induces ROS, which can lead to cell death (Melo et al., 2018; Lin et al., 2019). In addition, α-syn is associated with another mitochondrial dynamics, such as the regulation of morphology (fission and fusion), axonal transport and mitophagy (Pozo Devoto and Falzone, 2017).

DLB (Dementia With Lewy Bodies)

Lewy body dementia (LBD) consists of DLB and Parkinson's disease dementia (PDD), and it shares pathological characteristics with PD, such as the accumulation of α-syn, leading to neuronal loss (Kim et al., 2014; Gomperts, 2016; Surendranathan et al., 2020). These patients commonly show parkinsonism motor symptoms, neuropsychiatric symptoms, cognitive defects, sleep disorders, and visual hallucinations (McKeith et al., 2017; Velayudhan et al., 2017). Moreover, depressive symptom is also common in DLB patients. Several studies indicated that depression is associated with AD and DLB which cause cognitive and memory dysfunction (Fritze et al., 2011; Rapp et al., 2011). However, the frequency and severity of depression are more severe DLB than AD (Fritze et al., 2011; Chiu et al., 2017). Although LBD and PD have some common features, such as α-syn aggregates and clinical symptoms, they are distinguished by the relative onset and prognosis. DLB is diagnosed as dementia within 1 year after parkinsonian onset. On the other hand, PDD is diagnosed with dementia 1 year after parkinsonian onset. PDD patients show severe movement dysfunctions, whereas DLB patients show severe cognitive impairments (Hansen et al., 2019). DLB is caused by the abnormal accumulation of α-syn in neurons, called Lewy bodies (LBs) and Lewy neurites (LNs; Cummings, 2004; Walker et al., 2015). Most DLB cases occur sporadically, but several papers have suggested that DLB has a genetic cause, such as APOE, SNCA, and LRRK2 (Walker et al., 2015; Orme et al., 2018). Excessive accumulation of α-syn in DLB is associated with the loss and dysfunction of dopaminergic neurons and cholinergic neurons (Tiraboschi et al., 2000; Gomperts, 2016). Cholinergic neurons participate in memory function and age-related dementia including Alzheimer's disease (Haam and Yakel, 2017). In addition to LB and LN pathology, most DLB patients also have amyloid plaque and neurofibrillary pathology in their brains (Perry et al., 1993; Bohnen and Albin, 2011). Unfortunately, the clinical diagnosis of DLB remains difficult. Because the symptoms of DLB are similar to those of other diseases, such as AD, PD, and PDD (Tousi, 2017), DLB symptoms can overlap with those of AD and PDD at the same time (McKeith et al., 2017). Therefore, DLB is usually diagnosed after death.

MSA (Multiple System Atrophy)

Multiple system atrophy is an uncommon neurodegenerative disease that is pathologically characterized by a combination of parkinsonism, cerebellar ataxia, and autonomic dysfunction (Lopez-Cuina et al., 2018; Monzio Compagnoni and Di Fonzo, 2019). MSA is divided into two subtypes, MSA with parkinsonism features (MSA-P), which exhibits bradykinesia, rigidity, postural instability, and tremor, or MSA with cerebellar features (MSA-C), which exhibits gait ataxia, ataxic dysarthria, limb ataxia, and sustained gaze-evoked nystagmus (Gilman et al., 1999). MSA is caused by the aggregation of α-syn in oligodendrocytes, which are called GCIs and are regarded as a key feature of this disease (Asi et al., 2014; Djelloul et al., 2015; Monzio Compagnoni and Di Fonzo, 2019). Moreover, one case report suggested that α-syn co-localize with phosphorylated tau in GCIs in MSA patients (Piao et al., 2001). Normally, oligodendrocytes exhibit very low-level expression of α-syn (Miller et al., 2005). The mechanisms of α-syn accumulation in oligodendrocytes are not fully understood. Recently, some researchers suggested that the interaction between neurons and oligodendrocytes affects GCIs. The overexpression of α-syn in neurons induces extracellular secretion of α-syn. Then, oligodendrocytes absorb α-syn into the cell in clathrin-dependent manners. In addition, intracellular aggregated α-syn in neurons also increases α-syn release from neurons and this process is mediated by vesicle trafficking (Kisos et al., 2012; Konno et al., 2012; Monzio Compagnoni and Di Fonzo, 2019). Originally, prion-like α-syn propagation was examined in PD (Chu and Kordower, 2015). Monomeric α-syn aggregates into β-sheet structure, which are important features of the self-propagation of fibrillar α-syn aggregates (Chu and Kordower, 2015; Woerman et al., 2018). There are several pathological features of MSA, such as inflammation (Ishizawa et al., 2004; Jellinger, 2014) and impairments in protein degradation (Schwarz et al., 2012; Tanji et al., 2013). Moreover, mitochondrial impairment is implicated in MSA pathogenesis. A previous study showed that the coenzyme Q2 (COQ2) mutation induces MSA. The COQ2 mutation inhibits the synthesis of COQ10, which located in the mitochondrial inner membrane. COQ10 plays a role in the mitochondrial respiratory chain. Thus, loss-of-function of COQ10 increases oxidative stress and induces apoptotic cell death (Quinzii and Hirano, 2010; Multiple-System Atrophy Research Collaboration, 2013). It is already known that oxidative stress is a major contributor to the loss of oligodendrocytes (Bradl and Lassmann, 2010).

PAF (Pure Autonomic Failure)

Unlike PD, DLB, and MSA, Pure autonomic failure (PAF) is a rare sporadic neurodegenerative disease induced by dysfunction of autonomic nervous system (Thaisetthawatkul, 2016). It is slowly progressive disease and caused by abnormal accumulation of α-syn (Thaisetthawatkul, 2016; Coon et al., 2019; Coon, 2020). PAF patients showed orthostatic hypotension, syncope, falls, sexual and bladder dysfunction, and constipation with no motor dysfunction (Stubendorff et al., 2012; Allan, 2019). The pathological characteristic of PAF is the degeneration of peripheral autonomic neurons which have α-syn aggregates (Singer et al., 2017). Furthermore, accumulation of cytoplasmic α-syn inclusion in brainstem nuclei contributes to PAF (Garland et al., 2013; Coon et al., 2019). Donadio et al. (2013) suggested that α-syn aggregation founded in sympathetic nerve fiber using skin biopsy, it might be a biomarker for PAF diagnosis. However, α-syn induced autonomic dysfunction is not clearly understood and there is no cure for PAF (Thaisetthawatkul, 2016). Recent studies suggested that abnormal α-syn in autonomic neurons of PAF patients is able to propagate into central nerve. Wang X.L. et al. (2020, Wang X. J. et al. 2020) reported that injection of α-syn preformed fibrils (PFF) into stellate and celiac ganglia in mutant form α-syn-expressing mice produces propagation of α-syn throughout autonomic pathway into CNS. They also showed that the PFF injected mice have PAF-related pathology. Moreover, about 70% of PAF patients showed rapid eye movement sleep behavior disorder (RBD) strongly associated with the function of

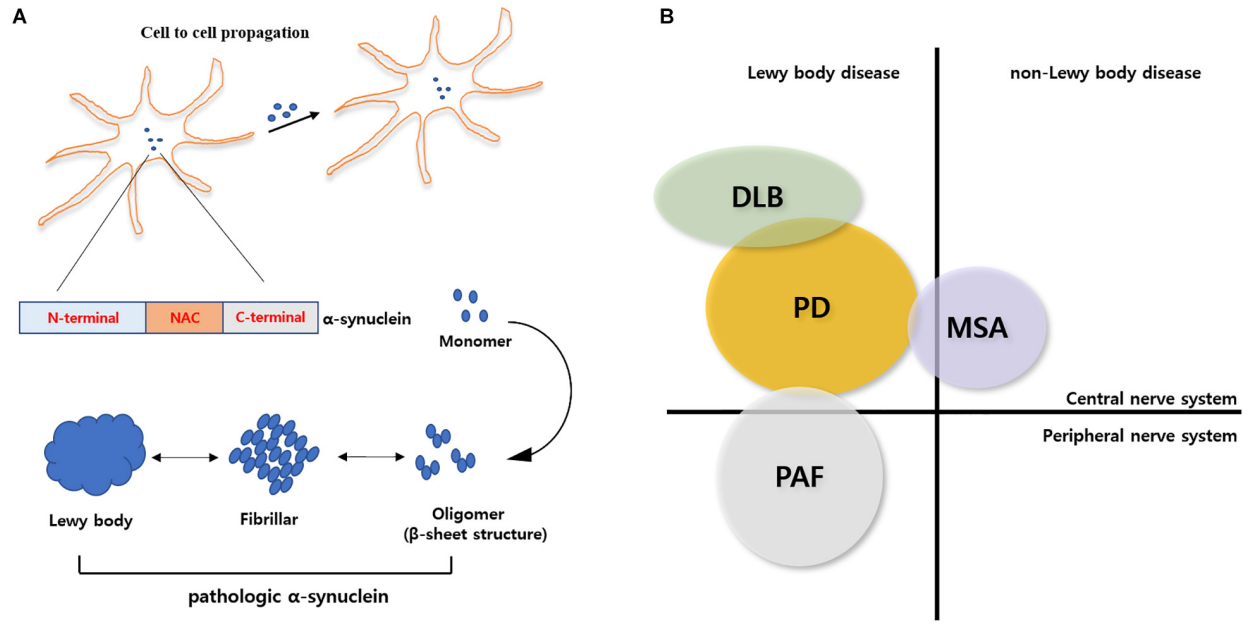

FIGURE 1 | Schematic overview of α-synuclein physiology and pathology. **(A)** Pathological conformation change of α-syn. α-syn composed of N-terminal, NAC, and C-terminal. Originally, α-syn exist as native or misfolded monomeric state. Monomeric α-syn can aggregate into cytotoxic oligomer or fibrillar α-syn. Aggregated α-syn forms Lewy body and it can propagate into other cells. **(B)** α-Synucleinopathies in neurodegenerative diseases. Aggregated α-syn is associated many neurodegenerative diseases such as Parkinson's disease, Dementia with Lewy Body, Pure Autonomic Failure and Multiple System Atrophy. PD, DLB and PAF belong to Lewy body disease.

the CNS ("Consensus statement on the definition of orthostatic hypotension, PAF, and MSA. The Consensus Committee of the American Autonomic Society and the American Academy of Neurology" No authors listed, 1996; Coon et al., 2019). Besides, more than 10% of patients initially diagnosed with PAF developed CNS diseases such as PD, DLB, and MSA (Muppidi and Miglis, 2017; Singer et al., 2017). Converting ratio of PAF to CNS diseases is twice as higher MSA than PD or DLB, and it might be associated with low circulating level of norepinephrine in the plasma of MSA and PAF patients (Goldstein et al., 1989).

The accumulation of α-syn aggregates might be the major cause of neurodegeneration in α-Synucleinopathy (**Figure 1**). Thus, there are several α-syn-mediated therapeutic approaches, such as prevention of α-syn aggregates, increasing of α-syn degradation and α-syn immunotherapy, in all α-syn-mediated diseases (Brundin et al., 2017).

α-SYNUCLEIN-INDUCED PATHOLOGICAL CHANGES IN GLIAL CELLS

Oligodendroglia (Oligodendrocytes)

Oligodendrocytes, together with astrocyte and microglia, are glial cells in CNS (Jakel and Dimou, 2017). Oligodendrocytes are myelin-forming cells in the CNS, accounting for approximately 5–8% of all cells in the adult brain (Levine et al., 2001). Myelin is essential for the transmission of electrical signals along the axon and additionally plays a role in supporting neuronal activity by regulating axon and neuro homeostasis through the supply of neurotrophic factors (Dawson, 2003; Wilkins et al., 2003; Bean, 2007; Peferoen et al., 2014; Kuhn et al., 2019). Demyelination can be caused by damage to oligodendrocytes or the myelin sheaths maintained by these cells (Arenella and Herndon, 1984); moreover, demyelination induces axonal degeneration and oligodendrocyte death, ultimately promoting neuronal death (Bradl and Lassmann, 2010; Kuhn et al., 2019). MS is a demyelination disease caused by inflammation in the CNS. In a previous study, overexpression of wild-type human α-syn in oligodendrocytes induced mitochondrial dysfunction in transgenic mice, leading to oxidative stress *in vivo* and neuronal cell death (Stefanova et al., 2005). The pathological features of oligodendrocytes are associated with ROS and the inflammatory response (Thorburne and Juurlink, 1996; Jurewicz et al., 2005). Normally, oligodendrocytes cooperate with astrocytes to regulate the immune response. A recent study suggested that nuclear factor κB (NF-κB) is a main inflammatory modulator in MS, and plays a role in myelin formation (Nickols et al., 2003; Stone et al., 2017). NF-κB predominantly localizes in the cytoplasm, and activated NF-κB translocate to the nucleus and activates the transcript of inflammatory genes including cytokines, chemokines, and adhesion genes (Lawrence, 2009). Moreover, another study showed that NF-κB activation in oligodendrocytes could drive experimental autoimmune encephalomyelitis (EAE) and the expression of interferon-γ (IFN- γ) to exacerbate the inflammatory response in an *in vivo*

model (Lin et al., 2012, 2013; Stone et al., 2017). Additionally, oligodendrocytes contact surrounding nerve cells to provide neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor 1 (IGF-1) for protection (Bradl and Lassmann, 2010). Oligodendrocytes do not express α-syn under normal conditions (Kahle et al., 2002), but aggregated α-syn was found in oligodendrocytes in midbrains of PD and DLB patients (Wakabayashi et al., 2000). Furthermore, phosphorylated α-syn was observed in GCIs (Fujiwara et al., 2002). Abnormal accumulation of α-syn occurs with the demyelination and brain atrophy associated with neurodegeneration (Yoon et al., 2020). We already mentioned that MSA is caused by aggregated α-syn in GCIs. A recent paper showed that α-syn aggregation was observed in the oligodendrocytes of human MSA-P patients (May et al., 2014).

Several papers have shown that the aggregation of α-syn is accelerated by heparin and heparan sulfate (HS; Cohlberg et al., 2002; Ihse et al., 2017; Maiza et al., 2018). HS is a linear polysaccharide expressed on the cell surface or as an extracellular matrix protein (Iozzo, 1998; Medeiros, 2000). Heparan sulfate proteoglycans (HSPGs) are internalized by α-syn in the MO3.13 human oligodendrocyte cell line (Ihse et al., 2017). Furthermore, the presence of HSPGs initiates a conformational change in α-syn forms a native state into an oligomeric state, including β-sheet formation, through protein unfolding (Motamedi-Shad et al., 2009; Maiza et al., 2018). Aggregate α-syn uptake relies on HS by binding to the plasma membrane (Holmes et al., 2013; Ihse et al., 2017). Additionally, several hypotheses of α-syn aggregation exist cell-to-cell propagation of aggregated α-syn from neurons to oligodendrocytes and/or the secretion of α-syn by oligodendrocyte.

Astroglia (Astrocytes)

Astrocytes are the most common type of glial cell in the CNS and account for at least one third of the brain mass. Astrocytes support the physiological function of the neuronal system by regulating blood flow, maintaining the blood-brain barrier and regulating synaptic plasticity (Sofroniew and Vinters, 2010; Chavarria et al., 2018). Moreover, astrocytes are the main cells responsible for glucose metabolism in the brain and release gliotransmitters, including γ-aminobutyric acid (GABA) and glutamate (Lee et al., 2010a). In addition, astrocytes also play major roles in maintaining neuronal homeostasis through the absorption of glutamate and potassium ions (Thorburne and Juurlink, 1996; Simard and Nedergaard, 2004).

A recent study found that astrocytes isolated from neuroinflammatory and ischemic brains showed two reactive phenotypes, A1 and A2 (Liddelow and Barres, 2017). A1 astrocytes are activated by pro-inflammatory microglia, impair synaptic formation, and kill both neurons and oligodendrocytes, while A2 astrocytes appear to produce substances that promote growth and neuronal survival. Although the number of A1 astrocytes are increased in the affected brain regions of many neurodegenerative diseases, including AD, HD, PD, ALS, and MS (Liddelow and Barres, 2017), detailed mechanisms of A1 and A2 induction have not been clearly identified.

In contrast to neurons, astrocytes express α-syn at very low levels. However, cytoplasmic immunoreactivity of α-syn in astrocytes has been clearly observed in the normal human brain (Mori et al., 2002). Some studies suggested that the number of reactive astrocytes did not increase in the SN and putamen regions in PD patients (Mirza et al., 1999; Song et al., 2009). However, other postmortem studies of PD patient brains reported massive increase in reactive astrocytes and the loss of dopaminergic neurons in the SN (Hirsch et al., 2005), while others reported that reactive astrogliosis was mild (Vila et al., 2001). In most postmortem studies using the brains of PD patients (Mythri et al., 2011; Lastres-Becker et al., 2012), increased expression of glial fibrillary acidic protein (GFAP) was used as a marker for reactive astrocytes (Eng et al., 1971; Dahl and Bignami, 1976). However, since a significant level of GFAP is already present in non-reactive astrocytes, it seems difficult to accurately measure the number of reactive astrocytes with GFAP immunostaining (Sofroniew and Vinters, 2010; Rusnakova et al., 2013).

Astrocytes also have phagocytic activity to degrade cellular debris and synaptic elements (Morizawa et al., 2017). Accumulating evidence suggests that the loss of proper phagocytic functions in astrocytes may contribute to neurodegenerative processes (Jones et al., 2013; Tong et al., 2014; Chung et al., 2016). Unfortunately, whether astrocytic receptors are involved in the recognition of α-syn aggregates and/or their delivery to lysosomes is still unknown.

Astrocytes respond to pathological stimuli by secreting inflammatory cytokines and increasing the level of GFAP. However, in the context of PD, it is still not clear whether astrocyte activation plays a positive or negative role in disease progression (Tremblay et al., 2019).

Both microglia and astrocytes can take up monomeric and fibrillar forms of α-syn (Stefanova et al., 2011; Fellner et al., 2013). In microglia, TLR4 has been implicated in α-syn clearance, the secretion of pro-inflammatory cytokines, and the production of ROS (Fellner et al., 2013). However, astrocytes do not require TLR4 for α-syn uptake (Fellner et al., 2013). Interestingly, α-syn oligomers are rapidly internalized by astrocytes, but internalized α-syn is not completely degraded by lysosomes. Thus, α-syn oligomers accumulate intracellularly and this accumulated α-syn induces mitochondrial defects (Lindstrom et al., 2017). Another study has also reported that the accumulation of α-syn in human astrocytes affects their phagocytic-lysosomal mechanism and that aggregated α-syn is transported through newly formed tunneling nanotubes (Rostami et al., 2017). Evidence suggests that astrocytic α-syn accumulation is implicated in neurotoxic alterations in astrocytes, but the underlying mechanism is still not clear.

Microglia

Although microglia account for the smallest proportion (10% or less) of healthy mammalian brain glia (von Bartheld et al., 2016), they play an important role in the immune response in the CNS. Microglia also regulate neuronal activity and viability in the adult brain through direct contact with neurons (Li et al.,

2012; Kohman et al., 2013) and the secretion of soluble factors (Ramirez et al., 2005; Tichauer et al., 2007).

The morphology and transformative ability of microglia are similar to those of peripheral macrophages/monocytes (Kreutzberg, 1996). The phagocytic activity of microglia is important for the developmental process, as well as injury repair in the adult brain (Neumann et al., 2009).

During brain development, a significant proportion of cells must be eliminated by programmed cell death, while the remaining cells differentiate and migrate. Microglia engulf dying cells, and this phagocytic activity contributes to the proper patterning of the developing brain (Marın-Teva et al., 2004; Frost and Schafer, 2016). In addition, microglia remove excess synapses in the developing brain and appear to play an important role in the wiring of the CNS (Jakel and Dimou, 2017). Under pathological conditions such as neurodegenerative diseases, infection and physical injury, microglia are activated. Activated microglia are polarized into two different states, M1 (pro-inflammatory), and M2 (anti-inflammatory), according to the signals in the surrounding environment and function in immune regulation (Moehle and West, 2015). M1 microglia release several pro-inflammatory cytokines, including TNF-α, IL-6, IL-12, and IL-1β, followed by CCL2 and CXCL10. In contrast to M1 cells, M2 microglia are associated with anti-inflammatory functions and promote wound healing and tissue repair. M2 microglia secrete major anti-inflammatory cytokines, such as IL-4, IL-13, IL-10, and TGF-β (Du et al., 2018). In PD, affected DA neurons in the brains of PD patients are surrounded by M1 microglia (Tang and Le, 2016). Moreover, microglia-mediated neuroinflammation shows an inverse relationship to the survival of dopaminergic neurons in PD patients (Tang and Le, 2016). Increased microglial activation has been predominantly found in the substantia nigra (SN) and other brain regions associated with pathological α-syn accumulation (Doorn et al., 2014). In addition, overexpression of the mutant α-syn in microglia induces polarization to the M1 phenotype, which is characterized by increased levels of pro-inflammatory cytokines (such as TNF-α and NO; Rojanathammanee et al., 2011). The role of M2 microglia in PD is not well understood, but the shift from the M1 pro-inflammatory state to the anti-inflammatory M2 state might be more beneficial in neuroprotection than simply blocking microglial activation (Subramaniam and Federoff, 2017). Thus, a therapeutic approach promoting M1 to M2 polarization could be promising in PD.

Toll-like receptors (TLRs) are known to be involved in the innate immune response, cell survival and death. TLRs are expressed in neurons (Tang et al., 2007), astrocytes (Bowman et al., 2003), microglia (Olson and Miller, 2004), and oligodendrocytes (Lehnardt et al., 2002) in the CNS. Many studies have reported an association between TLRs, especially TLR2, 4 and 9, and neurodegenerative diseases such as PD, AD and ALS. Several studies have shown that increased expression of various receptors, including TLR2 (Kim et al., 2013), TLR4 (Fellner et al., 2013), cluster of differentiation (CD) 11b (Hou et al., 2018), and CD36, (Su et al., 2008) by α-syn aggregation can lead to receptor-mediated activation of the inflammatory signaling cascade. TLR2 is one of the most well-studied TLRs in the context of neurodegenerative diseases. In mice, overexpression of α-syn increased TLR2 expression and microglial activation (Drouin-Ouellet et al., 2014).

The α-syn released by neurons acts as an endogenous ligand for TLR2 on microglia. TLR2-α-syn binding activates the inflammatory responses of microglia, which eventually produce toxic molecules that cause neurodegeneration (Kim et al., 2013, 2016). Previous studies have shown that TLR4 also plays an important role in α-Synucleinopathies.

Stimulation of TLR4 by monophosphoryl lipid A, a selective TLR4 agonist, in an α-syn-overexpressing mouse model, increased α-syn clearance by microglia and suppressed disease-like phenotype, such as motor deficiency (Venezia et al., 2017). In BV2 microglia-like cells, the TLR4-dependent immune response is modulated by Nurr1 and NF-κB signaling, and this TLR4-mediated neuroinflammation pathway is mediated by α-synuclein (Shao et al., 2019). In addition, Choi et al. (2020) showed that TLR4-NF-κB-p62-mediated selective autophagy activation in microglia was implicated in the clearance of neuron-secreted α-syn and this clearance mechanism was important for neuroprotective functions of microglia.

GLIAL MITOCHONDRIAL DYSFUNCTION IN α-SYNUCLEINOPATHIES

Cell Death

Severe mitochondrial damage can induce cell death via necrosis or apoptosis (Cillero-Pastor et al., 2011). Several studies have shown that the interaction between α-syn and cytochrome C oxidase (COX, mitochondrial complex IV) induces both α-syn aggregation and mitochondrial dysfunction, causing neurodegeneration (Irrinki et al., 2011; Al-Mansoori et al., 2013; Ciccone et al., 2013).

α-syn acts as a regulator of mitochondrial homeostasis, and mitochondrial deficiency and impairment are a key pathological features of PD (Gao et al., 2017; Pozo Devoto and Falzone, 2017). Complex 1 deficiency or dysfunction associated with mitochondrial dysfunction was found in the SN region of PD patients (Schapira et al., 1989; Parker et al., 2008). Furthermore, many α-Synucleinopathy models have shown that the level of α-syn expression is associated with cell death and mitochondrial deficiency (Hsu et al., 2000; Bellucci et al., 2008; Trancikova et al., 2012). Numerous studies have revealed that the interactions between mitochondria and α-syn play important roles in both physiological and pathological conditions, but most of these studies focused on their role in neurons (Faustini et al., 2017). However, some recent studies have suggested that α-syn-induced mitochondrial defects in glia are also implicated in various pathogenic features, such as neurodegeneration and neuroinflammation.

Accumulating evidence suggests that astrocytes might be a major contributor to the progression of α-Synucleinopathies. For example, α-syn overexpression in astrocytes induces astrocyte activation, and these reactive astrocytes cause neuronal death (Chavarria et al., 2018) and susceptibility to oxidative stress

(Stefanova et al., 2001). Chavarria et al. (2018) investigated the effects of the different species of α-syn (monomer, oligomer, and fibrillar) protein on primary rat cortical astrocyte. All of α-syn species can activate astrocytes, and astrocytes treated with the different α-syn species induced cell death of hippocampal neuron in neuron-astrocyte co-culture. In particular, only oligomeric α-syn induce mitochondrial dysfunction and increased hydrogen peroxided generation in astrocytes, whereas fibrillar α-syn treated astrocytes enhanced the secretion of pro-inflammatory cytokines. Stefanova et al. (2001) showed that overexpression of wild-type α-syn or C-terminal truncated α-syn in U373 astrocytoma cells induced apoptotic cell death and increased susceptibility to oxidative stress, and that it was partially present in the cytoplasmic inclusion. In addition, *in vitro* and *in vivo* model studies have demonstrated that α-syn can spread from neurons to glial cells either through secretion to the extracellular space or direct cell-to-cell transfer (Angot et al., 2012; Lee et al., 2012; Reyes et al., 2015). α-syn inclusions are mainly found in neurons, but also frequently detected in glial cells. Lindstrom et al. (2017) tried to elucidate the clearance mechanism of toxic α-syn in glial cells. This study examined the uptake, degradation, and toxic effects of oligomeric α-syn in the co-culture system of primary mouse neurons, astrocytes, and oligodendrocytes. Astrocytes rapidly and extensively absorb α-syn from the extracellular space and sequester it. In the early stage of α-Synucleinopathy, this sequestration has been shown to play a role in preventing toxicity and disease progression (Lindstrom et al., 2017; Lin et al., 2019). Astrocytes degrade absorbed α-syn oligomer via the autophagy lysosomal degradation pathway, but remaining intracellular deposition of α-syn causes mitochondrial dysfunction and cell death (Lindstrom et al., 2017). Another study showed that overexpression of wild-type and disease associated mutant (A30P and A53T) α-syn in immortalized astrocytes decreased LC3-II and increased p62 protein levels, suggesting the suppression of autophagy. In addition, mitochondrial membrane potential loss and increased parkin expression were evident in mutant α-syn expressing astrocytes (Erustes et al., 2018). Moreover, a recent study demonstrated that overexpression of mutant α-syn (A30P and A53T) in primary rat astrocytes induced an unfolded protein response through the PERK/eIF2alpha signaling pathway. In particular, dysfunction of the ER-Golgi compartment in astrocytes overexpressing wild type α-syn can inhibit neurite growth by reducing GDNF levels (Liu et al., 2018). In another study, α-syn was transfected into ONL-t40 cells, an oligodendroglial cell line. This study showed that α-syn overexpression induces mitochondrial dysfunction, impaired autophagic flux, and the formation of α-syn aggregates in ONL-t40 cell lines (Pukass et al., 2015). It is clear that mitochondrial impairment is implicated in α-syn-induced glial cell death, but the molecular mechanisms of glial mitochondrial dysfunction and cell death in α-Synucleinopathies are not fully understood.

Inflammation

Mitochondrial dysfunction is associated with the induction of the inflammatory response. Mitochondrial impairment increases mtROS production, extracellular ATP levels and mtDNA release, all of which induce a vicious inflammatory response cycle that exacerbates mitochondrial dysfunction (Lopez-Armada et al., 2013).

Among the various putative factors contributing to PD etiology, the neuroinflammatory mechanism serves as a major contributor. Increased pro-inflammatory cytokines (including IL-1β, IL-6, TNF, and IFN-γ) have been observed in postmortem brains and cerebrospinal fluid from PD patients (Mogi et al., 1994a,b), moreover, the blood serum of PD patients also showed increased concentration of inflammatory cytokines (including IL-2, IL-6, TNF-α, and IFN-γ; Reale et al., 2009). Neuroinflammation is primarily mediated by the activation of glial cells and is accompanied by the production of inflammatory cytokines. Microglia have traditionally been considered to play a major role in the immune response in the CNS (Soulet and Rivest, 2008). Pathological α-syn aggregation in PD causes microglial activation, which is known to strongly induce microglial neuroinflammatory responses in the brain (Zhang et al., 2005). Microglia that absorb α-syn aggregates via phagocytosis promote NADPH oxidase activation and ROS production, leading to the secretion of pro-inflammatory neurotoxic cytokines (Zhang et al., 2005; Walter and Neumann, 2009) and chemokines (Fellner et al., 2013; Roodveldt et al., 2013). In addition, during the inflammatory response, changes in mitochondrial metabolism contribute to microglial activation.

The levels of inflammatory cytokines, such as IL-6, and the number of activated microglia are increased in the hippocampus in patients with PD and DLB (Imamura et al., 2005). M1-related cytokines, such as TNF-α (Mogi et al., 1994b) and IL-6 (Muller et al., 1998), were increased in the cerebrospinal fluid of PD patients, and the levels of these cytokines correlated with poor prognosis. A number of studies have shown that α-syn induces the pro-inflammatory response in microglia or macrophage to produce the inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Klegeris et al., 2008; Lee et al., 2009). Inflammatory mediators such as iNOS and COX2 were also upregulated in activated microglia by wild-type α-syn treatment. This result suggested that that CD36 scavenger receptor and downstream kinases are involved in α-syn-mediated microglial activation. (Su et al., 2008). Hoenen et al. (2016) also showed that a disease associated α-syn mutant (A35T) more strongly induced reactive microglia than the human wild-type protein in a transgenic mouse model. The researchers also found that the NF-κB/AP-1/Nrf2 pathway was implicated in A35T α-syn-induced microglial activation via MAPK. In addition, Rannikko et al. (2015) reported that overexpression of exogenous wild type α-syn in primary mouse astrocytes can induce inflammatory responses such as induction of NO synthase and COX-2 expression.

Furthermore, in the Fellner (2013; Fellner et al., 2013) study, purified microglia and astrocytes from wild-type (TLR4 +/+) and TLR4-deficient (TLR4-/-) mice were treated with α-syn (full length soluble, fibrillized, and C-terminally truncated), and phagocytic activity, NF-κB nuclear translocation, cytokine release and ROS production were measured. Treatment of TLR4+/+ glial cells with C-terminally truncated α-syn induced upregulation of phagocytic activity, NF-κB translocation, ROS production, and the release of IL-6, CXCL1, and TNF-α (Fellner

et al., 2013). The stefanova (2011; Stefanova et al., 2011) study reported that α-syn clearance was reduced in TLR4 deficiency murine. Moreover, in an animal model study of MSA based on oligodendroglial α-syn overexpression, TLR4 deficiency increased motor impairment, and increased loss of nigrostriatal dopaminergic neurons. However, the role of TLR4 in α-syn-dependent activation of microglial and astrocytes has not been clearly elucidated.

To date, many studies have shown that activated astrocytes also play an important role in neuroinflammation in the aged brain and most neurodegenerative diseases. Activated astrocytes release inflammatory cytokines and chemokines that can cause neuronal damage (Komine et al., 2018; Yu et al., 2018). Although the exact pathogenic mechanism of PD is unclear, accumulating evidence suggests that reactive astrogliosis and astrocyte-mediated neuroinflammation play important roles in the pathogenesis of PD (Wang et al., 2015; Liddelow et al., 2017). In another study, inflammation-related factors such as GFAP, COX-2, iNOS, TNF-α, and IL-1β were increased in primary rat astrocytes by A53T α-syn aggregate treatment via the NF-κB and c-Jun N-terminal kinase signaling pathways, and the upregulation of heat shock protein 70 effectively suppressed the α-syn-induced neuroinflammatory response (Yu et al., 2018).

Calcium Homeostasis

Calcium is essential for cellular signaling, and calcium homeostasis is important for neuronal integrity because it is involved in neuronal plasticity, synaptic transmission, and cell survival. In neurons, calcium channels are the key signaling elements that regulate the release of biological factors, such as neurotransmitters and hormones, through calcium sensing (Leandrou et al., 2019). Among the causes of α-syn pathology, the breakdown of calcium homeostasis promotes aggregation and abnormal secretion of α-syn (Wojda et al., 2008; Hettiarachchi et al., 2009; Yang et al., 2019). Cell culture model studies suggested that a temporary increase in intracellular calcium concentration induced cytoplasmic α-syn aggregates (Nath et al., 2011; Follett et al., 2013).

Nath et al. (2011) showed that the addition of calcium to the recombinant α-syn monomer *in vitro* triggers oligomer formation in a dose-dependent manner. Besides, in 1321N1 glioma cells expressing α-syn-GFP, when treated with thapsigargin or calcium ionophore to induce a temporary

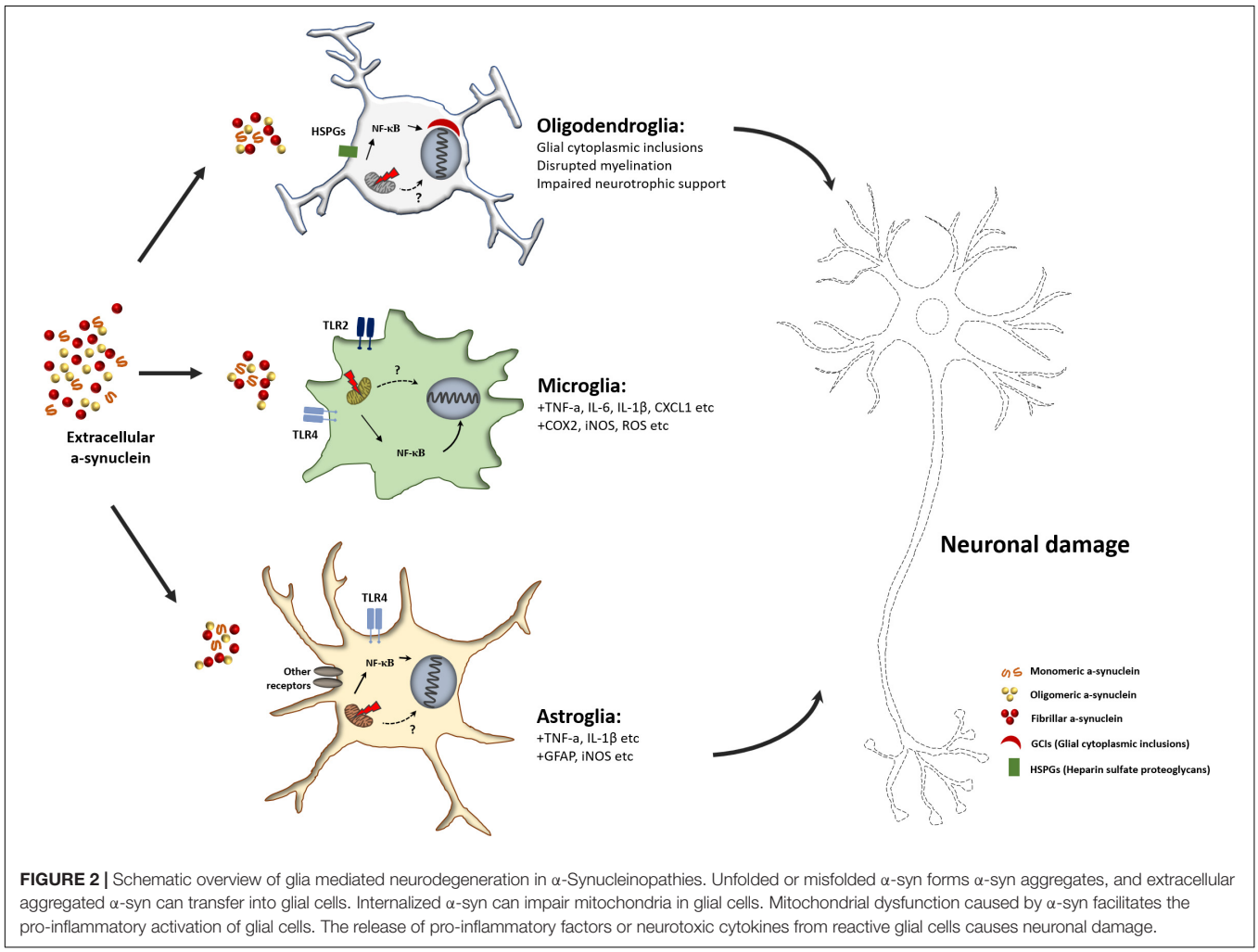

FIGURE 2 | Schematic overview of glia mediated neurodegeneration in α-Synucleinopathies. Unfolded or misfolded α-syn forms α-syn aggregates, and extracellular aggregated α-syn can transfer into glial cells. Internalized α-syn can impair mitochondria in glial cells. Mitochondrial dysfunction caused by α-syn facilitates the pro-inflammatory activation of glial cells. The release of pro-inflammatory factors or neurotoxic cytokines from reactive glial cells causes neuronal damage.

increase in intracellular free calcium, α-syn aggregates in the cytoplasm were significantly increased. Another study show that raised intracellular Ca^{2+} mediated by K^{+} depolarization can lead to cytoplasmic α-syn aggregation (Follett et al., 2013).

Dufty et al. (2007) demonstrated that cleavage of α-syn by calpain I, a calcium-dependent protease, can facilitate the aggregation of α-syn. Moreover, Luth et al. (2014) investigated the relationship between mitochondrial Ca^{2+} stress and α-syn using isolated mitochondria and purified recombinant human α-syn. Interestingly, the mitotoxic effect of α-syn was particularly dependent on electron flow through complex I and the mitochondrial uptake of exogenous Ca^{2+}. Consequently, the soluble prefibrillar α-syn oligomer induces several mitochondrial phenotypes (changes in membrane potential, disruption of Ca^{2+} homeostasis, mitochondrial complex I dysfunction, and enhancement of cytochrome c release) that have been observed *in vitro* and *in vivo* models of PD. However, the mechanism by which calcium promotes α-syn aggregation is not clear, and the interaction between calcium and α-syn aggregation in glial cells is not been clearly characterized.

CONCLUSION

Links Between Glial Mitochondrial Dysfunction and Neurodegeneration in α-Synucleinopathies

Many *in vitro* and *in vivo* animal studies have focused on neuron-to-neuron propagation of α-syn aggregates (Lee et al., 2012; Dehay et al., 2016). However, studies on α-syn in the context of neuronal dysfunction and studies on the association of α-syn accumulation and propagation in glial cells have been largely overlooked. Some recent studies have provided evidence for the pathogenic response of glial cells to neuron-derived α-syn aggregates. α-syn secreted from neurons induces mitochondrial impairment in microglia, and α-syn-induced mitochondrial dysfunction can activate the microglia-mediated neuroinflammatory response (**Figure 2**). Pro-inflammatory M1 microglia secrete neurotoxic agents that cause neurodegeneration. In addition, it has been found that the neurotoxic response of microglia is mediated by the activation of TLR2, a receptor for neuron-derived α-syn (Kim et al., 2013, 2016). Similar to microglia, pathogenic α-syn-treated astrocytes activate pro-inflammatory responses, including the production of pro-inflammatory cytokines and chemokines (Lee et al., 2010b). Although mitochondrial dysfunction in glial cells is one of the major contributors to neuroinflammation, which causes neurodegeneration in α-Synucleinopathies, the molecular mechanism underlying α-syn-induced mitochondrial impairment is not fully understood. Thus, further studies are warranted to elucidate the non-cell autonomous neurotoxic crosstalk between glial cells and neurons, and this crosstalk mechanism may be a promising target for α-Synucleinopathy-associated neurodegenerative disease treatment.

AUTHOR CONTRIBUTIONS

SL, YK, SK, MJ, and Y-MJ provided ideas for the project and participated in data collection. Y-MJ, YK, and H-JK wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.548283/full#supplementary-material

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REVIEW
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When Friendship Turns Sour: Effective Communication Between Mitochondria and Intracellular Organelles in Parkinson's Disease

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Parkinson's disease (PD) is a complex neurodegenerative disease with pathological hallmarks including progressive neuronal loss from the substantia nigra pars compacta and α-synuclein intraneuronal inclusions, known as Lewy bodies. Although the etiology of PD remains elusive, mitochondrial damage has been established to take center stage in the pathogenesis of PD. Mitochondria are critical to cellular energy production, metabolism, homeostasis, and stress responses; the association with PD emphasizes the importance of maintenance of mitochondrial network integrity. To accomplish the pleiotropic functions, mitochondria are dynamic not only within their own network but also in orchestrated coordination with other organelles in the cellular community. Through physical contact sites, signal transduction, and vesicle transport, mitochondria and intracellular organelles achieve the goals of calcium homeostasis, redox homeostasis, protein homeostasis, autophagy, and apoptosis. Herein, we review the finely tuned interactions between mitochondria and surrounding intracellular organelles, with focus on the nucleus, endoplasmic reticulum, Golgi apparatus, peroxisomes, and lysosomes. Participants that may contribute to the pathogenic mechanisms of PD will be highlighted in this review.

Keywords: Parkinson's disease, mitochondria, interorganelle communication, mitophagy, lysosome, endoplasmic reticulum, peroxisome, golgi apparatus

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (Poewe et al., 2017), affecting ∼1% of the population over the age of 60, resulting in a significantly shorter life span for PD patients (Abbas et al., 2018). This progressive neurodegenerative disease has two histopathological hallmarks: loss of dopaminergic neurons

in the substantia nigra pars compacta and the presence of intraneuronal α-synuclein (α-syn) protein inclusions called Lewy bodies (Klemann et al., 2017). Decreased dopamine secretion leads to cardinal PD motor phenotypes including resting tremor, bradykinesia, rigidity, and postural instability. These may be preceded by a prodromal phase of up to decades, characterized by specific non-motor symptoms such as rapid eye movement (REM) sleep behavior disorder, depression, constipation, hyposmia, and cognitive impairment (Chaudhuri et al., 2006). Although tremendous progress has been made in the pharmaceutical treatment of PD, the exact causes of PD remain unclear, although important risk factors have been identified, which include aging, genetics, and environmental factors (Poewe et al., 2017; Abbas et al., 2018). Most PD cases are sporadic, while only 5–10% are of the familial form. However, recent genetic discoveries have provided us with valuable insight into the molecular pathways involved in the neurodegenerative process of PD. Several biological processes, including mitochondrial dysfunction, α-syn aggregation, oxidative stress, defective endo-lysosomal functioning, and immune response activation, have been suggested to contribute to the pathogenesis of PD, and these molecular pathways appear to overlap at multiple points (Cipolla and Lodhi, 2017; Grunewald et al., 2019; Nguyen et al., 2019).

An important pathological characteristic of PD is the abnormal accumulation of the misfolded protein, α-syn (encoded by the SNCA gene). Being a major component of Lewy bodies in PD patients, α-syn aggregates have been suggested to play a critical role in PD pathogenesis. Although the physiological role of α-syn awaits elucidation, the detrimental outcome of α-syn oligomers and its aggregates has been the focus of extensive study. In pathological conditions, α-syn can form insoluble fibrils through oligomerization (Hijaz and Volpicelli-Daley, 2020). The α-syn oligomer induces overproduction of ROS due to mitochondrial respiratory complex I inhibition, leading to mitochondrial dysfunction. The link between mitochondrial dysfunction and PD dates back to 1982 when seven adolescents developed parkinsonism shortly after injection of synthetic heroin containing byproduct 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a specific mitochondrial respiratory chain complex I inhibitor (Nonnekes et al., 2018). Since then, a myriad of studies have demonstrated the causal role of mitochondrial dysfunction in PD, including many established PD models inhibiting respiratory complex I such as MPTP, the pesticide rotenone, and herbicide paraquat (Blesa and Przedborski, 2014). More recent genetic discoveries have underlined the importance of mitochondrial integrity in the neurodegenerative process of PD, as many familial PD genes encode proteins essential in mitochondrial homeostasis. Genome-wide association studies (GWAS) and transcriptome-wide association study (TWAS) have, respectively, identified 41 and 66 susceptible loci associated with PD, including autosomal recessive PD genes *PRKN/PARK2*, *phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1)/PARK6*, *Daisuke-Junko-1 (DJ-1)/PARK7*, *ATPase type13A2 (ATP13A2)/PARK9*, and *F-box only protein 7 (FBXO7)/PARK15*, and autosomal dominant PD genes *SNCA/PARK1*, *leucine-rich repeat kinase 2 (LRRK2)/PARK8*, and *vacuolar protein sorting 35 (VPS35)/PARK17* (Li et al., 2019b). All of these genes have been shown to affect mitochondrial biogenesis, morphology, trafficking, and elimination (Chang et al., 2017; Li et al., 2019b), further supporting the role of mitochondrial dysfunction in the pathophysiology of both sporadic and familial forms of PD.

In order to exert their complex activities, mitochondria must actively signal and interact with other subcellular compartments. This coordination between mitochondria and different organelles requires frequent communication via direct contact or indirect interorganelle signaling (Giacomello et al., 2020). Recent

Abbreviations: ABCD1, ATP binding cassette (ABC) transporter 1; mtDNA, Mitochondrial DNA; ACBD2(ECI2), Acyl-coenzyme A-binding domain; mTERF, Mitochondrial transcription termination factor; ADP, Adenosine diphosphate; nDNA, Nucleus DNA; APAF1, Apoptotic peptidase-activating factor 1; NCLX, Na^+/ Ca^{2+} exchanger; ATF4, Activating transcription factor; NRF1, Nuclear respiratory factor 1; ATP, Adenosine triphosphate; OPA1, Optic dominant atrophy; ATP13A2, ATPase type13A2; ORP5/8, Oxysterol-binding protein-related proteins 5 and 8; α-syn, α-synuclein; OXPHOS, Oxidative-phosphorylation; BAK, BCL2-antagonist/killer; PACS2, Phosphofurin acidic cluster sorting protein 2; Bap31, B-cell receptor associated protein 31; PD, Parkinson's disease; BiFC, Bimolecular fluorescence complementation; Pex, Peroxin; Ca^{2+}, Calcium; PGC, Peroxisome proliferator-activated receptors (PPAR) γ coactivator; DLP1, Dynamin like protein 1; PI3KC3-C1, Class III phosphatidylinositol 3-kinase complex I; DJ-1, Daisuke-Junko-1; PINK1, Phosphatase and tensin homologue (PTEN)-induced putative kinase 1; Drp1, Dynamin-related GTPase protein 1; PI(3)P, PI3KC3-C1 assists phosphatidylinositol 3-phosphate; ER, Endoplasmic reticulum; POLRMT, Mitochondrial RNA polymerase; ERGIC, ER-Golgi intermediate compartment; PPARs, Peroxisome proliferator-activated receptors; ERMES, ER-mitochondria encounter structure; PTPIP51, Phosphatase-interacting protein 51; ERRs, Estrogen-related receptors; PTS1/2, Peroxisome targeting signals 1 and 2; ETC, Electric transport chain; PXMP2, Peroxisomal membrane protein 2; FBXO7, F-box only protein 7; Rab7 GAP, Rab7 GTPase-activating proteins; Fis 1, Mitochondrial fission 1 protein; ROS, Reactive oxygen species; FUNDC1, FUN14 domain containing 1; Redox, Reduction-oxidization; GABPα, GA-binding protein-α (also called NRF2); RRBP1, Ribosome- binding protein 1; GRP75, Glucose-regulated protein 75; rRNA, Ribosomal RNAs; GWAS, Genome-wide association studies; SERCA2b, Sarcoplasmic/endoplasmic reticulum Ca^{2+} pump 2b; HIF-1, Hypoxia inducible factor 1; SNARE, Soluble N-ethylmaleimide-sensitiveăfactor attachment protein receptor; IMS, Intermembrane space; SOD, Superoxide dismutase; INF2, Inverted formin 2; SREBF1, sterol regulatory element-binding transcription factor 1; IP3R, Inositol 1,4,5 trisphosphate receptor; SYNJ2BP, Synaptojanin 2 binding protein; LC3, Microtubule-associated protein 1A/1B-light chain 3; TCA, Tricarboxylic acid; LIR, LC3-interacting region; TFAM, Transcription factor A; LRRK2, Leucine rich repeat Kinase 2; TFB1M/ TFB2M, Transcription specificity factors; MAM, Mitochondria-associated membranes; TMEM175, Transmembrane protein 175; MCU, Mitochondrial $Ca2^+$ uniporter; TOM20, Translocase of outer mitochondrial membrane 20; Mdm, Mitochondrial distribution and morphology; tRNA, Transfer RNAs; MDVs, Mitochondrial derived vesicles; TRPP2, Transient receptor potential protein 2; MERCs, Mitochondria-ER contacts; TWAS, Transcriptome-wide association study; Mff, Mitochondria fission factor; Ub, Ubiquitin; Mfn1/2, Mitofusin 1 and 2; ULK1, Unc-51-like kinase 1; Miro1/2, Mitochondrial Rho (Miro) GTPases; UPR, Unfolded protein response; MIM, Mitochondrial inner membrane; VAPB, Vesicle-associated membrane protein associated protein B; Mmm1, Maintenance of mitochondrial morphology protein 1; V-ATPase, Vacuolar ATPase; ΔΨm, Mitochondrial membrane potential; VDACs, Voltage-dependent anion-selective channel proteins; MOM, Mitochondrial outer membrane; VPS35, Vacuolar protein sorting 35; mPTP, Mitochondrial permeability transition pore; X-ALD, X-linked adrenoleukodystrophy; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

evidence has shown how interorganelle communication impacts mitochondrial and cellular functions from susceptible PD genes. Close communication between the nucleus and mitochondria through anterograde and retrograde signaling pathways plays a pivotal factor in maintaining mitochondrial integrity in response to cytosolic/mitochondrial stress for cellular survival (Schumacher and Vijg, 2019). Furthermore, the interactions between mitochondrial and endoplasmic reticulum (ER) are essential for normal mitochondrial functioning. These two organelles form tight contact sites for molecular transfers, which includes Ca^{2+} and metabolites such as lipids (Lee and Min, 2018). Silencing of various PD genes causes disruption of mitochondria/ER contact sites, increased ER stress, mitochondrial fragmentation, inhibition of mitophagy, and neuronal death (Tapias, 2019). The Golgi apparatus has also been observed to interact with mitochondria and ER for regulating Ca^{2+} homeostasis, and mutant α-syn has been shown to disturb ER–Golgi trafficking. The susceptible-PD-gene-encoded trafficking protein VPS 35 has been shown to be localized at the Golgi apparatus, suggesting their involvement in mitochondria-related protein transport (Ebanks et al., 2019). Recently, mounting evidence has indicated the interaction between peroxisomes and mitochondria. Both organelles play important roles in lipid β-oxidation metabolism, and both play a redox scavenging role to maintain cellular reactive oxygen species (ROS) homeostasis (Uzor et al., 2020). Interdependence between the two organelles has been noted, as dysfunction in one leads to dysfunction in the other. Evidence suggests that both peroxisomal and mitochondrial dysfunction contribute to organismal aging and is involved in neurodegenerative diseases (Cipolla and Lodhi, 2017). Moreover, since the early 2000's, evidence has demonstrated that lysosomal and mitochondrial dysfunctions in PD are inseparable, as lysosomal degradation of damaged mitochondria is crucial in mitochondrial quality control. Further, studies have also demonstrated the reciprocal relationship between mitochondria and lysosomes (Deus et al., 2020). Thus, interorganelle communication, with mitochondria as a hub, is important in intracellular function, and when friends become foes, pathophysiological connections to diseases ensue. In the following sections, we will review the role of mitochondria in cellular functions and the distinct interactions of the interorganelle network and discuss how this crosstalk may impact PD pathogenesis.

MITOCHONDRIAL BIOLOGY

Mitochondria have been a major focus for research endeavors for over 30 years due to their involvement in cellular ROS production and the induction of apoptosis (Pfanner et al., 2019). This is in part due to a growing acknowledgment of the role of mitochondria in multiple functions other than energy production, including Ca^{2+} homeostasis, generation of ROS, regulation of apoptosis, activation of endoplasmic reticulum (ER)-stress response, and other consequences of mitochondrial dysfunction (Giorgi et al., 2018; Spinelli and Haigis, 2018). Mitochondria are also implicated in many neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, as well as in the aging process. The organelle is under the control of both mitochondrial and nuclear genomes, leading to a potential mosaic of pathogenic mutations (Quirós et al., 2016). A majority of mitochondrial proteins are encoded by nuclear genes and follow the usual pattern of Mendelian inheritance. A few [2 ribosomal RNAs, 22 tRNAs, and 12 subunits of the electron transport chain (ETC)] are encoded by mitochondrial DNA (mtDNA), a circular genome within the mitochondria that is 13,794 nucleotides in length (Giacomello et al., 2020).

The mitochondria contain two membranes, the mitochondrial outer membrane (MOM) and the mitochondrial inner membrane (MIM), with the intermembrane space (IMS) between the two and a central mitochondrial matrix. The MIM is highly folded, creating deep invaginations called cristae, which provide a large surface area harboring ETC complexes I–IV and F_1F_0-ATP synthase for ATP production (Capaldi and Aggeler, 2002). Reducing equivalents (NADH and $FADH_2$) generated from the tricarboxylic acid (TCA) cycle in the matrix pass electrons on to the ETC in the MIM, with O_2 serving as the terminal electron acceptor to form water. The ETC enzyme complexes I–IV use energy generated from the redox reactions to pump protons across the MIM into the IMS. Complexes I and IV are proton pumps, while complex III is not considered a true pump but rather a Mitchellian proton-loop machine (Stuchebrukhov, 2018). Impermeable to protons, the MIM acts as a functional barrier and establishes a proton-motive force comprised of electrical and chemical potentials across the MIM, termed mitochondrial membrane potential ($\Delta\psi m$). This proton gradient generated from electron transportation (oxidation) then drives protons across the MIM through the F_1F_0-ATP synthase (complex V) and ultimately phosphorylate adenosine diphosphate (ADP) into ATP (phosphorylation), called oxidative-phosphorylation (OXPHOS) coupling (Lu, 2011). $\Delta\psi m$ is also a crucial indicator for mitochondrial health and, if dissipated, may signal the cell to perform various stress responses or even mitochondrial mediated apoptosis (Mitchell, 2011). However, ROS are generated from up to 12 different enzymes associated with nutrient metabolism and OXPHOS, including several flavoproteins and respiratory complexes I–III (Gorrini et al., 2013; Mailloux, 2020). ROS are highly reactive and can imbalance cellular reduction–oxidization (redox) and readily oxidize proteins, lipids, carbohydrates, DNA, and RNA causing oxidative damage if in excess. To protect organelles from ROS oxidative damage, mitochondria develop their own antioxidative system, such as the protective antioxidative manganese superoxide dismutase 2 (SOD2) enzymes and the glutathione/glutathione peroxidase/glutathione reductase axis (Beer et al., 2004; Lin et al., 2018; Mailloux, 2020) With its close association with the OXPHOS system, the mitochondrial genome is subject to ROS assault (Saki and Prakash, 2017). Thus, mitochondria are heavily dependent on antioxidative enzymes encoded by the nuclear genome (Kazak et al., 2012). Both mtDNA mutation and nuclear DNA repair defects are considered cellular mechanisms of aging, and a recent study has also found that mtDNA can destabilize nuclear genome

maintenance (Hamalainen et al., 2019). In the normal aging process, mtDNA mutations cause respiratory chain deficiencies and deficient protein homeostasis (proteostasis) (Schumacher and Vijg, 2019). These findings are observed in the PD brain in association with α-syn aggregation, especially in the substanstia nigra (Dolle et al., 2016; Rango and Bresolin, 2018).

Mitochondria also play major roles in the decision regarding cell fate through intrinsic mitochondrial apoptotic pathways. Regulating both cellular and mitochondrial Ca^{2+} distribution, mitochondria sense alterations of intracellular Ca^{2+} homeostasis. Uncontrolled mitochondrial Ca^{2+} overloading and ROS overproduction induce cell death by triggering mitochondrial permeability transition pore (mPTP) opening (Feno et al., 2019). Sustained mPTP opening leads to the collapse of $\Delta\psi m$, swelling of the mitochondria, cytochrome c and proapoptotic mediators release from the mitochondrial intermembrane space to the cytosol, and eventual cellular apoptosis. The release of cytochrome c from the mitochondria to the cytosol activates downstream proapoptotic mediators including apoptotic peptidase-activating factor 1 (APAF1), pro-caspase-9, and apoptosome-dependent activation of caspase 3, caspase 6, and caspase 7, the executioners of apoptosis. Mitochondrial Ca^{2+} homeostasis is regulated by electrogenic Ca^{2+}uptake [via mitochondrial Ca^{2+} uniporter (MCU)] and efflux (in excitable cells via Na^{+}/Ca^{2+} exchanger NCLX) (Dupont and Combettes, 2016). The mitochondria are strategically placed throughout the cell, and mitochondrial Ca^{2+} influx stimulates Ca^{2+}-dependent dehydrogenases, which use NADH/FADH2 and activate the electron. NCLX inhibition has been indicated in a familial form of Parkinson's disease, in which PINK-1 deficiency leads to a delayed Ca^{2+} efflux and mitochondrial Ca^{2+} overload in response to physiological Ca^{2+} stimulation (Glancy and Balaban, 2012).

With an array of mitochondrial functions, the maintenance of a healthy pool of mitochondria is critical, and this versatile organelle has developed protective measures for dysfunctional mitochondria through quality control (Eisner et al., 2018). These processes include mitochondrial dynamics of fusion, fission, trafficking, and clearance through mitophagy. Morphological changes in the mitochondrial network to fusion provides macromolecular exchange between the neighboring mitochondria and complements mtDNA. Fusion processes restore functional proteins and non-damaged mtDNA to dysfunctional mitochondria, decreasing the occurrence of mitophagy. Major fusion machinery includes three GTPases: the mitofusins 1 and 2 (Mfn1 and Mfn2) mediate MOM fusion, and the optic dominant atrophy (OPA1) mediates MIM fusion (Picca et al., 2018). The fission of the mitochondria segregates dysfunctional parts of the mitochondria, and these fragmented mitochondria can subsequently be degraded through mitophagy. This fragmented morphology allows for more efficient engulfment by autolysosome machinery, while the interconnected tubular mitochondrial morphology is protective. Master mitochondrial fission proteins include the dynamin-related GTPase protein 1 (Drp1), the mammalian Drp1 homolog dynamin-like protein 1 (DLP1), and mitochondrial fission 1 protein (Fis1) (Fonseca et al., 2019). As defective mitochondria can be detrimental to the cell, the elimination of the entire or partial mitochondria is essential and mainly mediated via three pathways. First, mitophagy via the selective autophagy lysosomal pathway is initiated to remove the entire organelle either in dysfunction or as superfluous. Second, mitochondrial-derived vesicles (MDVs) are released from mitochondria to target selectively chosen damaged mitochondrial portions to the lysosome for degradation in a mitophagy-independent manner. Third, the proteolytic control of mitochondrial protein misfolding facilitates clearance of misfolded proteins to allow for replacement with newly synthesized polypeptides (Pickles et al., 2018). There are a number of mitophagy mechanisms, and the most well-known is mediated by the familial PD gene-encoded proteins, PINK1 and parkin (Palikaras et al., 2018). PINK1 is localized to the MOM, and in a normal functioning mitochondria, PINK1 is rapidly imported into mitochondria to be cleaved by proteases and further degraded by the ubiquitin proteasome system. During mitochondrial dysfunction, $\Delta\psi m$ is dissipated, protein import is hindered, and PINK1 are stabilized on the MOM (Gladkova et al., 2018). Auto-activated on MOM, PINK1 recruits parkin to MOM and phosphorylate/activates parkin. Parkin then works to ubiquitinate MOM proteins, while PINK1 phosphorylates the ubiquitin (Ub), and poly-Ub chains are formed. The poly-Ub chains mark the damaged mitochondria for degradation and are recognized by the autophagic adaptor proteins (p62, OPTN, NDP52), which bind with microtubule-associated protein 1A/1B-light chain 3 (LC3) on the growing phagophore membrane through LC3-interacting region (LIR) (Runwal et al., 2019). The strip of phagophore encircles the damaged mitochondria and forms a double-membraned autophagosome, which further fuses with lysosomes to facilitate defective mitochondria elimination (Dikic and Elazar, 2018). Another mitochondrial dynamic characteristic is the trafficking of mitochondria, which is essential for energy and Ca^{2+} distribution and is dependent on mitochondrial Rho (Miro) GTPases. Miro 1 and 2 localize to MOM and form complexes with the TRAK adaptors and dynein or kinesin motors to transport mitochondria along microtubules (Modi et al., 2019).

Therefore, the mitochondria are cellular energy providers, damage sensors, Ca^{2+} regulators, and cell death initiators. The versatility of mitochondrial functions relies upon effective communication between the organelle and the entire cellular community. Below, we investigate mitochondrial interactions with other cellular organelles (*Abbreviations* and **Figure 1**).

MITOCHONDRIAL COMMUNICATION WITH THE NUCLEUS

As it has been hypothesized that mitochondria evolved from an independent α-proteobacteria (Degli Esposti, 2014), they retained prokaryotic characteristics such as the double-membraned structure, the ability to aerobically synthesize ATP, and the possession of their own DNA. In evolution, autonomy was lost, and mitochondria became dependent on their host cells; thus, mitochondrial biogenesis and bioenergetics became nuclear

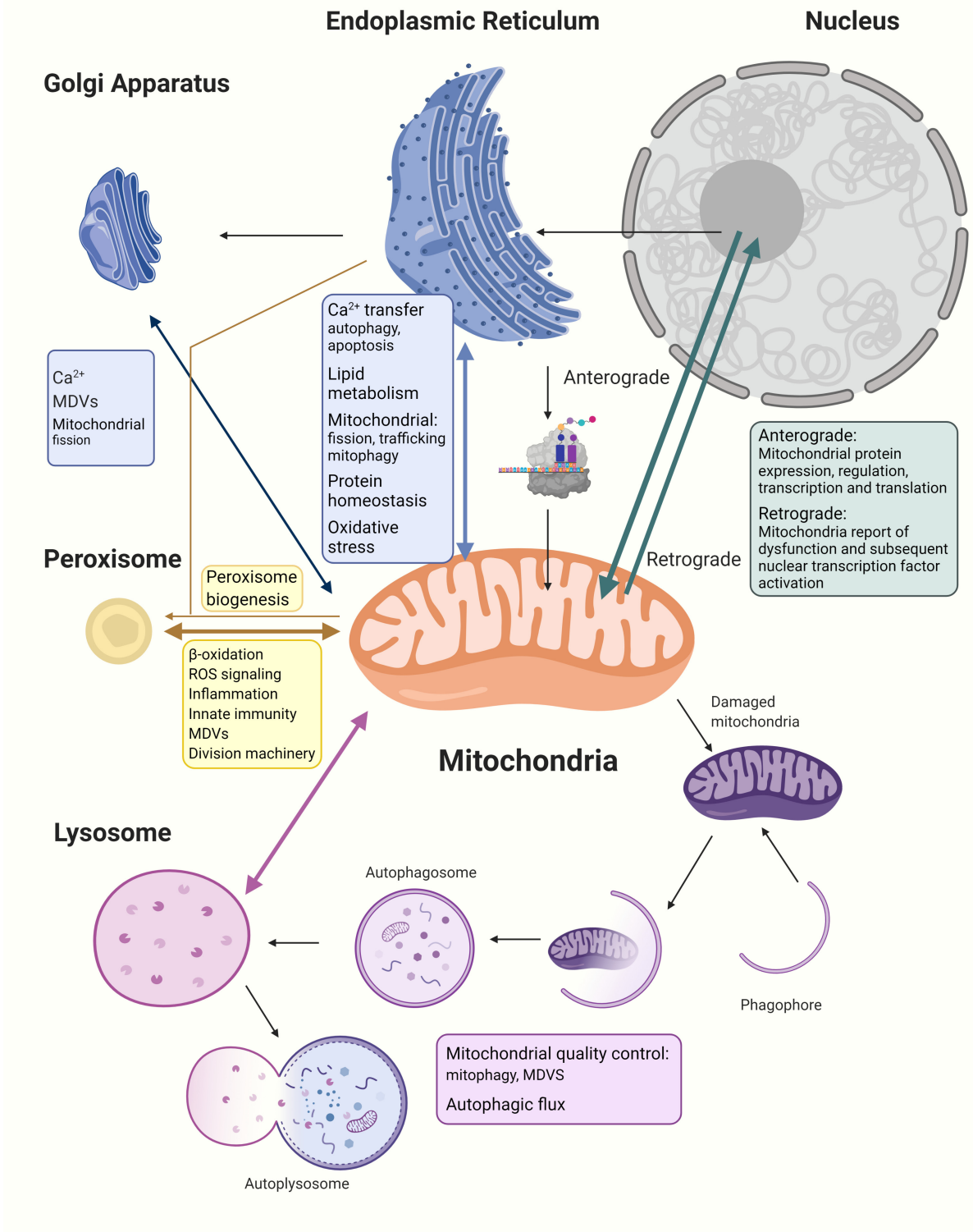

FIGURE 1 | The mitochondria establish networks of communication with other organelles within the cellular community. (1) The nucleus regulates expression of all mitochondrial proteins, including the 99% nucleus-encoded and the remaining mitochondria-encoded 13 peptides manufactured within the mitochondria.

(Continued)

FIGURE 1 | Anterograde signaling are nucleus-controlled genetic expressions of mitochondrial proteins through transcription and nuclear factors to regulate mitochondrial biogenesis, while retrograde signaling pathways allow the stressed organelle to report and regulate nuclear gene transcription to decrease the need for energy and allow repairing of dysfunctional mitochondria. (2) In communicating with the ER, Ca^{2+} and lipids are transferred between the mitochondria and ER, and the MERCs regulate mitochondrial fission, trafficking, and mitophagy; moreover, the two organelles provide feedback to the nucleus about protein homeostasis and oxidative stress. (3) The Golgi apparatus has been shown to participate in intracellular Ca^{2+} transport with mitochondria and ER as well as share cargo proteins with mitochondria possibly to do with MDVs trafficking. There has also been evidence of Golgi participation in late-stage mitochondrial fission. (4) The mitochondria and peroxisomes cooperate in lipid oxidation, signaling of reactive oxidative species, regulation of inflammation, and innate immunity. Division machinery of both organelles are overlapped. Mitochondria are also discovered to be involved in peroxisome biogenesis. (5) Lysosomes govern important parts of mitochondrial quality control including whole mitochondrial degradation through mitophagy and the degradation of mitochondrial proteins through MDVs. Dysfunctional mitochondria are shown to decrease the autophagic flux and lysosome health, while lysosome dysfunction also affects mitochondria morphology and function. The interdependency between different organelles and mitochondria highlight the importance of intricate communications and balance within the cellular community for the maintenance of normal functioning cells.

regulated (Cherry and Piantadosi, 2015). As has been succinctly reviewed by Quirós et al. (2016), the communication between the mitochondria and the nucleus is a requirement not only to coordinate mitochondrial protein synthesis during biogenesis but also to communicate eventual mitochondrial malfunctions, triggering compensatory responses in the nucleus. The majority of mitochondrial proteins required in mitochondrial biogenesis is encoded by nuclear genes, synthesized on cytosolic ribosomes, directed to mitochondria via targeting signals, and imported into the mitochondria through mitochondrial membrane translocase complexes (Pfanner et al., 2019). Therefore, mitochondrial biogenesis is under dual genetic control from both the nucleus and mitochondria; while all factors that activate mitochondria transcription and translation are encoded in the nucleus, it is mainly the nuclear respiratory factor 1 (NRF1) and GA-binding protein-α (GABPα; also known as NRF2α) that regulate mitochondrial transcription and translation in both the nucleus and mitochondria (Gureev et al., 2019). Additionally, there are nuclear receptors such as the peroxisome-proliferator-activated receptors (PPARs) and estrogen-related receptors (ERRs) that activate the expression of nuclear-encoded mitochondrial proteins. Coactivators for stimulating mitochondrial biogenesis include the PPAR-γ coactivator (PGC) family, which are master regulators of mitochondrial biogenesis and play central roles in the coordination and driving of energy metabolism, fatty acid oxidation, gluconeogenesis, peroxisomal remodeling, and oxidative phosphorylation (Scarpulla et al., 2012). Among them, PGC-1α integrates and coordinates the activity of multiple transcription factors, including NRFs, ERRs, and PPARs, and mitochondrial transcription factor A (TFAM), which are all involved in mitochondrial biogenesis (Gleyzer and Scarpulla, 2011). Additionally, NRF1 integrates the mtDNA gene expression through direct control of the expression of important mitochondrial transcription machinery proteins: the mitochondrial RNA polymerase (POLRMT), TFAM, transcription specificity factors (TFB1M and TFB2M), and transcription termination factor (mTERF) (28). These machineries are made in the cytosol, shipped to the mitochondria, and aid in mtDNA transcription, maintenance, replication, and repair.

A delicate balance between nuclear- and mitochondria-encoded mitochondrial proteins is under continuous monitoring for organelle health (Eisenberg-Bord and Schuldiner, 2017). The nucleus controls mitochondrial gene expression and posttranslational modifications, the so-called anterograde signaling, and the mitochondria modulate nuclear gene expression and cellular protein activity through signal transport originating from the mitochondria, termed retrograde signaling. Additionally, in response to mitochondrial stress, the mitochondria can also send extracellular cues known as mitokines to affect nuclear regulation and modulate cellular or organismal homeostasis. For example, a second messenger such as hydrogen peroxide can be utilized by the mitochondria for hypoxia-inducible factor 1 (HIF-1) and NRF2 signaling for adaptive responses (Quirós et al., 2016).

The signaling between the mitochondria and nucleus is crucial for the homeostasis of the intracellular community, especially in response to cellular stress. Tsou *et al.* demonstrated that moderate physical exercise activated NRF2-dependent mitochondrial biogenesis and improved Parkinson's disease symptoms in MPTP models (Tsou et al., 2015). Parkin is also shown to regulate the PGC-1α-mediated transcription of nuclear genes encoding for TFAM, TFB2M, and complex subunits, favoring mitochondrial biogenesis (Shin et al., 2011). The transcriptional activities and the expressions of *PINK1* and Parkin genes are positively regulated by NRF-1 in dopaminergic cell SH-SY5Y, which activate mitochondrial quality control through mitophagy (Lu et al., 2020). NRF2-dependent transcription of the hereditary PD gene *PINK1* has also been identified in helping to save oxidative-stress-induced cell death (Murata et al., 2015). Moreover, NRF2 plays a part in parkin-mediated mitophagy by regulating the expression of p62/SQSTM1, an adapter molecule for linking ubiquitinated cargo directly with the autophagosome (Yang et al., 2019b). The disruption of p62/SQSTM1-dependent mitophagy has been shown to be pathological to PD (Sanchez-Martin and Komatsu, 2018). Thus, multiple signaling components involved in transcriptional adaptations governing the mitochondria and nuclear communication should provide potential targets for salvaging damaged mitochondrial networks and possibly offer therapies for PD (Glaab and Schneider, 2015; Blaudin de The et al., 2016; Kelly et al., 2019).

MITOCHONDRIA, ENDOPLASMIC RETICULUM AND PD

The ER consists of the nuclear envelope and a reticulated interconnected network of tubules and sheets. These ER sheets

are studded with ribosomes and provide the entryway for proteins into the secretory pathway (Salvador-Gallego et al., 2017). ER tubules are observed to move dynamically on microtubules and tether to membranes of other organelles but do not fuse. Communication between ER and mitochondria is essential for the eukaryotic cells to integrate cellular physiology under changing environments. Mitochondria form close physical contacts (15–50 nm) with a specialized domain on the ER membrane, known as the mitochondria-associated membrane (MAM) (Martinvalet, 2018). These contact sites between the two organelles are called the mitochondria–ER contacts (MERCs), and up to 5–20% of mitochondrial surface is distributed in opposition to the smooth or rough ER (Bartok et al., 2019). This association constitutes a key signaling hub to regulate several fundamental cellular processes including lipid metabolism, inflammation, Ca^{2+} signaling, cell survival, autophagy, intracellular motility of both organelles, and protein homeostasis through close physical contacts and protein tethers (Gomez-Suaga et al., 2018; Barodia et al., 2019). Mounting evidence indicates that perturbed ER–mitochondria signaling contributes to many neurodegenerative diseases, including PD.

MERCs Tethers

Several proteinaceous tethers that bind the two organelles together with specific biological functions have recently been identified (**Figure 2**). Among these, one of the most well-known tethers that mediates Ca^{2+} transfer from the ER to mitochondria is the ternary binding complex, composed of the ER Ca^{2+} channel inositol 1,4,5-trisphosphate receptor (IP_3R) and the major MOM Ca^{2+} channel voltage-dependent anion channel isoform 1 (VDAC1), with the mitochondrial molecular chaperone glucose-regulated protein 75 (GRP75) linked between (Leipnitz et al., 2018). Other tethering proteins include the interaction of the ER B-cell receptor-associated protein 31 (Bap31) with the mitochondrial Fis1 protein, which is associated with promotion of apoptosis. Another proposed tethering complex is the homotypic and heterotypic link of ER Mfn2 with MOM Mfn1/2, the functioning of which remains unclear (Filadi et al., 2018). As commonly proposed, groups including Naon et al. confirmed Mfn2 as a bona fide ER–mitochondria tether whose ablation decreases interorganellar juxtaposition and communication (Naon et al., 2016; Basso et al., 2018); meanwhile, groups including Leal et al. and Filadi et al. revealed that Mfn2 knockdown showed a contradictory increase in the ER–mitochondria association (Filadi et al., 2015; Leal et al., 2016). Another important tether is the binding of integral ER protein vesicle-associated membrane protein associated protein B (VAPB) to MOM protein tyrosine phosphatase-interacting protein 51 (PTPIP51), which is associated with Ca^{2+} regulation and autophagy (Gomez-Suaga et al., 2017b). PTPIP51 also binds to other mitochondrial proteins localized in the MAM, the oxysterol-binding protein-related proteins (ORP5/8) (Lee and Min, 2018). The phosphofurin acidic cluster sorting protein 2 (PACS2), a key ER protein required for MAM assembly and activity, has also been suggested to be involved with ER–mitochondria association integrity, influence MAM lipid metabolic enzymes activity, and regulate localization of the ER chaperone calnexin and the Ca^{2+} channel transient receptor potential protein 2 (TRPP2) at the MAMs (Rodriguez-Arribas et al., 2017). In yeast cells, a structural protein complex that connects ER and mitochondria called the ER–mitochondria encounter structure (ERMES), has been reported to contain Mdm12, Mdm34, Mdm10, and Mmm1 proteins, which allow for efficient lipid transport (Lee and Min, 2018). Most ER–mitochondrial tethers are found on smooth ERs; however, Victoria Hung *et al.* identified an interesting MOM protein synaptojanin 2 binding protein (SYNJ2BP) that joins with ER partner ribosome-binding protein 1 (RRBP1), whose overexpression dramatically increased rough ER–mitochondria association (Hung et al., 2017).

Mitochondria and ER Communication Regulating Calcium Transfer and Mitochondrial Dynamics

ER is the major intracellular Ca^{2+} store of cells, while mitochondria shape and decode cellular Ca^{2+} signals by taking up and then releasing Ca^{2+} ions at specific positions throughout the cell (Santo-Domingo and Demaurex, 2010). Ca^{2+} transfer from ER to mitochondria has an important function in regulating cellular health, since basal Ca^{2+} concentration is necessary to maintain mitochondrial ATP and protein production due to three TCA cycle dehydrogenases and FAD-glycerol phosphate dehydrogenase, which are activated by Ca^{2+} (Giorgi et al., 2018). The MERCs are hotspots for Ca^{2+} signaling and are enriched with Ca^{2+} channels such as the IP3R–GRP75–VDAC complex and IP3R-related chaperones (calnexim and calreticulum), the VAPB–PTPIP51 complex, the Mfn complex, while other regulators include the ER Ca^{2+} transport ATPase (SERCA2b) (McDonnell et al., 2019). Ca^{2+} freely passes MOM through the VDAC, while the main control of mitochondrial matrix Ca^{2+} concentration is through the MIM mitochondrial Ca^{2+} uniporter complex (Steffen and Koehler, 2018). However, in the situation of Ca^{2+} transfer blockage, ATP production decreases and autophagy is triggered (Tubbs and Rieusset, 2017). Furthermore, in prolonged Ca^{2+} overload, mitochondrial Ca^{2+} concentration surpasses a threshold, and mitochondrial permeability transition pore (mPTP) opens permanently, leading to dissipation of the $\Delta\psi m$ and subsequent apoptosis (Krols et al., 2016). Thus, mitochondrial Ca^{2+} plays a dual role, and its homeostasis is intimately linked to both cell survival and death (Ilacqua et al., 2017).

Mitochondrial function can also be affected by ER; for example, MAM on ER influences mitochondrial dynamics including fission, trafficking, and mitophagy (Prudent and McBride, 2016). Friedman et al. noted that ER tubules wrap and constrict around mitochondria, which recruit Drp1 to these MERCs and promote MOM fission (Friedman et al., 2011). Later studies by Chakrabarti et al. showed that actin polymerization through ER-bound inverted formin 2 (INF2) stimulated fission of both MIM and MOM via increased mitochondrial matrix Ca^{2+} and Drp1 recruitment, respectively (Chakrabarti et al., 2018). Other mitochondrial fission-involved proteins that recruit Drp1 including mitochondria fission

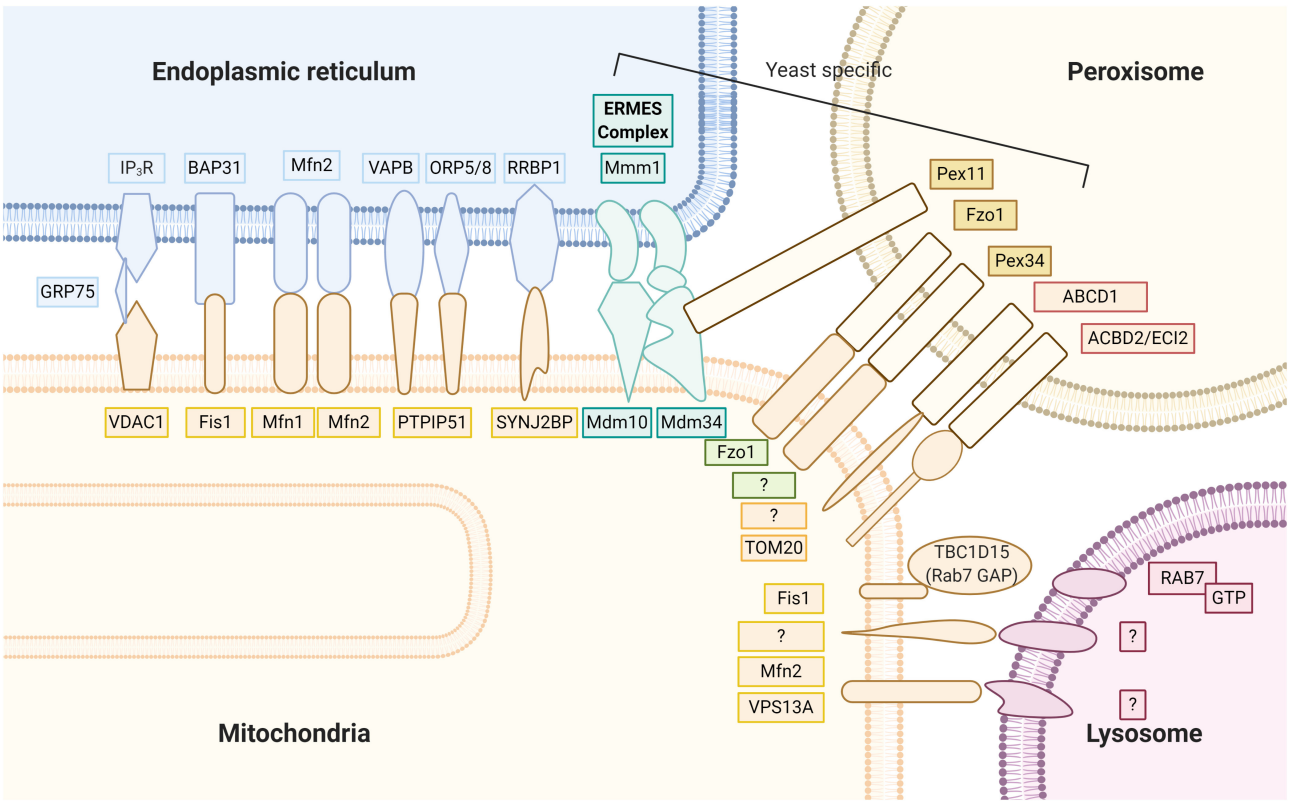

FIGURE 2 | Mitochondria keep close contact with intracellular organelles within the cellular community in order to coordinate different intracellular functions. The MAM of the ER harbor at least seven proteinaceous tethers that bridge to the mitochondria, including (1) the ER Ca^{2+} channel IP3R and the MOM Ca^{2+} channel VDAC1 with the mitochondrial molecular chaperone GRP75 linked between; (2) the ER Bap31 with the mitochondrial Fis 1; (3) the ER Mfn2 with MOM Mfn1/2; (4) the integral ER protein VAPB to MOM protein PTPIP51; (5) the ER ORP5/8 also binds to mitochondrial protein PTPIP51; (6) the ER RRBP1 partners with MOM protein SYNJ2BP; (7) the yeast cell protein complexes containing Mdm12, Mdm34, Mdm10, and Mmm1 proteins that connect ER and mitochondria, called the ER–mitochondria encounter structure (ERMES). Peroxisomes partner with the mitochondria through (1) yeast Pex 11 to ERMES Mdm34; (2) yeast mitofusin homolog, Fzo1 on both peroxisome and MOM; (3) yeast Pex 34 on peroxisomes to unknown MOM partner; (4) mammalian ABCD1 on the peroxisomal membrane, whose loss of function causes X-linked adrenoleukodystrophy, is a peroxisome-mitochondria tether; (5) mammalian ACBD2/ECI isoform A with mitochondrial import receptor subunit TOM20. Mitochondrial contact with lysosomes was recently noted; however, exact tethers are unclear. Studies have clarified a major contact tethering promoter, lysosomal GTP-bound Rab7. Conversely, recruitment of cytosolic TBC1D15 (Rab7 GAP) to the mitochondria outer membrane via Fis1 leads to hydrolysis of GTP-bound Rab7. The resultant GDP-bound Rab7 dislodges from the lysosomal membrane and causes mitochondria and lysosome untethering. At the interface between the mitochondria and lysosome, studies have suggested MOM proteins VPS13A and Mfn2 to play functional roles.

factor (Mff), Fis1, and syntaxin 17 are also localized to the MERCs (Arasaki et al., 2015). For cells under high energy demand such as neurons, being the primary generators of ATP, the mitochondria are dynamically transported intracellularly, presumably for appropriate distribution to cellular regions of high metabolic demand and elevated intracellular calcium. ER has been found to play a role in mitochondrial trafficking. Mitochondrial trafficking along microtubules relies on the Miro GTPases, which localize to MOM and form complexes with the TRAK adaptors and dynein/kinesin motors (Murley et al., 2013). These molecular machineries are tightly regulated by Ca^{2+} sensors, and increased Ca^{2+} levels have been shown to slow down or stop mitochondrial motility, which is reversible by the return of Ca^{2+} concentrations to a normal range. Souvik et al. have shown Miro clusters to increase MERCs, and other reports also have shown Miro regulation of the IP3R–GRP75–VDAC complexes for Ca^{2+} transfer (Lee et al., 2018; Modi et al., 2019). Under pathological conditions, high levels of Ca^{2+} in mitochondria have been shown to reduce both ER and mitochondrial trafficking and tighten the ER–mitochondria alignment, causing mitochondrial arrest (Grimm, 2012; Lee et al., 2016). This arrest will enhance Ca^{2+} transfer from ER and induce mitochondrial fragmentation in order to remove damaged organelles through mitophagy, initiated with membranous structures contributed by nearby MERCs (Jeyaraju et al., 2009; Hom et al., 2010; Steffen and Koehler, 2018). The VAPB–PTPIP51 complex regulate autophagy by tightening the MERCs and affecting Ca^{2+} transfer. Knockdown of either VAPB or PTPIP51 loosens the MERCs and stimulates autophagosome formation, whereas overexpression of either inhibits autophagosome formation (Gomez-Suaga et al., 2017a). Hamasaki et al. demonstrated in mammalian cells that pre-autophagosome/autophagosome marker ATG14 (also known as ATG14L) relocalizes to the MERCs after starvation, and the

autophagosome-formation marker ATG5 also localizes at the MERCs until autophagosome formation is complete (Hamasaki et al., 2013). They also showed that disruption of the ER–mitochondria contact site prevents the formation of ATG14 puncta, emphasizing the importance of MERCs during early stages of autophagy (Dikic and Elazar, 2018). The implication of MERCs in mitophagy includes the ubiquitination-mediated PINK1 and parkin-related pathway and the mitophagy receptor-mediated pathway through the FUN14 domain containing 1 (FUNDC1). Wu et al. demonstrated that, under hypoxic conditions, FUNDC1 initiates mitophagy at the MERCs via interaction with calnexin and recruitment of upstream autophagy protein Unc-51-like kinase 1 (ULK1) (Wu et al., 2014). Wu et al. also observed FUNDC1 mediation of mitochondrial fission in hypoxia by binding to Drp1 (Wu et al., 2016). An interesting soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein syntaxin 17 has been shown to play multiple roles between the mitochondria, ER, Golgi, and lysosome. Syntaxin 17 localizes at the MERCs to promote mitochondrial fission by recruitment of Drp1 (Arasaki et al., 2015). During starvation, syntaxin 17 translocalizes to MERCs and binds and recruits ATG14 to initiate the formation of phagophores (Hamasaki et al., 2013; Wang et al., 2019). In the late stage of autophagy, syntaxin 17 present on autophagosomes, mediating the fusion of autophagosomes with lysosomes (Arasaki et al., 2017). Xian et al. identified the syntaxin 17–Fis1 interaction control of syntaxin 17 shuttling between mitochondria and ER and that Fis1 loss results in aberrant syntaxin 17 accumulation in the mitochondria, further triggering mitophagy (Xian et al., 2019). Another response noted concerning MERCs is the unfolded protein response (UPR)-ER, which is triggered by ER stress and accumulation of misfolded proteins aiming to maintain cellular proteostasis (Hetz and Papa, 2018; Kopp et al., 2019). Many UPR-ER-related chaperones are found in the MAM, and the disruption of ER–mitochondrial signaling by the array of tethering proteins can induce UPR-ER including PACS2, Mfn2, and VAPB (Paillusson et al., 2016; Chu et al., 2019).

The Role of MERCs in PD

The role of MERCs in PD involves Ca^{2+} regulation, proteostasis, and several familial PD-related proteins (Kazlauskaite and Muqit, 2015; Basso et al., 2018). The α-syn is a well-known PD pathogenic protein and has been observed in membrane compartments of synaptic vesicles, mitochondria, and ER in neurons. α-Syn is enriched in the MAMs, and Cali et al. have reported that overexpression of either wild-type or familial mutant α-syn decreases ER–mitochondria communication, damages Ca^{2+} homeostasis, induces mitochondrial fragmentation, and augments autophagy (Cali et al., 2012). Supporting this, Paillusson *et al.* used structured illumination and electron microscopy to quantify MERCs and revealed that α-syn binds to VAPB and that overexpression of wild-type or mutant α-syn disrupts the VAPB-PTPIP51 tethers to loosen ER–mitochondria associations and further affect Ca^{2+} signaling (Paillusson et al., 2017). The autosomal recessive PD risk genes *PINK1* and *parkin* help to maintain mitochondrial health through several mitochondrial quality control mechanisms: the turnover of MOM proteins by the proteasome, the generation of mitochondrial-derived vesicles, and whole-organellar degradation by mitophagy. More recently, PINK1 and parkin have been found to localize at MERCs to modulate organellar crosstalk. At MERCs, the PINK1/parkin-dependent mitophagy pathway is involved in disturbed Ca^{2+} transferring, mitochondrial fragmentation, trapped mitochondrial movement, loosening ER/mitochondrial contact, recruitment of autophagic machinery to dysfunctional mitochondria, and acquirement of membrane components from the ER for autophagosome membrane formation (Amadoro et al., 2014; Barazzuol et al., 2020). The importance of mitochondria–ER association in the initiation stage of autophagosome has been revealed by Yang et al., such that parkin-mediated mitophagy recruits autophagosome precursors to the MERCs at damaged mitochondria and LC3-marked autophagic structures emerge from the ER–mitochondria contact sites (Yang and Yang, 2013). Gelmetti et al. noted PINK1 and parkin to relocalize at MERCs, where autophagosome originate, and that *PINK1* silencing impaired pro-autophagic protein enrichment at MERCs (Gelmetti et al., 2017). At the start of PINK1/parkin-dependent mitophagy, MERCs-localized Mfn2 is phosphorylated by PINK1, and the phosphorylated Mfn2 recruits parkin, which ubiquitinates Mfn2. Accumulation of ubiquitinated Mfn2 on the MOM acts as a signal to mark damaged mitochondria and initiates mitophagy (Bockler and Westermann, 2014). After mitophagy initiation, mitochondria–ER contacts are dissociated, as PINK1/parkin catalyzes Mfn2 to dissemble Mfn2 from the MOM (Basso et al., 2018). The loosening of ER/mitochondrial contact in mitophagy pathways has been shown to increase the rate of mitochondrial degradation, and McLelland et al. demonstrated that MERCs tethers suppress mitophagy (McLelland et al., 2018). Parkin has also been shown to accumulate at MERCs to modulate ER–mitochondrial crosstalk, and overexpression or silencing of *parkin* is correlated to Ca^{2+} dyshomeostasis (Cali et al., 2013; Gautier et al., 2016). In *Drosophila* models of PD, mutations in *parkin* and *PINK1* induce ER stress through activating PERK (Celardo et al., 2016). Another PD recessive risk gene is *PARK7*, encoding the DJ-1 protein that may be important in both sporadic and familial PD (Blackinton et al., 2009). In most contexts, DJ-1 plays multiple protective roles in cells as redox sensors, antioxidants, chaperone with protease activity, and transcription regulator. Liu *et al.* reported that DJ-1 physically interacts with and is an essential component of the IP3R3–Grp75–VDAC1 complexes at MAM. Loss of DJ-1 disrupted the IP3R3–Grp75–VDAC1 complex and led to loosened ER–mitochondria association and disturbed function of MAM and mitochondria in neuronal cells and *in vivo* (Liu et al., 2019b). Although DJ-1 is often suggested to be protective, Yang et al. noted that deficiency of DJ-1 ameliorates death in the context of acute ER stress *in vitro* and *in vivo*. By contrast, overexpression of wild-type and PD-associated pathogenic DJ-1 mutant forms of PARK7 L166P enhance ER-stress-induced neuronal death by regulating activating

transcription factor 4 (ATF4) transcription and translation (Yang et al., 2019a).

The Golgi Apparatus, ER, Mitochondria, and PD

Working closely with ER, the main function of the Golgi apparatus is to modify and package proteins and carbohydrates into membrane-bound vesicles and dispatch these cargoes for exportation. The Golgi apparatus and mitochondria also communicate with each other by physical interaction. The existence of Ca^{2+} gradients from the Golgi apparatus to mitochondria has been discovered. Structurally, apposition of the Golgi apparatus and mitochondria has been demonstrated by microscopy techniques; however, the molecular features of this interaction in PD pathophysiology remain poorly understood (Valm et al., 2017). The involvement of ER/Golgi in the α-syn pathology has been further elucidated recently by Paiva *et al.* who reported that PD pathogenic A30P mutant α-syn causes alteration of Golgi morphology and increases the susceptibility of dopaminergic neurons to ER stress (Paiva et al., 2018). It has also been observed that overexpression of PD pathogenic A53T mutant α-syn delays ER–Golgi transport by up to 50% via inhibiting ER/Golgi vesicle fusion-related SNARE complex assembly (Thayanidhi et al., 2010). The SNARE protein, syntaxin 17, assists in vesicle fusion and is also a receptor at the ER membrane that mediates trafficking between the ER and ER–Golgi intermediate compartment (ERGIC) as well as localizes to MERCs for mitochondrial fission (McLelland et al., 2016; Sugo et al., 2018). The interesting interorganelle communication of MDVs are a means of vesicular transport of selectively incorporated protein cargoes from the mitochondria to other subcellular compartments, mainly lysosomes or peroxisomes, thereby transferring mitochondrial proteins to these organelles (Sugiura et al., 2014; Giacomello et al., 2020). The finding that syntaxin 17 participates in PINK1/parkin-dependent MDVs fusion within the endolysosomal compartments suggests a role of ER/Golgi interorganelle communication in mitochondrial dynamic control (Muppirala et al., 2011; Arasaki et al., 2015). Another participator of MDVs transport is the vacuolar protein sorting-associated protein 35 (VPS35), a gene product of autosomal dominant late-onset PD gene *VSP35*, which is involved in retrograde transport of proteins from endosomes to the trans-Golgi network (Yun et al., 2017). This retromer complex is also found to regulate MDVs cargo transport from the mitochondria to peroxisomes (Grunewald et al., 2019). More recently, there has been the discovery of Golgi apparatus involvement in late-stage mitochondrial fission. Nagashima et al. documented that microdomains of phosphatidylinositol 4-phosphate [PI(4)P] on trans-Golgi network vesicles were recruited to MERCs during late stage of mitochondrial division, the loss of which impeded fission, causing extended mitochondrial constriction sites with hyperfused and enlarged mega-mitochondria. This indicated that these Golgi-derived vesicles may drive the final events of mitochondrial division downstream of Drp1, leading to mitochondrial scission (Nagashima et al., 2020).

MITOCHONDRIA, PEROXISOMES, AND PD

Peroxisomes are ubiquitous, single membraned cellular organelles that do not contain DNA or RNA. They perform important roles in biosynthesis and signal transduction, including phospholipid biosynthesis, fatty acid α- and β-oxidation, bile acid and docosahexaenoic acid synthesis, glyoxylate metabolism, amino acid catabolism, polyamine oxidation, the metabolism of reactive oxygen and nitrogen species, inflammation, and innate immunity (Islinger et al., 2018). Peroxisomes are dynamic, interconnected, and actively contribute to signaling, developmental decisions, aging, and defense against pathogens. To exert these activities, peroxisomes must interact both functionally and physically with other cellular organelles. Numbers, morphology, and activity are modulated to adapt to diverse environments in different tissues, organs, and nutritional states (Farre et al., 2019).

The crosstalk between mitochondria and peroxisomes is essential for several metabolic processes including redox-(ROS scavenging), lipid-(β-oxidation), inflammatory-, and innate immune-(antiviral responses) signaling networks (Fransen et al., 2017). The efficient exchange of molecules between mitochondria and peroxisomes are poorly understood but are likely to involve shuttle mechanisms such as the carnitine system, membrane pores, vesicle transport, and contact sites, which have been shown to localize at MERCs (Giacomello et al., 2020). The existence of peroxisome–mitochondria contact sites was confirmed by bimolecular fluorescence complementation (BiFC), and Shai et al. discovered yeast peroxisome–mitochondria contact site tether proteins: Fzo1 (yeast mitofusin protein) and peroxin (Pex) 34 (Shai et al., 2018; Farre et al., 2019). A handful of other tethers between peroxisomes and mitochondria have also been discovered, including peroxisome protein Pex11 to mitochondrial Mdm34, an ERMES component in yeast; mammalian peroxisome ATP-binding cassette subfamily D member 1 (ABCD1); and enoyl-CoA delta isomerase 2 (ECI2, also known as ACBD2) isoform A in mammals, which is linked to translocase of outer mitochondrial membrane 20 (TOM20) (Fan et al., 2016; Giacomello et al., 2020) (**Figure 2**). The passage of molecules through the peroxisome membrane is achieved through nonselective channels such as mammalian peroxisomal membrane protein 2 (PXMP2). Solutes with molecular masses smaller than 300 Da have free transmembrane movement, while larger molecules such as fatty acids, acetyl-CoA, and ATP pass through specific transporter proteins (Theodoulou et al., 2013). The integrity and stability of peroxisomes are important for the maintenance of normal mitochondrial function. Peroxisome dysfunction seriously affects mitochondrial metabolism, morphological stability, and biosynthesis, which directly or indirectly lead to rare genetic diseases, such as X-linked adrenoleukodystrophy (X-ALD), acatalasemia, and Zellweger syndrome, and age-related disorders such as PD (Muntau et al., 2000; Pascual-Ahuir et al., 2017; Uzor et al., 2020).

A Role for Mitochondria in Peroxisome Biogenesis

A characteristic feature of peroxisomes is that they proliferate, change internal enzymes, and dissipate in response to external

cues; the subsequent degradation, once they are excessive or non-functional, is through selective autophagy called pexophagy (Cho et al., 2018). The biogenesis of peroxisomes implicates important peroxisome biogenesis proteins known as peroxins, which modulate import of peroxisome matrix proteins and help target peroxisomal membrane proteins to the peroxisome membrane. The proteins destined for peroxisomes possess peroxisome targeting signals (PTS1 and PTS2) and are translated on free cytosolic ribosomes and then transported directly or indirectly into peroxisomes as completed polypeptide chains (Kim and Hettema, 2015). Two main models of peroxisome biogenesis in mammals have been proposed: one is by growth and division of existing peroxisomes (South et al., 2000); the other is *de novo* peroxisome biogenesis by the insertion of peroxisome membrane proteins into a specific region of ER or mitochondria membrane, which later buds to form pre-peroxisomal vesicles. These pre-peroxisomal vesicles containing different subsets of peroxisomal membrane proteins then fuse and mature into peroxisomes (Farre et al., 2019). In either peroxisomal biogenesis or growth, there are two proposed routes for peroxisomal membrane protein insertion into peroxisomes: one is through direct insertion of peroxisomal membrane proteins into membranes of pre-existing peroxisomes, and the other involves indirect trafficking of peroxisomal membrane proteins via ER/mitochondria followed by their subsequent sorting into peroxisomes (Agrawal and Subramani, 2016). In support of mitochondrial participation in peroxisome biogenesis, experiments to observe *de novo* peroxisome biogenesis were performed on human fibroblasts lacking peroxisome, revealing that integral peroxisomal membrane proteins Pex3, Pex12, Pex13, Pex14, Pex26, PMP34, and ALDP were imported into mitochondria at the beginning of peroxisome biogenesis (Halbach et al., 2006; Kim et al., 2006). These mitochondrial-derived pre-peroxisomal vesicles then fuse with ER-derived pre-peroxisomal vesicles to form newly born peroxisome, as reported by McBride *et al.* who used a human fibroblast cell line lacking Pex3 obtained from a patient with Zellweger syndrome (Sugiura et al., 2017). The fusion of mitochondria-derived pre-peroxisomal vesicles in peroxisomal formation allows transfer of functional proteins, which may be a reason for the similar functions of the peroxisomes and mitochondria (Schrader and Pellegrini, 2017).

Brotherly Traits Between Peroxisomes and Mitochondria

Consistency amid diversity is observed between mitochondria and peroxisomes (Chipuk and Luna-Vargas, 2017). The two share biogenesis transcriptional programs, which are both triggered by PPAR-γ and its coactivator PGC1-α, including genes involved in peroxisomal β-oxidation. In addition, peroxisomal and mitochondrial membranes share the same division machinery, including Drp1. While mitochondria have Fis1 and MFF as Drp1 receptors, peroxisomes have also their own specific Drp1 receptors, Pex11 (Otera et al., 2013; Shai et al., 2016). However, although peroxisomes and mitochondria share a dynamic nature in fission, trafficking, and degradation, unlike mitochondria, mature peroxisomes cannot fuse with one another (Schrader and Pellegrini, 2017). The localization of proapoptotic regulator protein BCL2-antagonist/killer (BAK) has been found in both MOM and peroxisome membranes, modulating membrane permeability and peroxisomal enzyme deficiency (Chipuk and Luna-Vargas, 2017; Hosoi et al., 2017).

Another well-known example of cooperation between these two organelles is the β-oxidation of fatty acids. Mitochondria and peroxisomes each own a distinct set of substrate-specific enzymes with peroxisomes only able to shorten, but not completely degrade, the fatty acid chains; while mitochondria are able to β-oxidize down to H_2O and CO_2 (Poirier et al., 2006). Thus, dietary and very long chain fatty acids target peroxisomes for β-oxidation, and the medium chain fatty acids metabolites as well as acetyl-CoA are guided to the mitochondria where further oxidation and ATP production in the TCA cycle take place (Reddy and Hashimoto, 2001).

Evidence has shown that mitochondria and peroxisomes are sophisticated redox signaling hubs, and most of the cellular redox reactions occur within mitochondria, peroxisomes, and ER, which are the main generators of H_2O_2 and other ROS (Lismont et al., 2015). Redox-regulatory enzymes are thought to assemble at a "redox triangle" formed by the three organelles, assembling "redoxosomes" that sense ROS accumulations and redox imbalances (Yoboue et al., 2018). Each organelle harbor their own antioxidative system (Fransen et al., 2012, 2017).

Importance of Peroxisomal Health on Mitochondrial Integrity

Normal peroxisomal function is crucial for maintaining the health of the mitochondrial network. Multiple studies have shown deficiencies in peroxisome to damage mitochondrial integrity including morphology, mitochondrial proteostasis, redox balance, mitochondrial biogenesis, and even leading to cell death (Walton and Pizzitelli, 2012; Lismont et al., 2015; Schrader et al., 2015; Cipolla and Lodhi, 2017). Supporting this, increased mitochondrial protein oxidative damage and impairment of mitochondrial OXPHOS was observed by Lopez et al. in the spinal cord of mice with inactivated ABCD1, a peroxisome very-long-chain-fatty-acid transporter causative for X-ALD. The very long chain fatty acid substrate accumulates in the cytosol due to hindered import into the peroxisome for degradation and leads to progressive demyelination/neurodegeneration in the central nervous system (Lopez-Erauskin et al., 2013). Wang et al. demonstrated that excessive ROS production by peroxisomes led to mitochondria-induced cell death in Pex19p-deficient human fibroblasts and that this process may be countered by targeted overexpression of select antioxidant enzymes: peroxisomal glutathione S-transferase Kappa 1, SOD1, and mitochondrial catalase (Wang et al., 2013). Similarly, Peeters et al. demonstrated disruption of peroxisome biogenesis in hepatocyte-selective Pex5 knockout mice to damage MIM, deplete mtDNA, reduce or incomplete respiratory chain complexes I, III, and V, increase oxidative stress, increase mitochondrial membranes permeability and fluidity, and increase mitochondrial biogenesis (Peeters et al., 2015; Tanaka et al., 2019). Both peroxisome and mitochondrial dysfunction and dysregulated ROS balance is important in the occurrence of age-related disease (Cipolla and Lodhi, 2017). Several reports support that peroxisomal function progressively declines during aging and that, in cultured human cells catalase,

is observed to be increasingly excluded from peroxisomes after repeated cell passage, thereby impeding the breakdown of H_2O_2. Concurrently, old cells accumulate old peroxisomes, increasing the ROS burden and ultimately may accelerate aging (Giordano and Terlecky, 2012; Pascual-Ahuir et al., 2017). Koepke et al. demonstrated that altering the catalase peroxisome targeting signal to the more effective serine–lysine–leucine (SKL) sequence results in a catalase molecule that more strongly interacts with its receptor and is more efficiently imported in both *in vitro* and *in vivo* assays. The catalase-SKL stably expressed in cells was able to repolarize mitochondria and reduce the number of senescent cells in a model of late-passage human fibroblast cell cultures, which may provide a potential strategy for rejuvenation (Koepke et al., 2007). Nell et al. showed genetically engineered derivative of the peroxisomal antioxidant enzyme catalase CAT-SKL usage to reduce neuroinflammation and long-term reference memory deficits induced by beta-amyloid in the mature rat brain (Nell et al., 2017).

Peroxisomes and Mitochondria Relationship With PD

Peroxisomes have shown interdependency with mitochondria, and experiments have been conducted to investigate possible connections between peroxisomes and PD (Lazarou et al., 2012; Uzor et al., 2020). Lazarou et al. reported PD-related PINK1 and parkin ectopic localization to peroxisomes where they initiated pexophagy (Lazarou et al., 2012). Potential effects of peroxisomal dysfunction on α-syn-related pathogenesis were demonstrated on inactivated Pex2–/–, Pex5–/–, and Pex13–/– mouse models presenting increased α-syn oligomerization and deposition in cytoplasmic inclusions. Yakunin et al. further showed that α-syn abnormalities correlate with the altered lipid metabolism and specifically, with accumulation of long chain, n-6 polyunsaturated fatty acids, which occurs in peroxisome biogenesis dysfunctional models (Yakunin et al., 2010). These data demonstrate a role of peroxisomes in the prevention of α-syn aggregation, a pathological hallmark of PD. The mitochondria are integrated in the cellular endolysosomal system via MDVs, which transfers mitochondrial proteins and lipids by fusing with peroxisomes, endosomes, and lysosomes. The late-onset familial PD gene, *VPS35*, encodes a key component of the retromer complex for cellular protein trafficking, which also participates in generation and intracellular trafficking of MDVs to lysosomes (Braschi et al., 2010). Taken together, these findings support peroxisome participation in mitochondrial morphology dynamics, PINK1/parkin autophagic pathways, and cooperation with the mitochondria in shared stress responses such as ROS balancing in the pathogenesis of PD with aging.

MITOCHONDRIA AND LYSOSOMES AND PD

Quality control of damaged mitochondria conducted by lysosomes is essential for maintaining normal mitochondrial functions. Lysosomes are single membrane-enclosed organelles that govern terminal degradation and act as sopisticated signaling centers of growth, division, and differentiation (Lim and Zoncu, 2016). They contain an array of ∼50 acid hydrolases capable of breaking down all types of biological polymers—proteins, nucleic acids, carbohydrates, and lipids (Lawrence and Zoncu, 2019). Lysosomal hydrolases are activated under an acidic enviroment and rely upon vacuolar ATPase (V-ATPase) pumps in the lysosomal membrane to actively transfer protons into the lysosome, maintaning a pH of ∼5. With the participation of hydrolases, lysosomes digest large molecules through autophagy and pass the fragments on to other parts of the cell for recycling. As the degradative endpoint for intracellular and exogenous biomacromolecules, these cellular quality controllers continuously fuse and fission with each other as well as other organelles, including late endosomes, phagosomes, and autophagosomes for autolysosome formation (Wong and Cuervo, 2010). The chemistry between mitochondria and lysosomes facilitate sensing the availability of nutrients and energy, coordinating anabolic and catabolic processes, as well as coping with cellular stress in autophagy, proliferation, and cell death (Raimundo et al., 2016). The means of communication between the two organelles include signaling pathways, MDVs, fusion with damaged mitochondria for degradation, and "kiss and go" membrane contacts dynamically formed with healthy mitochondria. The formation of these physical contact sites are promoted by lysosomal active GTP-bound Rab7 and are dissociated by deactivated Rab7 GTP via Rab7 GTPase-activating protein TBC1D15 (Rab7 GAP); however, the tethers that bridge the contact sites in mammals are as yet unclear (Wong et al., 2018) (**Figure 2**).

Mitochondria Quality Control and Lysosomes

The most well-known interaction between mitochondria and lysosomes is in the selective autophagy of mitochondria, called mitophagy. This housekeeping mechanism of the mitochondrial network is critical for mantaining efficient working organelles within the cell and prevent excessive production of ROS from malfunctioning organelles. Autophagy is a process in which lysosomes degrade unneeded or damaged large molecules in cells through segregation in a double-membraned vesicle and lysosomal fusion with the vesicle enabling hydrolase function. There are three types of autophagy, which include microautophagy, chaperone-mediated autophagy, and the most common macroautophagy. Henceforth, we refer to macroautophagy as autophagy, which is also the process involved in mitophagy. Steps of autophagy include initiation, nucleation, elongation, lysosomal fusion, and degradation. The initiation stage involves activating ULK1 complex, which phosphorylates the class III phosphatidylinositol 3-kinase complex I (PI3KC3-C1) (Feng et al., 2019). The PI3KC3-C1 assists phosphatidylinositol 3-phosphate [PI(3)P] production at the isolation membrane of the ER, from which transient double-membraned phagophores are formed (Hurley and Young, 2017). Downstream proteins and complexes work to enhance autophagy-related proteins ATG8-family proteins (ATG8s) binding to the phosphatidylethanolamine (PE) on

the membrane. ATG8s [including the microtubule-associated protein light chain 3 (LC3) proteins] assist in the nucleation process, which recruits LC3-interacting-region (LIR)-motif-bearing autophagy factors, and selectively sequester specifically tagged cargo via LIR cargo receptors (Wirth et al., 2019). While ATG8s also facilitate elongation and closure of the phagophore membrane to form the autophagosome, the insertion of lipidated-ATG8s to the autophagosome membrane drives autophagosome maturation (Johansen and Lamark, 2019). Thus, the binding of lipidated ATG8s family member LC3-II to the autophagic membrane is suggested to be a signature characteristic of autophagic activation (Wild et al., 2014). The elongation of autophagosomal membranes is facilitated by ATG9-containing vesicles bringing lipid bilayers from the plasma membrane, the mitochondria, recycling endosomes, and the Golgi complex. Autophagosomes are trafficked to and fuse with a lysosome, forming an autolysosome, and trapped cargo is subsequently degraded (Klionsky et al., 2014). This, therefore, is the well-known incinerating function of lysosomes in cellular degradation; the elimination of perturbed organelles via selective autophagy in the case of mitochondria is mitophagy (Raimundo et al., 2016). The warning signal for damaged mitochondria is membrane depolarization, after which MOM proteins are ubiquitinated, mitophagy receptors are recruited, autophagosomes encircle the damaged mitochondria, and finally fuse to lysosomes for degradation (Palikaras et al., 2018; Liu et al., 2019a). A second type of mitochondrial quality control involving lysosomes is the excision of MDVs from the mitochondria, which fuse with lysosomes to degrade damaged parts of mitochondria (Sugiura et al., 2014). Both mitochondria and lysosomes are self-aware of their functional status and constantly relay organelle conditions and stresses to the rest of the cell (Tai et al., 2017). Lysosomes achieve this by retaining Ca^{2+}, iron, cholestrol, or sphingomyelin within the lysosomal lumen (Audano et al., 2018). The mitochondria do this by mediating Ca^{2+} uptake, ROS signaling, slowing protein input, effluxing peptides, vesiclular signaling, and regulating mtDNA expression (Haynes et al., 2010; Lin and Haynes, 2016; Shpilka and Haynes, 2018). Apart from initiation, the termination of stress responses is especially important to prevent extreme reactions that could lead to irreversible damage. When stress signals elevate over a threshold, housekeeping mechanisms may initiate the removal of the damaged organelle (Palikaras et al., 2018).

Functional Crosstalk Between Lysosomes and Mitochondria

Intimate functional connections between the mitochondria and lysosomes are demonstrated by investigating the interactions between these two organelles. First, the removal of entirely damaged mitochondria through mitophagy involves autolysosome formation. Second, MDVs released from the mitochondria in response to acute mitochondrial damage are targeted for digestion to lysosomes. MDVs harbor selectively chosen mitochondrial proteins and detach from the mitochondria independently of Drp1 but require proteins that affect mitochondrial dynamics, such as parkin and PINK1 (Sugiura et al., 2014). Third, damaged proteins and lipids are compartmentalized in a specific area of mitochondria called mitochondria-derived compartments (MDCs) and are released involving Drp1, the mitochondrial fission machinery, to be degraded in the lysosome (Hughes et al., 2016). MDCs differ from MDVs in protein composition and have only been detected under chronic conditions, such as aging. In addition, MDCs primarily function in selective cargo degradation, while MDVs also play a role in protein exchange. Lastly, direct physical contacts are formed between these two organelles via membrane contact sites, allowing for the exchange of lipids and metabolites between these compartments (Giacomello et al., 2020).

Dysfunctional mitochondria have an impact on lysosomes (Demers-Lamarche et al., 2016; Fernandez-Mosquera et al., 2019). For example, knockout of the mitochondrial apoptosis-inducible factor (AIFM1) required for the assembly of respiratory chains leads to lysosomal impairment, evidenced by enlargement of specific lysosomal vesicles that become non-acidic and lose their hydrolytic activity (Demers-Lamarche et al., 2016). In reaction to imminent danger and to chronic stress, the two organelles collaborate on the modulation of intracellular metabolic processes via two major opposing metabolic regulators: the AMP-dependent protein kinase (AMPK) and mechanistic target of rapamycin complex 1 (mTORC1) signaling (Rabanal-Ruiz and Korolchuk, 2018). AMPK is the regulator that activates key catabolic pathways for the generation of ATP and is activated in response to low AMP/ATP, such as in a starvation condition (Mihaylova and Shaw, 2011). Meanwhile, mTORC1 is the kinase that coordinates most anabolic pathways, promoting synthesis of proteins, cholestrol, and nucleotides for cell growth and proliferation (Fernandez-Mosquera et al., 2019). Studies have revealed lysosomal biogenesis to increase in acute mitochondrial stress but not in chronic mitochondrial stress. In response to acute mitochondrial stress, AMPK is activated, which increases fission of damaged mitochondria, induces autophagosome formation through ULK1 and ULK2, increases lysosomal biogenesis through transcription factor EB (TFEB) and microphthalmia-associated transcription factor (MITF), leading to eventual mitophagy and increased autophagic flux (Nezich et al., 2015; Carroll and Dunlop, 2017). Under chronic mitochondrial stress, AMPK signaling is shut down, and TFEB returns to basal levels (Fernandez-Mosquera et al., 2017). This suggests that under chronic mitochondrial stress, a cellular protective mechanism is activated to stop mitochondrial cleanup, and the cell chooses to live with inefficient mitochondrial rather than without (Fernandez-Mosquera et al., 2019). AMPK also plays an important part in Ca^{2+} release from the lysosome into the cytoplasm by increasing the channel mucolipin-1 (MCOLN1), a key Ca^{2+}-conducting channel on the lysosomal membrane that is essential for lysosomal biogenesis and autophagy (Medina et al., 2015). Elevated ROS production caused by mitochondrial dysfunction triggers AMPK activation with subsequent MCOLN1 activity stimulation, increased lysosomal activity, and potentially enhancing cellular capability to turn over damaged mitochondria (Zhang et al., 2016).

Genetic Evidence of Lysosome, Mitochondria, and PD Connections

Recent genetic studies have revealed that mutations in the glucocerebrosidase (GBA1) gene cause Gaucher disease (GD), the most common lysosomal storage disorder, and increase susceptibility to PD and α-syn pathology (Klein and Mazzulli, 2018). The lysosomal storage diseases are caused by loss-of-function variants in genes that encode lysosome-digesting enzymes, leading to lysosomal dysfunction and consequential intralysosomal buildup of undegraded substrates (so-called "storage") (Li et al., 2019a). The participation of lysosomal dysfunction on PD pathogenesis was first suggested in the recessively inherited lysosomal storage disorder, GD (Klein and Mazzulli, 2018). GD patients harbor homozygous mutations in the GBA1 gene, which encodes the lysosomal hydroxylase β-glucocerebrosidase for degrading the lipid glucosylceramide into ceramide and glucose. Typical clinical presentations include hepatomegaly, pancytopenia, osteoporosis, and neurological impairment in olfactory, neuromuscular, and cognitive systems (Magalhaes et al., 2018). Recently, Sidransky et al. discovered that GD patients presented with symptoms of PD (Sidransky, 2005). Lewy body pathology with α-syn-positive inclusions were also found in cortical and brain stem autopsies of GD patients (Bembi et al., 2003; Hruska et al., 2006). Furthermore, epidemiology studies demonstrated that family members of GD patients carrying heterozygotic-mutated GBA1 gene have significantly higher incidence of parkinsonism (Riboldi and Di Fonzo, 2019). Later studies demonstrated that PD patients had increased incidence of GBA1 mutations in comparison with control, while PD patients with GBA1 mutations had an earlier onset age (Robak et al., 2017). The clearance of α-syn is primarily mediated through autophagic-lysosomal systems, in which aggregated forms are degraded through (macro)autophagy and soluble forms through chaperone-mediated autophagy (Webb et al., 2003; Martinez-Vicente and Vila, 2013). In chaperone-mediated autophagy, the KFERQ domain of α-syn is recognized by the heat shock cognate 71 kDa protein (Hsc70) chaperone that targets α-syn to the lysosome. Upon arrival at the lysosome membrane, the lysosome-associated membrane protein type 2A (LAMP2A) receptor assists in the docking and internalization of α-syn into the lysosome, where α-syn is degraded by hydrolases (Kaushik and Cuervo, 2018). Lysosomal dysfunction decreases lysosomal enzymatic function, impairing α-syn autophagic clearance, and increases α-syn misfolding leading to an increase in damaged α-syn accumulation (Sidransky and Lopez, 2012; Mehra et al., 2019). Alternatively, α-syn accumulation disrupts GCase trafficking to lysosomes and decreases GCase activity, which further exacerbates the vicious cycle of protein misfolding underlying GBA-associated PD Lewy body formation, thereby forming a bidirectional feedback loop between α-syn and lysosomes (Bras et al., 2008; Wong and Krainc, 2016). Recently, extracellular transfer of misfolded α-syn throughout the brain has been suggested to involve neuron to neuron prion-like propagation between neuroanatomically connected areas. Jakob et al. demonstrated in a 3D-matrix differentiated human neuroblastoma model that, following autophagic failure, α-syn aggregates accumulate within the cell and the lysosomal system releases partially degraded α-syn via exocytosis to be taken up by neighboring cells through endocytosis. They also revealed that α-syn was colocalized with the lysosomal system, both pre- and postsynaptically (Domert et al., 2016). These findings suggest the potential of lysosomal involvement in the processes of interneuron spreading of α-syn pathology observed in the Braak pathology staging (Steiner et al., 2018).

Strong mitochondria-lysosomal interactions have also been found in other PD-susceptible genes. For example, deletion of *PINK1* of MOM in mouse cortical neurons resulted in lysosomal dysfunction including defective lysosomal acidification, decreased lysosomal activity, and large cytoplasmic late-endosome-marker-positive vacuole formation. Meanwhile, further addition of antioxidants to these mitochondria-dysfunctional neurons exhibited improvement of decreased lysosomal activity (Demers-Lamarche et al., 2016; Gomez-Sanchez et al., 2016). To preserve the integrity of oxidative-stressed mitochondria, PINK1/parkin are noted to be involved in the rapid lysosomal targeting of oxidized mitochondrial proteins via MDVs, a process that is PINK1/parkin dependent, autophagy independent, and lysosome targeted (McLelland et al., 2014; Roberts et al., 2016). DJ-1, the gene product of autosomal recessive PD-related *DJ-1*, has been shown to decrease α-syn aggregation via the lysosomal system. Xu et al. demonstrated that *DJ-1* knockout/down repressed α-syn degradation through inhibiting chaperone-mediated autophagy by lysosomes (Xu et al., 2017). DJ-1 has also been shown to regulate the proteolytic machinery 20S proteasome under an oxidizing environment, which is involved in protein homeostasis and ubiquitin-independent autophagosome–lysosome fusion (Tanaka, 2009; Moscovitz et al., 2015; Kumar Deshmukh et al., 2019; Njomen and Tepe, 2019). The most frequent PD gene, *LRRK2*, interacts with many proteins in the endo-lysosomal system and is involved in the steps of lysosome formation, trafficking, and autophagosome formation (Hockey et al., 2015). Meanwhile, *LRRK2* mutations are noted to interfere with mitochondria fission factor DLP1, causing mitochondrial dynamic imbalance and disturbing mitochondrial quality control. These combined effects lead to the eventual accumulation of damaged mitochondria (Salašová et al., 2017; Singh et al., 2019). A recent study by Ysselstein et al. discovered that the inhibition of LRRK2 kinase activity results in increased glucocerebrosidase activity in DA neurons with either *LRRK2* or *GBA1* mutations. This increase in glucocerebrosidase activity partially rescues accumulation of oxidized dopamine and α-syn in PD patient neurons (Ysselstein et al., 2019).

The autophagic-lysosomal pathway is also implicated in some less common familial PD genes (Pitcairn et al., 2019). Grünewald et al. and Gusdon et al. demonstrated that depletion of the ATP13A2, the gene responsible for a form of autosomal recessive juvenile-onset parkinsonism, is associated with impaired lysosomal acidification, decreased autophagic flux, diminished lysosomal-mediated clearance of autophagosomes, mitochondrial fragmentation, increased ROS production, higher frequency of mtDNA lesions, and

decreased mitochondrial turnover (Grunewald et al., 2012; Gusdon et al., 2012). Supporting this, Veen et al. showed that ATP13A2 is an important lysosomal polyamine exporter, and defective lysosomal polyamine export causes lysosome-dependent cell death (Estrada-Cuzcano et al., 2017; van Veen et al., 2020). The lysosomal protein, VPS35, a key component of the retromer complex for cellular protein trafficking, mediates MDVs trafficking between mitochondria and other cellular compartments and is suggested to be associated with rare familial PD (Olszewska et al., 2016; Yun et al., 2017). The pathogenic D620N PD mutation in *VPS35* was shown to disrupt endosomal protein trafficking, autophagosome formation, and cause mitochondrial fragmentation and respiratory chain defects (Follett et al., 2014; Zavodszky et al., 2014; Zhou et al., 2017). As reported, *VPS35* and PINK1/parkin interaction occurred in the formation of mitochondria-derived vesicles, in which overexpression of *VPS35* salvaged parkin mutant phenotypes (Malik et al., 2015). In addition, depletion of *VPS35* in mouse neurons reduced mitochondrial fusion protein Mfn2 stabilization, impeded mitochondrial fusion, and resulted in mitochondrial fragmentation. In the same study, Tang et al. demonstrated that deletion of the *VPS35* gene in mouse DA neurons caused neuronal loss and α-syn accumulation (Tang et al., 2015). The mechanisms of mutant *VPS35* involvement in mitochondrial fission was also noted in the interaction with mitochondrial fission factor DLP1, as *VPS35* regulate recycling of DLP1 complexes. Mutant *VPS35* (D620N)–DLP1 interaction was shown to cause excessive mitochondrial fission and neuronal death, which are enhanced in oxidative stress (Wang et al., 2016). Recently, over 41 genetic susceptibility loci have been associated with late-onset PD in the largest genome-wide association studies (GWAS) meta-analysis of PD to date (Chang et al., 2017). One identified gene is the human transmembrane protein 175 (TMEM175), encoding the lysosomal K^+ channel transmembrane protein (Jinn et al., 2019). Deficiency of TMEM175 has been revealed to impair lysosomal acidification, causing mitochondrial dysfunction, influencing α-syn phosphorylation, and impairing autophagy (Jinn et al., 2017). Also noted through the GWAS study was the sterol regulatory element-binding transcription factor 1 (SREBF1) that links lipogenesis to PD (Do et al., 2011; Ivatt and Whitworth, 2014). SREBF1 is a transcriptional activator imperative for the regulation of lysosomal lipid, and cholesterol accumulation and knockdown of the SREBF1 have been shown to block the translocation of parkin to mitochondria, consequently decreasing mitophagy (Gan-Or et al., 2015; Redensek et al., 2017). These findings indicate a reciprocal functional relationship between the mitochondria and lysosomes, with defects in one impacting the function of the other (Plotegher and Duchen, 2017).

CONCLUSION

A major hurdle in the development of neuroprotective therapies for PD is the limited comprehension of key molecular pathways and targets in the pathogenesis of the disease. The involvement of the energy and metabolic factory, mitochondria, has long been associated with PD progression. Recent identification of physical contact sites between the mitochondria and multiple intracellular organelles has provided critical insight into clarifying how normal mitochondrial functioning extends beyond the organelle itself, exhibiting a complex array of dynamic behaviors. These highlight the bidirectional crosstalk as well as intracellular alarms and protective systems maintained by mitochondrial communication within the intracellular community. The interorganelle communication network expands the scope of investigation: mitochondrial dysfunction in PD pathogenesis is no longer an isolated event but impacts the entire cellular community. Despite recent insights, we lack a clear understanding at the molecular level of how hindered mitochondrial interorganelle communication affects PD pathogenesis.

Mitochondrial damage in the pathology of PD involves several key characteristics: morphological changes, loss of $\Delta\psi m$, protein misfolding and accumulation, decreased ATP production, Ca^{2+} dyshomeostasis, ROS imbalance, mtDNA mutation, autophagy, apoptosis, and lipid oxidation. Research aimed at strategies to maintain effective and efficient interorganelle balance and communication networks will be necessary to develop treatments for this neurodegenerative disease in the future.

AUTHOR CONTRIBUTIONS

T-KL contributed to concept generation, data interpretation, graphic drawing, and drafting of the manuscript. K-JL contributed to concept generation, graphic drawing, and drafting of the manuscript. K-LL contributed to concept generation and drafting of the manuscript. C-WL contributed to concept generation, data interpretation, and approval of the article. S-DC contributed to concept generation, data interpretation, and drafting of the manuscript. Y-CC, P-WW, J-HC, and T-JW contributed to concept generation, data interpretation, and approval of the article. All authors contributed to the article and approved the submitted version.

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Dysfunction of Mitochondrial Ca^{2+} Regulatory Machineries in Brain Aging and Neurodegenerative Diseases

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Calcium ions (Ca^{2+}) play critical roles in neuronal processes, such as signaling pathway activation, transcriptional regulation, and synaptic transmission initiation. Therefore, the regulation of Ca^{2+} homeostasis is one of the most important processes underlying the basic cellular viability and function of the neuron. Multiple components, including intracellular organelles and plasma membrane Ca^{2+}-ATPase, are involved in neuronal Ca^{2+} control, and recent studies have focused on investigating the roles of mitochondria in synaptic function. Numerous mitochondrial Ca^{2+} regulatory proteins have been identified in the past decade, with studies demonstrating the tissue- or cell-type-specific function of each component. The mitochondrial calcium uniporter and its binding subunits are major inner mitochondrial membrane proteins contributing to mitochondrial Ca^{2+} uptake, whereas the mitochondrial Na^{+}/Ca^{2+} exchanger (NCLX) and mitochondrial permeability transition pore (mPTP) are well-studied proteins involved in Ca^{2+} extrusion. The level of cytosolic Ca^{2+} and the resulting characteristics of synaptic vesicle release properties are controlled *via* mitochondrial Ca^{2+} uptake and release at presynaptic sites, while in dendrites, mitochondrial Ca^{2+} regulation affects synaptic plasticity. During brain aging and the progress of neurodegenerative disease, mitochondrial Ca^{2+} mishandling has been observed using various techniques, including live imaging of Ca^{2+} dynamics. Furthermore, Ca^{2+} dysregulation not only disrupts synaptic transmission but also causes neuronal cell death. Therefore, understanding the detailed pathophysiological mechanisms affecting the recently discovered mitochondrial Ca^{2+} regulatory machineries will help to identify novel therapeutic targets. Here, we discuss current research into mitochondrial Ca^{2+} regulatory machineries and how mitochondrial Ca^{2+} dysregulation contributes to brain aging and neurodegenerative disease.

Keywords: mitochondria, calcium regulation, aging, neurodegenerative disease, synaptic regulation

INTRODUCTION

Mitochondria affect cellular functions *via* their roles in ATP production, lipid synthesis, reactive oxygen species (ROS) generation, and Ca^{2+} regulation. Recent studies of mitochondria-dependent Ca^{2+} handling have revealed the molecular identities of Ca^{2+}-control components, including the mitochondrial calcium uniporter (MCU) and its auxiliary subunits (Mammucari et al., 2017). Furthermore, the development of enhanced Ca^{2+} sensors has enabled subcellular investigations of how mitochondria contribute to synaptic transmission. In addition, mitochondrial matrix-targeting sequence-tagged genetically encoded calcium indicators (GECIs) have allowed direct monitoring of mitochondrial Ca^{2+} dynamics (Kwon et al., 2016a).

In aged animals and humans, mitochondrial functional impairment is a key hallmark of brain aging (Grimm and Eckert, 2017; Mattson and Arumugam, 2018; Muller et al., 2018). Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and other aging-related neurodegenerative diseases also show mitochondrial defects. However, the detailed molecular mechanisms underlying these defects, particularly those related to mitochondrial Ca^{2+}, have not yet been studied in depth.

Here, we describe mitochondrial Ca^{2+}-related features and unveiled mitochondrial Ca^{2+} regulatory molecular mechanisms of brain aging and neurodegenerative disease models, and discuss experimental methods and controversies within the current research.

MITOCHONDRIAL Ca^{2+} REGULATORY COMPONENTS AND THEIR PHYSIOLOGICAL ROLES IN NEURONS

Ca^{2+} ions enter neurons through ionotropic glutamate receptors and voltage-dependent Ca^{2+} channels, with the imported Ca^{2+} then affecting various cellular processes, including the modulation of synaptic strength and Ca^{2+}-mediated cell death (Ghosh and Greenberg, 1995). At the presynapse, Ca^{2+} triggers synaptic vesicle exocytosis, and residual Ca^{2+} alters synaptic release properties toward asynchronous release. Moreover, short-term synaptic plasticity can be controlled by presynaptic Ca^{2+} dynamics, while in dendrites, Ca^{2+} influences various signaling cascades involved in long-term synaptic plasticity and gene transcription (Hayashi and Majewska, 2005; Higley and Sabatini, 2008; Sudhof, 2012; Kaeser and Regehr, 2014; Kwon et al., 2016a).

Cytosolic Ca^{2+} is controlled by plasma membrane Ca^{2+} pumps and intracellular organelles, including mitochondria and the endoplasmic reticulum (ER). The ER imports Ca^{2+} through sarco/endoplasmic Ca^{2+} ATPase and releases it *via* ryanodine receptors or IP_3 receptors (IP_3Rs) (Verkhratsky, 2005). ER is partially tethered to mitochondria by mitochondria-associated membrane (MAM) or mitochondria-ER contact sites (MERCs) proteins such as Mitofusin 2, Sigma-1 receptor, vesicle-associated membrane protein-associated protein B (VAPB)/ protein tyrosine phosphatase-interacting protein 51 (PTPIP51), IP3R/glucose-regulated protein (Grp75)/Voltage-dependent anion-selective channel 1 (VDAC1), and PDZ domain containing 8 (PDZD8), enabling ER-to-mitochondria Ca^{2+} transfer (Rapizzi et al., 2002; Szabadkai et al., 2006; Hayashi and Su, 2007; De Vos et al., 2012; Hirabayashi et al., 2017). The involvement of MAM in neurodegenerative diseases is a major topic in the field, but previous reviews wonderfully covered this scope (Paillusson et al., 2016; Liu and Zhu, 2017; Area-Gomez et al., 2018; Bernard-Marissal et al., 2018; Gomez-Suaga et al., 2018; Lau et al., 2018). Therefore, only a part of studies using direct observation of mitochondrial Ca^{2+} dynamics will be discussed here.

Several Ca^{2+} regulatory proteins have been identified in the outer and inner mitochondrial membranes. VDACs located in the outer mitochondrial membrane (OMM) are responsible for importing various ions and metabolites (Colombini, 2016). In the inner mitochondrial membrane, MCU mediates mitochondrial membrane potential-dependent Ca^{2+} influx into the mitochondrial matrix (Kirichok et al., 2004; Baughman et al., 2011; De Stefani et al., 2011). Reduced MCU-dependent Ca^{2+} uptake at presynaptic sites elevates cytosolic Ca^{2+} and alters short-term synaptic plasticity and synchronous release (Kang et al., 2008; Kwon et al., 2016b). In addition, a recent study showed upregulation of mitochondrial fission and dendritic mitochondrial Ca^{2+} transients following chemically induced long-term potentiation (LTP), with the interference of fission impairing mitochondrial Ca^{2+} uptake and LTP (Divakaruni et al., 2018).

Mitochondrial calcium uniporter forms complexes with other proteins, which regulate its opening dynamics (Mammucari et al., 2017; Pallafacchina et al., 2018). Mitochondrial calcium uptake protein 1/2/3 (MICU1/2/3) are the first MCU binding proteins to be characterized. MICU1 and MICU2 serve as molecular gatekeepers that negatively regulate MCU under low Ca^{2+} but positively regulate it under high cytosolic Ca^{2+} (Csordas et al., 2013; Patron et al., 2014; Liu et al., 2016). MICU3 is abundant in the brain and enhances mitochondrial Ca^{2+} uptake, with silencing of MICU3 in cortical neurons causing a reduction in stimulation-induced mitochondrial Ca^{2+} levels (Patron et al., 2019). Furthermore, the presynaptic MICU3-dependent increase in Ca^{2+} sensitivity allows MCU to open without Ca^{2+} release from ER and facilitates Ca^{2+}-mediated mitochondrial ATP production and synaptic vesicle endocytosis (Ashrafi et al., 2020).

Essential MCU regulator (EMRE) is another MCU complex protein that bridges MCU and MICU1 and regulates the level of Ca^{2+} in the mitochondrial matrix. In addition, recent unveiled structural features of EMRE show that it triggers dimerization of MCU-EMRE complex and controls pore opening (Sancak et al., 2013; Vais et al., 2016; Wang et al., 2019). The MCU paralog MCUb exerts an inhibitory effect on MCU, with overexpression of MCUb completely abolishing MCU currents (Raffaello et al., 2013; Mammucari et al., 2017). Mitochondrial calcium uniporter regulator 1 (MCUR1) is a scaffold factor, whose absence results in the failure of MCU to form a complex (Tomar et al., 2016; **Supplementary Figure 1**).

The mitochondrial Na^+/Ca^{2+} exchanger (NCLX) is one of the primary Ca^{2+} efflux units in mitochondria (Palty et al., 2010). Genetic ablation of NCLX increases Ca^{2+} retention in mitochondria and causes mitochondria-dependent cell death

(Luongo et al., 2017). Also, H^+/Ca^{2+} exchanger is considered as a Ca^{2+} efflux component, although its molecular identity is arguable (Jiang et al., 2009; De Marchi et al., 2014). Additional Ca^{2+} release-associated protein, mitochondrial permeability transition pore (mPTP), is activated by Ca^{2+} overload and ROS, leading to apoptosis or necrosis (Giorgi et al., 2018). This pore has been mainly studied under pathological conditions, including aging and neurodegenerative diseases, and the roles of core components including ATP synthase, cyclophilin D (CyPD), and the adenine nucleotide translocators (ANTs), are recently updated, although there are debates (Kokoszka et al., 2004; Bonora et al., 2013; Alavian et al., 2014; Karch and Molkentin, 2014; Raffaello et al., 2016; He et al., 2017; Rottenberg and Hoek, 2017; Zhou et al., 2017; Bernardi, 2018; Muller et al., 2018; Carroll et al., 2019; Karch et al., 2019).

MITOCHONDRIAL Ca^{2+} DYSHOMEOSTASIS IN AGED BRAINS

Dysregulation of Ca^{2+} homeostasis is one of the hallmarks of brain aging (Mattson and Arumugam, 2018), with impaired Ca^{2+} control in aged brains resulting in various cellular and physiological deficits. Hippocampal CA1 pyramidal neurons in aged animals show elevated Ca^{2+} currents, as confirmed by Ca^{2+} imaging using multiple Ca^{2+} fluorophores (Landfield and Pitler, 1984; Disterhoft et al., 1996; Verkhratsky et al., 1998; Thibault et al., 2001; Lessmann et al., 2003). Age-associated Ca^{2+} changes have also been observed in other brain regions and in peripheral nerves (Verkhratsky et al., 1998).

Age-dependent dysregulation of Ca^{2+} results from various molecular changes, including increased voltage-gated Ca^{2+} channel expression, reduced Ca^{2+} binding protein expression, and impaired mitochondrial and ER Ca^{2+} handling (Mattson and Arumugam, 2018). Ca^{2+} isotope uptake by isolated synaptosomal mitochondria is significantly reduced in aged rat brains (Leslie et al., 1985). In addition, cytosolic Ca^{2+} dynamics in aged rodent brain slices or acutely dissociated neurons have been monitored using chemical Ca^{2+} dyes, such as Fura-2. Use of this dye in combination with a mitochondrial membrane potential indicator or mitochondrial uncoupler has revealed that the potential is disrupted in aged neurons, resulting in a decrease in mitochondrial Ca^{2+} uptake and an elevation of cytosolic Ca^{2+} upon stimulation (Xiong et al., 2002; Murchison et al., 2004). Mitochondrial Ca^{2+} buffering is also reduced in aged Rhesus monkeys, shown using isolated putamen mitochondria (Pandya et al., 2015).

ALTERED MITOCHONDRIAL Ca^{2+} DYNAMICS IN AD

Ca^{2+} dysregulation is a common feature of several neurodegenerative diseases, including AD and PD (Beal, 1998; Zundorf and Reiser, 2011; Liao et al., 2017; Pchitskaya et al., 2018). Disruption of Ca^{2+} homeostasis and mitochondrial Ca^{2+} overload have been observed before pathological features of these diseases appear, highlighting the importance of neuronal Ca^{2+} regulation (Lesne et al., 2008; Surmeier et al., 2017).

AD is characterized by the accumulation of amyloid beta (Aβ) peptide, which is produced by abnormal proteolytic cleavage of amyloid precursor protein (APP), as well as by the formation of neurofibrillary tangles composed of tau protein and by neuronal loss, leading to learning and memory impairment (Oddo et al., 2003; Mattson et al., 2008). Mutations in APP or in the γ-secretase components presenilin1 and 2 (PSEN1/2) are the most well-characterized alterations contributing to dominantly inherited familial AD (FAD) (Chen et al., 2017; Muller et al., 2018).

Increased Aβ expression in FAD models or exogenous application of Aβ leads to elevated cytosolic Ca^{2+}. In the past two decades, multiple underlying mechanisms have been suggested, including mitochondrial Ca^{2+} dysregulation (Du et al., 2008; Supnet and Bezprozvanny, 2010; Jadiya et al., 2019; Calvo-Rodriguez et al., 2020). *In-vivo* Ca^{2+} imaging with mitochondria-targeted Förster resonance energy transfer (FRET)-based GECI has directly demonstrated an Aβ-dependent mitochondrial Ca^{2+} increase in mouse cortex. This upregulation was observed prior to neuronal death, with blockade of MCU restoring the mitochondrial Ca^{2+} level in the APP/PS1 mutant mouse model (Calvo-Rodriguez et al., 2020).

Brain levels of NCLX protein are significantly reduced in human AD patients and in 3xTg-AD triple mutant mice (expressing mutations in APP, presenilin 1, and tau). Mutant APP-expressing-N2a cells also show decreased NCLX expression, resulting in impaired mitochondrial Ca^{2+} extrusion, consistent with the increased mitochondrial Ca^{2+} transients revealed by the mitochondria-localized GECI mito-R-GECO1. Alleviation of mitochondrial Ca^{2+} overload by NCLX expression in 3xTg-AD mice rescues cognitive decline and AD-related pathology (Jadiya et al., 2019). Another Ca^{2+} extrusion-related molecule, CypD, which is part of mPTP, is known to interact with mitochondrially transported Aβ. Inhibition or genetic ablation of CypD protects neurons from Aβ-triggered cell death and rescues impaired LTP and deficits in spatial learning and memory (Du et al., 2008).

Mitochondrial Ca^{2+} overload in AD can also result from impaired ER–mitochondria communication. Previous studies suggest that ER–mitochondria contacts are increased in AD models, promoting Ca^{2+} transfer to mitochondria (Zampese et al., 2011; Area-Gomez et al., 2012; Hedskog et al., 2013; Calvo-Rodriguez et al., 2019). ER-to-mitochondria Ca^{2+} transfer has been monitored using the mitochondria-localized chemical dye Rhod-5N or a mitochondrial matrix- or OMM-targeted protein Ca^{2+} sensors (Zampese et al., 2011; Hedskog et al., 2013; Calvo-Rodriguez et al., 2019). Opposite to these, some studies using electron microscopy (EM) and fluorescent imaging have reported reduced ER-mitochondria contacts in AD animal models and patients (Sepulveda-Falla et al., 2014; Martino Adami et al., 2019; Lau et al., 2020; **Figure 1**).

Apolipoprotein E4 (ApoE4) is the major risk factor for sporadic AD and it can increase ER–mitochondria contacts (Tambini et al., 2016; Orr et al., 2019). ApoE4-expressing cells show higher cytosolic and mitochondrial Ca^{2+}, and given that ApoE4 expression alters neuronal MAM-tethering protein

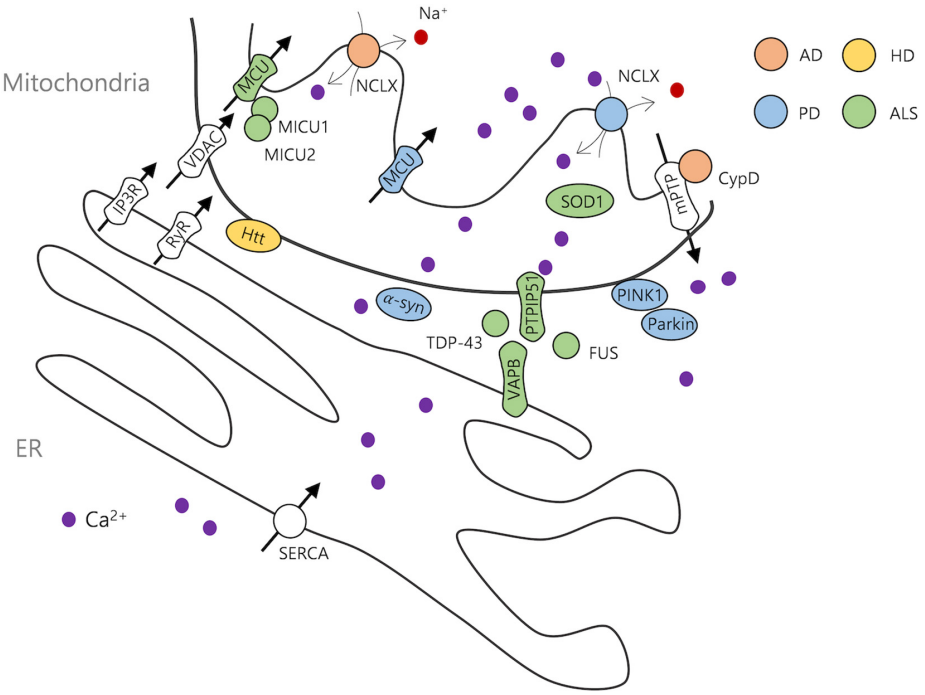

FIGURE 1 | Neurodegenerative disease-related ER and mitochondrial proteins. AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis.

composition, this could explain enhanced MAM activity in sporadic AD (Orr et al., 2019; **Table 1**).

Furthermore, mitochondria contribute to the presynaptic defects observed in AD. Increased insulin-like growth factor-1 receptor (IGF-1R) levels are found in AD patient and mouse model brain samples (Moloney et al., 2010; Zhang et al., 2013). IGF-1R regulates synaptic transmission by modulating presynaptic mitochondrial Ca^{2+} buffering and ATP production, as measured using the mitochondria-targeted GECI and the ATP sensor, although detailed mechanisms are not known. Interestingly, inhibition of IGF-1R reverses altered synaptic release in an APP/PS1 mutant model (Gazit et al., 2016).

PD-RELATED MITOCHONDRIAL Ca^{2+} DYSFUNCTION

Parkinson's disease is characterized at the cellular level by dopaminergic neuron loss in the substantia nigra. Several genes contributing to PD pathogenesis have been identified, including α-synuclein, leucine-rich repeat kinase 2 (LRRK2), PTEN-induced kinase 1 (PINK1), and parkin (Abou-Sleiman et al., 2006; Ferreira and Massano, 2017). Ca^{2+} regulation is especially important for dopaminergic neurons because of their steady and autonomous pacemaker function (Chan et al., 2007; Guzman et al., 2010).

Mutation of α-synuclein and its aggregation into Lewy bodies are well-known pathological processes in PD. Interestingly, α-synuclein is localized to ER, mitochondria, and MAM and contributes to regulating ER–mitochondria communication (Li et al., 2007; Cali et al., 2012; Guardia-Laguarta et al., 2014). In one study, overexpression of WT or mutant α-synuclein in HeLa and SH-SY5Y cells was found to increase mitochondrial Ca^{2+} by enhancing ER–mitochondria interaction (Cali et al., 2012, 2019). However, another study using mutant α-synuclein-overexpressing cells produced conflicting results in terms of ER–mitochondria interactions (Guardia-Laguarta et al., 2014). Furthermore, overexpression of α-synuclein (WT/mutant) in SH-SY5Y cells disturbed the interaction between VAPB and PTPIP51, and this was accompanied by reduced ER-to-mitochondria Ca^{2+} transfer (Paillusson et al., 2017). High dose of WT/mutant α-synuclein can form the aggregates, and this in turn reduces ER-mitochondria contacts (Cali et al., 2012, 2019). Therefore, the dose-dependent effect could be the possible cause of discrepancies (**Figure 1**).

PINK1, a mitochondrial serine/threonine kinase, and parkin, an E3 ubiquitin ligase, are proposed to underlie mitochondrial quality control, with mutations in either gene highly related to PD (Narendra and Youle, 2011; Pickrell and Youle, 2015). Dopaminergic neuron-specific mitochondrial Ca^{2+} imaging with mito-GCaMP, a mitochondria-targeted GECI, in *Drosophila* PD models revealed elevated mitochondrial Ca^{2+}. Pharmacological and genetic inhibition of IP_3R and MCU restore mitochondrial Ca^{2+} and dopaminergic neuron loss (Lee et al., 2018). In contrast, due to negative regulation of NCLX, PINK1-deficient cortical neurons show reduced mitochondrial Ca^{2+} capacity and higher mitochondrial Ca^{2+} accumulation (Gandhi et al., 2009).

TABLE 1 | Ca^{2+} dynamics in neurodegenerative disease models.

Disease	Model		Ca^{2+} level		Ca^{2+} indicator		Ca^{2+} inducer	ER-mito contact	References
			Cyto	Mito	Cyto	Mito			
AD	Aβ	APP/PS1 mut *in-vivo* cortex	↑	↑		mtYellow Cameleon3.6			Calvo-Rodriguez et al., 2020
	Aβ oligomer	Hippocampal neuron (DIV15-21)	↑	↓	Fura-2	Rhod-5N	Caffeine, ACh	↑	Calvo-Rodriguez et al., 2019
	APP_{swe} 3xTG-AD	Neuroblastoma N2a cell	↑	↑	Fura4-AM	mito-R-GECO1	KCl		Jadiya et al., 2019
	Aβ	$APP_{swe/Lon}$ SH-SY5Y	↔	↑	Cyt-AEQ	mit-AEQ	Bradykinin	↑	Hedskog et al., 2013
	PS2 (WT, T122R)	SH-SY5Y		↑ (ER-mito transfer)	Cyt-AEQ, N33D1cpv	4mtD1cpv, mit-AEQ	Bradykinin	↑	Zampese et al., 2011
	APOE4	Neuroblastoma N2a cell	basal ↔	↑	Fura-2 AM	Rhod-2AM	$CaCl_2$, Thapsigargin	↑ (MAM protein level)	Orr et al., 2019
PD	α–synuclein (WT)	SH-SY5Y HeLa cell	↔	↑	Cyt-AEQ	mit-AEQ	Bradykinin, Histamine	↑	Cali et al., 2012
	α–synuclein (WT, A53T, A30P)	SH-SY5Y Neuron derived from patient iPSC		↓		Rhod2-AM	Oxo-M	↓	Paillusson et al., 2017
	PINK1 mutant	Drosophila	↔	↑	GCaMP	mito-GCaMP, Rhod2-AM		↑	Lee et al., 2018
	PINK1 KD / KO	SH-SY5Y human neuron mouse neuron	↑	↑ (Capacity ↓)	Fluo-4, Fura-2		KCl, Ca-NPEGTA		Gandhi et al., 2009
	PINK1 KO	Isolated mitochondria		Capacity ↓		Extra mitochondria Ca^{2+}: Calcium Green 5N	KCl		Akundi et al., 2011
	Parkin KD / mutant	Drosophila Patient fibroblast	↔	↓	Cyt-AEQ	mit-AEQ	Histamine ATP	↓	Basso et al., 2018
	Parkin KO / mutant	PARK2 KO mouse Patient fibroblast	↓	↑	Fura-2	N33-D1cpv, pericam-mt	Bradykinin, ATP, Histamine	↑	Gautier et al., 2016
	LRRK2 (G2019S, R1441C)	Cortical neuron patient fibroblast	↑	↑	RCaMP	mt-GCaMP6m	KCl		Verma et al., 2017
HD	HTT (YAC72)	Isolated mitochondria (YAC72 brain)	↑ (slower recovery)	Capacity ↓		Extra-mitochondrial Ca^{2+}: Calcium Green 5N	$CaCl_2$		Panov et al., 2002
	HTT (YAC128)	Isolated mitochondria, striatal neuron	↑	Capacity ↑	Fura-2FF-AM	Extra-mitochondrial Ca^{2+} using electrode	$CaCl_2$ (w/o BSA), glutamate		Pellman et al., 2015
	HTT (YAC128)	Isolated forebrain mitochondria		↑		Extra-mitochondrial Ca^{2+}: Calcium Green 5N	$CaCl_2$		Oliveira et al., 2007
	R6/2 mice			↑					
	Hdh150 knock-in mice			↔					

(Continued)

TABLE 1 | Continued

Disease	Model		Ca^{2+} level		Ca^{2+} indicator		Ca^{2+} inducer	ER-mito contact	References
			Cyto	Mito	Cyto	Mito			
	STHdhQ111	Striatal cell line	↔ ↑(Bradykinin)	↓ ↔ (low Ca^{2+})	Fura-2AM	mit-AEQ	ATP, Bradykinin, Ca^{2+}		Lim et al., 2008
	STHdhQ111	Striatal cell line	↔	↓	Fluo-3AM	Rhod-2AM	Thapsigargin		Quintanilla et al., 2013
	HTT (YAC128)	Medium spiny neuron (MSN)	↑		Fura-2		Glutamate		Tang et al., 2005
	HTT (YAC128), HD patient	MEF, MSN, patient fibroblast		↑		2mt-cameleon	Bradykinin, DHPG		Wang et al., 2013
ALS	SOD1 (G37R)	Neuroblastoma N2a cell	↑	↓	Cyt-AEQ	mit-AEQ	Bradykinin		Coussee et al., 2011
	SOD1 (G93A)	Motor neuron	↓ (release from mito)		Fura-2AM		FCCP		Tadic et al., 2019
	SOD1 (G93A)	Motor neuron	↑	↓	Fura-2AM	Rhod-2AM	Glutamate		Kruman et al., 1999
	SOD1 (G93A)	Motor neuron	↑	↑	Fura-2AM	mt-pericam	No inducer		Tradewell et al., 2011
	SOD1 (G93A)	Isolated spinal cord mitochondria		↓endstage & presymptomatic		Calcium Green 5N			Parone et al., 2013
	SOD1 (G37R, G85R)			↓endstage ↔presymptomatic					
	TDP43 (M337V, Q331K, A382T, G348C)	HEK293	↑	↓	Fluo-4AM	Rhod-2AM	Oxo-M	↓	Stoica et al., 2014
	FUS (R521C, R518K)	HEK293	↑	↓	Fluo-4AM	Rhod-2AM	Oxo-M	↓	Stoica et al., 2016
	C9ORF72, TARDBP (M337V, I383T)	Patient fibroblast derived MN	↑ (recovery time)	↓	Fura-2AM	Rhod-2AM	KCl, Glutamate		Dafinca et al., 2020

Disease models show differential Ca^{2+} dynamics depending on disease models, Ca^{2+} sensors, and stimulation condition. Disease models: 3xTG-AD, Presenilin 1 (Psen1, M146V homozygous knock-in), amyloid beta precursor protein (APPswe, K670N/M671L transgene) and microtubule associated protein tau (MAPT, P301L transgene); $APP_{swe/Lon}$, Swedish (K670N/M671L) and London (V717I) mutations; YAC models, expanded number of polyQ in Htt; R6/2 mice, expressing a short N-terminal fragment of human Htt with 150 polyQ; Hdh150 knock-in mice, full-length Htt with 150 polyQ; STHdhQ111, striatal cell lines from HD knock-in mouse model; Ca^{2+} sensors: AEQ, aequorin (chemiluminescence-based genetically encoded Ca^{2+} sensor); D1cpv, FRET-based Ca^{2+} sensor (N33 for outer mitochondrial membrane, 4 mt for mitochondrial matrix); Etc: Ach, Acetylcholine; DHPG, (RS)-3,5-dihydroxyphenylglycine, a potent agonist of group I metabotropic glutamate receptors; MEF, mouse embryonic fibroblast.

Similarly, purified mitochondria from PINK1$^{-/-}$ mouse brain show a significantly decreased mitochondrial Ca^{2+} buffering capacity (Akundi et al., 2011).

Primary fibroblasts from PD patients with parkin mutations had reduced mitochondrial Ca^{2+} uptake due to loosened ER–mitochondria connectivity (Basso et al., 2018). Contrary to these results, other studies found that fibroblasts from Parkin-deficient mice or from PD patients with a parkin mutation show increased ER–mitochondria contacts (Gautier et al., 2016). In addition, OMM- and matrix-targeted Ca^{2+} sensors revealed higher ER-mitochondrial Ca^{2+} transfer (Gautier et al., 2016).

Dendrite shortening in LRRK2 mutant models is a well-known change related to mitochondrial dysfunction (Cherra et al., 2013). Abnormal mitochondrial function results from the stimulation-induced increase in mitochondrial Ca^{2+}, which is accompanied by upregulated MCU expression. Interestingly, chemical inhibition or knockdown of MCU successfully restores neurite length (Verma et al., 2017; **Table 1**).

DYSREGULATION OF MITOCHONDRIAL Ca^{2+} IN OTHER NEURODEGENERATIVE DISEASES

HD is a hereditary neurodegenerative disease characterized by involuntary movements, psychiatric abnormalities, and dementia. The major pathogenic features of the disease are progressive striatal neuronal loss,

particularly of GABAergic medium spiny neurons, and extension of the N-terminal polyglutamine (polyQ) stretch of the huntingtin protein (Walker, 2007; Brustovetsky, 2016).

In the mutant huntingtin transgenic mouse brain, polyQ-stretched huntingtin is associated with the mitochondrial membrane (Choo et al., 2004). Interestingly, deficits in mitochondrial Ca^{2+} buffering have been observed after applying Calcium Green-5N to isolated mitochondria from HD mouse brain to monitor extramitochondrial Ca^{2+} (Panov et al., 2002). Live imaging of HD model striatal cell lines with mitochondria-targeted aequorin or Rhod-2AM indicates that mitochondria are able to handle a low Ca^{2+} challenge, but that a higher Ca^{2+} concentration disrupts their buffering ability (Lim et al., 2008; Quintanilla et al., 2013).

However, other studies demonstrated increased Ca^{2+} uptake capacity in isolated mitochondria from HD model mouse forebrains (Oliveira et al., 2007; Pellman et al., 2015). Furthermore, mitochondrial Ca^{2+} influx is higher in primary medium spiny neurons of HD model mice (Tang et al., 2005) and in fibroblasts from HD patients (Wang et al., 2013), which leads to cell death or mitochondrial DNA damage. Interestingly, this excitotoxicity is prevented by MCU or mPTP inhibition (Tang et al., 2005; **Figure 1** and **Table 1**).

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive muscle paralysis resulting from the degeneration of upper and lower motor neurons. ALS exhibits multiple pathogenic features, including oxidative stress, mitochondrial dysfunction, and protein dysfunction of 43-kDa transactivating response region binding protein (TDP-43) and cytoplasmic Cu^{2+}/Zn^{2+}-superoxide dismutase 1 (SOD1) (Ferraiuolo et al., 2011; Lee et al., 2011; Tadic et al., 2014).

Monitoring with mitochondria-/ER-targeted ratiometric sensor proteins and Fura-2 identified elevated levels of mitochondrial, ER, and cytosolic Ca^{2+} in the motor neurons of ALS mutant transgenic mice ($SOD1^{G93A}$) (Tradewell et al., 2011). However, in another study, Rhod-2- and Fura-2-based Ca^{2+} imaging showed significantly decreased mitochondrial Ca^{2+} uptake and increased cytosolic Ca^{2+} in $SOD1^{G93A}$ mice motor neurons (Kruman et al., 1999). Decreased mitochondrial Ca^{2+} buffering capacity in $SOD1^{G93A}$-expressing mice can be restored by CypD deletion, which regulates mPTP opening, suppressing cell death (Parone et al., 2013). Other ALS mutant ($SOD1^{G37R}$-overexpressing) N2a cells also show significantly reduced mitochondrial Ca^{2+} uptake and elevated cytosolic Ca^{2+} (Coussee et al., 2011).

Multiple studies suggest that specific molecular processes underlie ALS progression, but their findings are contentious. Hypoglossal motor neurons of $SOD1^{G93A}$-transgenic mice show upregulated MCU and MICU1 expression at the end stage of the disease (P115–140) (Fuchs et al., 2013). However, in symptomatic cervical spinal cord motor neurons, MCU level is significantly decreased (Tadic et al., 2019).

Other ALS-associated genes have also been identified, including TDP-43, fused in sarcoma (FUS), VAPB, and expanded hexanucleotide repeats in intron 1 of the encoding chromosome 9 open reading frame 72 (C9ORF72). The OMM protein PTPIP51 is a known binding partner of the ER protein VAPB. VAPB–PTPIP51 interaction in mouse motor neurons is disrupted by overexpression of ALS mutant or wild-type TDP-43 and FUS, also leading to disruption of Ca^{2+} homeostasis in HEK293T cells (Stoica et al., 2014, 2016). ALS patient fibroblast-derived motor neurons with C9ORF72 and TDP-43 mutations show delayed clearance of cytosolic Ca^{2+}, lower mitochondrial buffering capacity, and imbalance of MICU1 and MICU2 expression (Dafinca et al., 2020) (**Figure 1** and **Table 1**).

DISCUSSION

In summary, brain aging and neurodegenerative diseases involve mitochondria- and ER-mitochondria contact-related Ca^{2+} regulatory defects. These alterations have been revealed using various experimental methods, including electrophysiological recording and live imaging. However, large part of *in-vitro* studies for neurodegenerative diseases have performed using cell lines and patient-derived fibroblasts rather than neurons. In addition, Ca^{2+} signals were triggered by various chemicals, and most conditions are not neurophysiological (**Table 1**). Depending on tissues and brain regions, mitochondrial Ca^{2+} uptake capacity and regulatory components can be different (Markus et al., 2016; Vecellio Reane et al., 2016; Patron et al., 2019). Therefore, application of recently advanced genetically encoded Ca^{2+} sensors for specific organelles will provide more precise neuron type-specific data (Kwon et al., 2016a).

Finally, mitochondrial Ca^{2+} regulatory molecular mechanisms have recently been revealed, with some studies showing that the composition of the MCU complex can change during disease progress and differ between mutant types (**Table 1**). Thus, revealing the detailed pathophysiological mechanisms of mitochondrial defects at the molecular level could lead to novel therapeutic targets that are specific for particular mutations and disease stages.

AUTHOR CONTRIBUTIONS

HJ, SYK, FC, and S-KK wrote the manuscript and created the figures and table. S-KK and YC provided guidance and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.599792/full#supplementary-material

Supplementary Figure 1 | MCU complex machinery. When the cytosolic Ca^{2+} level is low, MICU1/MICU2 heterodimer keeps MCU as a closed-form, whereas in high Ca^{2+} concentration, their conformational change helps MCU allow Ca^{2+} influx toward mitochondrial matrix. Otherwise, MICU1/MICU3 heterodimer has less gatekeeping function than MICU1/MICU2 dimer, which leads to opening the MCU complex in lower Ca^{2+} condition.

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REVIEW
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Targeting Mitochondrial Impairment in Parkinson's Disease: Challenges and Opportunities

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The underlying pathophysiology of Parkinson's disease is complex, but mitochondrial dysfunction has an established and prominent role. This is supported by an already large and rapidly growing body of evidence showing that the role of mitochondrial (dys)function is central and multifaceted. However, there are clear gaps in knowledge, including the dilemma of explaining why inherited mitochondriopathies do not usually present with parkinsonian symptoms. Many aspects of mitochondrial function are potential therapeutic targets, including reactive oxygen species production, mitophagy, mitochondrial biogenesis, mitochondrial dynamics and trafficking, mitochondrial metal ion homeostasis, sirtuins, and endoplasmic reticulum links with mitochondria. Potential therapeutic strategies may also incorporate exercise, microRNAs, mitochondrial transplantation, stem cell therapies, and photobiomodulation. Despite multiple studies adopting numerous treatment strategies, clinical trials to date have generally failed to show benefit. To overcome this hurdle, more accurate biomarkers of mitochondrial dysfunction are required to detect subtle beneficial effects. Furthermore, selecting study participants early in the disease course, studying them for suitable durations, and stratifying them according to genetic and neuroimaging findings may increase the likelihood of successful clinical trials. Moreover, treatments involving combined approaches will likely better address the complexity of mitochondrial dysfunction in Parkinson's disease. Therefore, selecting the right patients, at the right time, and using targeted combination treatments, may offer the best chance for development of an effective novel therapy targeting mitochondrial dysfunction in Parkinson's disease.

Keywords: Parkinson's disease, mitochondria, therapy, mitochondrial dysfunction, neurodegeneration

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INTRODUCTION

Parkinson's disease (PD) is a common disorder, with over 6 million individuals affected globally (Collaborators, 2019). With only symptomatic treatments available, the greatest current challenge is to develop an effective disease-modifying therapy based on an understanding of the underlying disease mechanisms. While the pathophysiology of PD is complex, mitochondrial dysfunction has an established central role. A growing body of research has provided insights

into the diversity of mechanisms governing mitochondrial dysfunction in PD. Thus, targeting mitochondrial dysfunction is a promising approach for the development of future therapies. Numerous trials focused on ameliorating mitochondrial dysfunction in PD have been conducted but overall have been unsuccessful to date.

We will review previous efforts, the development of new therapies and potential targets yet to be exploited for mitochondrial dysfunction in PD. We highlight the importance of targeting the right patients at the right time for clinical trials, and the need for relevant and accurate biomarkers to monitor response to therapy. We discuss how a combined approach targeting various aspects of mitochondrial dysfunction may serve as the best pathway forward to developing an effective treatment in PD.

Genetic Studies of PD Have Provided Insights Into Disease Pathophysiology

PD is a multifactorial disease with a diverse genetic, biological, and environmental background. The majority of PD is thought to be non-Mendelian and is termed "idiopathic PD" (IPD). The pathophysiology underlying the vast majority of IPD patients is complex and to date, only partially understood (Blauwendraat et al., 2020). Monogenic forms of PD (mPD) have been linked to multiple cellular pathways, the investigation of which has improved our understanding of the molecular basis of IPD in a reductionist manner (**Supplementary Table 1**). Many of the identified forms of mPD have been linked to impaired mitochondrial homeostasis, including pathways that are also relevant for IPD (Larsen et al., 2018). Furthermore, genome-wide association studies have identified IPD risk loci within mPD genes. Many mPD genes and IPD risk loci have proven relationships to mitochondrial dysfunction. For example, *PRKN*, *PINK1*, and *LRRK2*-related cases show distinct disturbances in mitochondrial-related pathways (Bose and Beal, 2016). *DJ-1*-related PD may relate to a disturbance of the cytoprotective role against oxidative stress with a plausible link to mitochondrial dysfunction, despite a lack of detail on mechanistic involvement (Dolgacheva et al., 2019). It is essential to recognise that these pathways often influence one another and that each of the PD-linked gene products, when dysfunctional, can impact multiple pathways either directly or indirectly. When considering the centrality of mitochondria in cellular and metabolic homeostasis and the predominance of mitochondrial dysfunction in PD pathogenesis, it would appear that a tapestry of interconnected pathways and events, rather than a single pathogenic pathway, is the conceptual hurdle that must be addressed by therapy. This may, in part, explain why the neuroprotective strategies trialled to date that are directed at a single target have generally been unsuccessful at producing clear benefits (**Supplementary Table 2**).

Mitochondrial Dysfunction Plays a Central and Multifaceted Role in Parkinson's Disease

Mitochondrial dysfunction plays a fundamental and complex role in many neurodegenerative disorders, including PD (Grimm and Eckert, 2017). PD-associated mitochondrial dysfunction can result from a number of causes, including impairment of mitochondrial biogenesis, increased reactive oxygen species (ROS) production, defective mitophagy, compromised trafficking, electron transport chain (ETC) dysfunction (**Figure 1**), variations to mitochondrial dynamics, calcium (Ca^{2+}) imbalance and possibly other indirect influences on mitochondrial function from unrelated pathways (Park et al., 2018; Grunewald et al., 2019). If these insults cannot be overcome by protective mechanisms, a relentless cycle of dysfunction will eventually evoke all these dysfunctions, leading to cellular impairment and ultimately cell death. Besides the mitochondrion's major function of generating cellular energy in the form of adenosine triphosphate (ATP), their involvement in the regulation of cell death via apoptosis, Ca^{2+} homeostasis, haem biosynthesis, and the formation and export of iron-sulphur (Fe-S) clusters, control of cell division, and growth have also been shown to be altered in IPD and mPD (Bose and Beal, 2016).

During the oxidative phosphorylation process, electrons can leak from the ETC, largely from complex I (C.I) and complex III (C.III), and react with molecular oxygen to form superoxide (O_2^-), one of the reactive oxygen species (ROS) (Rani and Mondal, 2020). Under physiological conditions, this production occurs at relatively low levels and is removed by mitochondrial antioxidants, such as manganese superoxide dismutase (MnSOD or SOD2), glutathione (GSH) and the peroxiredoxins. MnSOD converts O_2^- to hydrogen peroxide (H_2O_2), which is then converted to H_2O by GSH, as part of the network that removes H_2O_2 (Mischley et al., 2016). A reduction in the concentration of GSH in the substantia nigra pars compacta (SNpc) could be an early and modifiable event for PD. The provision of energy by oxidative phosphorylation is probably the most striking feature of mitochondria, and respective disturbances are common in mPD and IPD. Furthermore, environmentally-induced PD can occur as a result of compounds that inhibit the mitochondrial ETC, such as rotenone (Ramalingam et al., 2019). As most drug candidates target the ETC (usually by bypassing defective ETC complexes), the question arises whether this approach neglects the complexity and widespread function of this fundamental cell organelle. Mitochondria are composed of a double lipid bilayer with a permeable phospholipid outer membrane and an impermeable phospholipid inner membrane that surrounds the intra-compartmental matrix. This compartmentalization leads to additional pharmacodynamic challenges in drug delivery (Murphy and Hartley, 2018).

The Cause and Consequence Dilemma of Mitochondrial Dysfunction in Parkinson's Disease Aetiology

Mitochondrial dyshomeostasis is a significant factor in PD neuronal cell death (Liu et al., 2018). Since the initial discovery of C.I dysfunction in PD, our understanding of the complexity and interconnectedness of mitochondria in PD's pathogenesis has considerably expanded. The overall high prevalence of mitochondrial dysfunction in PD is likely underpinned by the fundamental role of mitochondria in

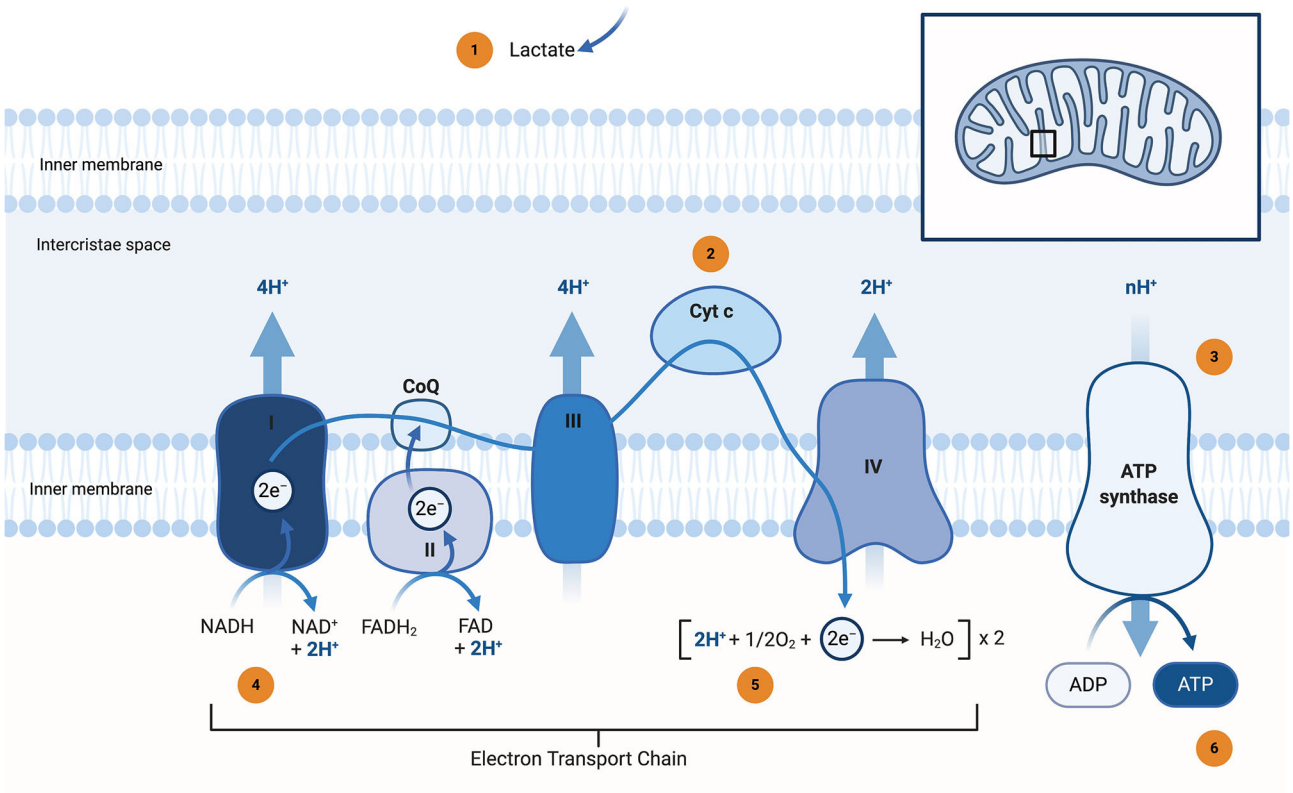

FIGURE 1 | Schematic representation of the Electron Transport Chain (ETC) and potential neuroimaging modalities to assess bioenergetic disturbances in Parkinson's disease (PD) patients. The localisation of the ETC complexes is highlighted in the right upper corner. Lactate (as a surrogate marker for mitochondrial dysfunction) can be assessed by ^{1}H-MRSI **(1)**. bNIRS devices can measure Cyt c oxidation state under dynamic conditions **(2)**. ADP and ATP can either be measured under dynamic (ATP synthase activity) by ^{31}P-MRSI magnetisation transfer **(3)** or static conditions (regular ^{31}P-MRSI) **(6)**. Complex I activity can be studied by ^{31}P-MRSI (by measuring the NAD+/NADH ratio) or via a radiolabeled PET tracer (^{18}F-BCPP-EF) **(4)**. Many different approaches can be used to study *in-vivo* oximetry and oxygen turnover. The most common non-invasive methods are ^{17}O-MRI (direct measurement) or a combination of BOLD and ASL imaging (indirect measurement by employing different physiological models) **(5)**. ^{1}H-MRSI, proton magnetic resonance spectroscopy imaging; ^{17}O-MRI, oxygen magnetic resonance imaging; ^{18}F-BCPP-EF, 2-tert-butyl-4-chloro-5-[6-[2-2[([^{18}F]fluoroethoxy)-ethoxy]-pyridin-3-ylmethoxy]-2H]-pyridazin-3-one; ^{31}P-MRSI, phosphorus magnetic resonance spectroscopy imaging; ADP, adenosine diphosphate; ASL, arterial spin labelling; ATP, adenosine triphosphate; bNIRS, broadband near-infrared spectroscopy; BOLD, blood oxygen-dependent imaging, functional MRI; CoQ, coenzyme Q; Cyt c. Cytochrome c; e^{-}, electron; FAD, flavin adenine dinucleotide (oxidised form); $FADH_2$, flavin adenine dinucleotide (reduced form); H^{+}, proton; H_2O, water; I, complex I; NADH, ubiquinone oxidoreductase, Type I NADH dehydrogenase; II, complex II, Succinate dehydrogenase, succinate-coenzyme Q reductase; III, complex III, coenzyme Q: cytochrome c—oxidoreductase; IV, complex IV, cytochrome c oxidase; MRI, magnetic resonance imaging; *n*, number; NAD^{+}, nicotinamide dinucleotide (oxidised form); NADH, nicotinamide dinucleotide (reduced form); O_2, molecular oxygen; PD, Parkinson's disease; PET, positron emission tomography. *Created with https://biorender.com/.*

cellular energy production, as well as their role in the majority of metabolic pathways, their centrality to cellular homeostasis, and their overall mediation of cell survival (Angelova and Abramov, 2018). Despite the overwhelming evidence for the pathophysiological role of mitochondria in PD and their pivotal role in diverse cellular pathways, the disentanglement of mitochondrial dysfunction as a causative or consequential factor and the identification of the seminal events leading to neurodegeneration remains challenging (Park et al., 2018).

Interestingly, inherited mitochondriopathies do not usually present with parkinsonian features [with some notable exceptions, such as in *POLG* mutation carriers (Ma et al., 2020)]. The primary evidence for a causative role of mitochondrial dysfunction are studies on mPD and environmental studies. In addition, phenotypes consistent with IPD can be induced by several endogenous and exogenous inhibitors of mitochondrial function, including rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP), paraquat, nitric oxide, and the dopamine metabolite aminochrome (Bellou et al., 2016). MPTP is a potent inhibitor of C.I in the ETC, yet paradoxically, parkinsonism is generally not present in patients with genetic C.I deficiency (Langston, 2017). Therefore, the extent to which mitochondrial dysfunction alone can be a precipitating factor in PD is still uncertain.

It is becoming increasingly apparent that the reductionist approach to mitochondrial dysfunction (by focusing solely on the ETC and its production of ATP) oversimplifies their complex role in controlling cellular homeostasis. However, the complexity could also provide an opportunity for drug development, as many

cellular alterations in PD (and other neurodegenerative disease for that matter) can be modulated by alleviating mitochondrial dysfunction. Genetic PD may be both primarily mitochondrially involved but also indirectly involved; therefore, targeting only the mitochondria will be insufficient as a stand-alone therapeutic approach. This may particularly be the case if mitochondrial dysfunction is the consequence rather than the cause of disease pathophysiology (e.g., in *ATP13A2* mutation carriers with Kufor-Rakeb Syndrome; Park et al., 2014, 2016; Wang Z. B. et al., 2019).

Recognising Prodromal Mitochondrial Dysfunction: Selection of Study Participants and the Role of Neuroimaging

The temporal dynamics of mitochondrial dysfunction in PD are unclear. For example, there is currently no experimental evidence to determine whether ROS production leads to dysfunctional ETC complexes or dysfunctional ETC complexes cause increased production of ROS (Singh et al., 2019). But the temporal aspects of mitochondrial dysfunction are crucial for understanding PD pathophysiology and accelerating future drug development. Identifying at-risk individuals and treating them in a prodromal phase based on their driving pathophysiological process could prevent the manifestation of PD, or at least alter the disease progression (Ascherio and Schwarzschild, 2016).

At present, two approaches could be feasible to investigate this matter: the a-priori stratification of study cohorts (based on assumed mitochondrial dysfunction) or the *in-vivo* assessment of mitochondrial dysfunction (by neuroimaging methods or blood biomarkers) (Prasuhn et al., 2019). It is desirable to enrich study cohorts with participants exhibiting mitochondrial impairment, to ensure the success of clinical trials directly targeting the mitochondria. Enrichment or stratification of study cohorts by mPD (e.g., *PRKN* mutation carriers) fosters this approach. However, the extension of potential findings to the vast majority of IPD patients remains challenging. Polygenic risk scoring of *in-silico* annotated single nucleotide polymorphisms (SNPs) could translate the concept of mitochondrial impairment found in monogenic PD to IPD (Prasuhn et al., 2019). However, for most useful SNPs, functional studies are lacking and require validation for humans in advance of clinical trials.

Secondly, the *in-vivo* assessment of key aspects of mitochondrial dysfunction via specific neuroimaging methods is likely to be highly advantageous. Magnetic resonance spectroscopy imaging (MRSI) could provide a non-invasive approach to assess mitochondrial bioenergetics. ^{31}Phosphorus-MRSI (^{31}P-MRSI) bears the potential to measure ATP among other phosphorus-containing metabolites (Forester et al., 2010; **Figure 2**). ^{31}P-MRSI has been used to measure the ratio of NAD^+/NADH, which offers unique opportunities for recent niacin-based clinical trials in PD [(Lehmann et al., 2017), REPAIR-PD: NCT03815916]. In addition, proton-MRSI (^{1}H-MRSI) can be applied to measure lactate levels as a surrogate marker of impaired oxidative phosphorylation (Henchcliffe et al., 2008).

Continuous methodological improvements of scanner hardware (by increasing the static magnetic field strengths) or MRI sequence optimization, offer great potential for MRSI studies (Henchcliffe et al., 2008). ^{1}H-MRSI can be used to measure glutathione levels, which helps assess the oxidative state of PD brain tissue (Mischley et al., 2016). Further proof is required to determine whether the latter approach might benefit studies investigating the potential therapeutic use of glutathione or other antioxidants in PD. However, a recent placebo-controlled and randomised clinical trial with intranasal administration of glutathione in unselected PD patients did not show superiority to placebo using the Unified PD Rating Scale as an endpoint (Mischley et al., 2017). Promisingly, *in-vivo* oximetry (as a combination of different MRI modalities, such as arterial spin labelling and BOLD imaging, susceptibility-weighted imaging, or ^{17}O-MRI) shows potential for discerning *in situ* mitochondrial impairment (Borghammer et al., 2008).

Besides MRI-based methods, broadband near-infrared spectroscopy (bNIRS) can be employed to study the oxidation status of cytochrome c by multiwavelength absorption and reflection of cortical cytochrome c in the near-infrared spectrum of light (Lange et al., 2019). Unfortunately, bNIRS devices are currently not commercially available, with limited application to date. Additionally, positron emission tomography (PET) provides additional insights into mitochondrial dysfunction, with a novel PET probe, ^{18}F-BCPP-EF, recently used to examine C.I activity in PD patients (Wilson et al., 2020).

Neuroimaging studies on mitochondrial impairment in PD are still scarce and lack intra-site reliability (e.g., by distinct hardware requirements), which is one major prerequisite for theranostic-accompanied drug trials (Dossi et al., 2019). The ongoing development of quantitative MRI/MRSI sequences and dual-calibrated fMRI (for MRI-based oximetry) will more than likely overcome the problem of inter-site reliability (Lara et al., 1993; Germuska et al., 2019). However, current neuroimaging studies often lack reference values to interpret findings, and longitudinal studies are needed to identify bioenergetic disturbances' temporal dynamics. Aside from the unknown temporal dynamics of mitochondrial dysfunction, clinical trials in neurodegenerative diseases, in general, face unique challenges with long interventional periods, the need for sophisticated trial designs (delayed-clinical trials or adaptive designs), and a priori patient-stratification. At present, the proposed neuroimaging methodology only addresses alterations in mitochondrial bioenergetics and neglects other (maybe earlier) pathophysiological hallmarks. Future studies in this area are urgently needed and will play a pivotal role in future clinical trials' success.

Blood Biomarkers of Mitochondrial Dysfunction

In addition to neuroimaging findings, blood-based biomarkers may also be helpful to assess mitochondrial dysfunction *in vivo*. One general concern of blood-based biomarkers is that they do not recapitulate mitochondrial dysfunction of neuronal cell populations. Gene mutations leading to mPD

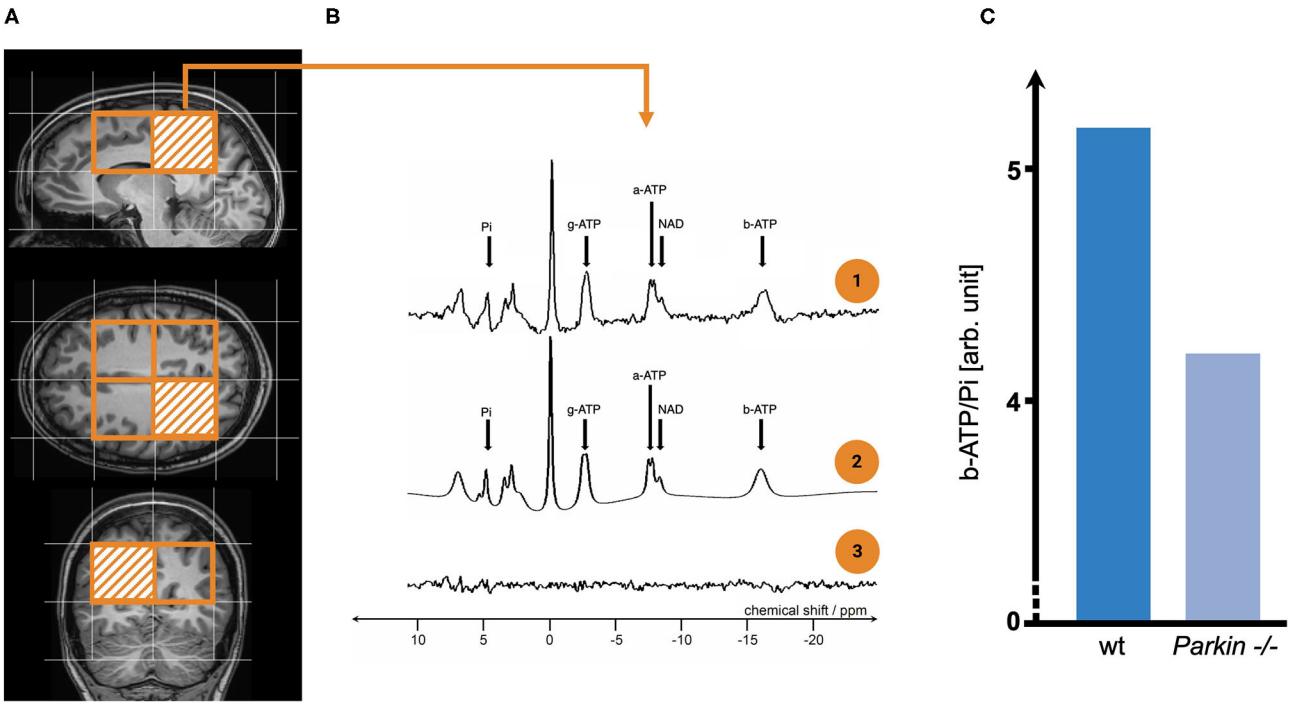

FIGURE 2 | The exemplary workflow of phosphorus-magnetic resonance spectroscopy imaging (^{31}P-MRSI) analysis and its potential role in imaging mitochondrial dysfunction in Parkinson's disease (PD) patients. **(A)** Depicts the voxel placement (orange bordered) for metabolic studies *in-vivo*. As seen here, one of the most significant limitations in ^{31}P-MRSI studies is the relatively poor spatial resolution. The arbitrarily chosen voxel-of-interest is highlighted by orange striation. We receive a spectrum of phosphorus-containing metabolites for each voxel (see **B.1**), which we marked by small black arrows. a/b/g-ATP do not reflect the number of covalently bound phosphate groups (by means of AMP/ADP, and ATP) but result, e.g., from electron-mediated interaction of two (or more) nuclear spins residing on the same molecule (among other quantum physical mechanisms). Usually, the raw spectrum (see **B.1**) is processed by mathematical operations that incorporate prior knowledge on investigated molecules' physical properties (see **B.2**). In **(B.3)**, one can see the 'unfitted' noise of the raw spectrum. To illustrate how ^{31}P-MRSI can be applied to study mitochondrial dysfunction in PD, two exemplary patients were investigated here (see **C**): One patient carrying two wild type (wt) alleles for the *Parkin* allele and, in contrast, one patient carrying two homozygous loss-of-function mutations (*Parkin*$^{-/-}$). The spectral integral of investigated metabolites (here b-ATP and Pi) is used for the (semi-)quantification of metabolite content. Commonly, metabolites are normalised (as a ratio to Pi) to account for the overall phosphorus amount in human brain tissue (which might be influenced by, e.g., nutritional intake). -/-, homozygous *Parkin* mutation carrier; ^{31}P-MRSI, phosphorus magnetic resonance spectroscopy imaging; a/b/g-ATP, alpha/beta/gamma-adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; arb. unit, arbitrary unit; NAD, nicotinamide adenine dinucleotide; PD, Parkinson's disease; Pi, inorganic phosphate; ppm, parts per million; wt, wild type.

often show tissue-specific expression patterns and therefore lack biological interpretability in peripheral blood cells, e.g., peripheral monoclonal blood cells (PMBCs; Dossi et al., 2019; He et al., 2019). The determination of mtDNA mutation load is altered by the different proliferation rates of distinct cell types, which hinders the insights gathered from PMBCs to assess neuronal dysfunction (O'Callaghan et al., 2015). This mainly applies to most of the current approaches in measuring mitochondrial dysfunction via the measurement of mitochondrial membrane potential, C.I activity, among other functional assays. Besides, it is still unclear which neuronal cell types are affected by mitochondrial dysfunction. As most (pre-)clinical studies investigate the influence of mitochondrial dysfunction on dopaminergic neurons, other neuronal cell types and neurotransmitter systems are likewise involved in PD pathogenesis. The use of mitochondria-specific blood biomarkers would most likely not assess the selective loss of dopaminergic neurons, comparing the small size of the substantia nigra to the overall large human blood volume. Supporting evidence comes from a study comparing the amounts of FGF-21 and GDF-15 (as well-established biomarkers of mitochondrial disease), showing negligible diagnostic value in assessing mitochondrial dysfunction of PD patients (Davis et al., 2020).

THERAPEUTIC APPROACHES

Recovering Physiological ATP Production by Targeting the ETC and Antioxidative Treatment Strategies

Based on the close interconnectedness of ETC complex dysfunction and increased oxidative stress, most compounds trialled to date essentially influence both aspects of mitochondrial dyshomeostasis. Coenzyme Q10 (ubiquinone; CoQ10) and its derivatives are still the most studied investigational products (Negida et al., 2016; Zhu et al., 2017). The proposed method

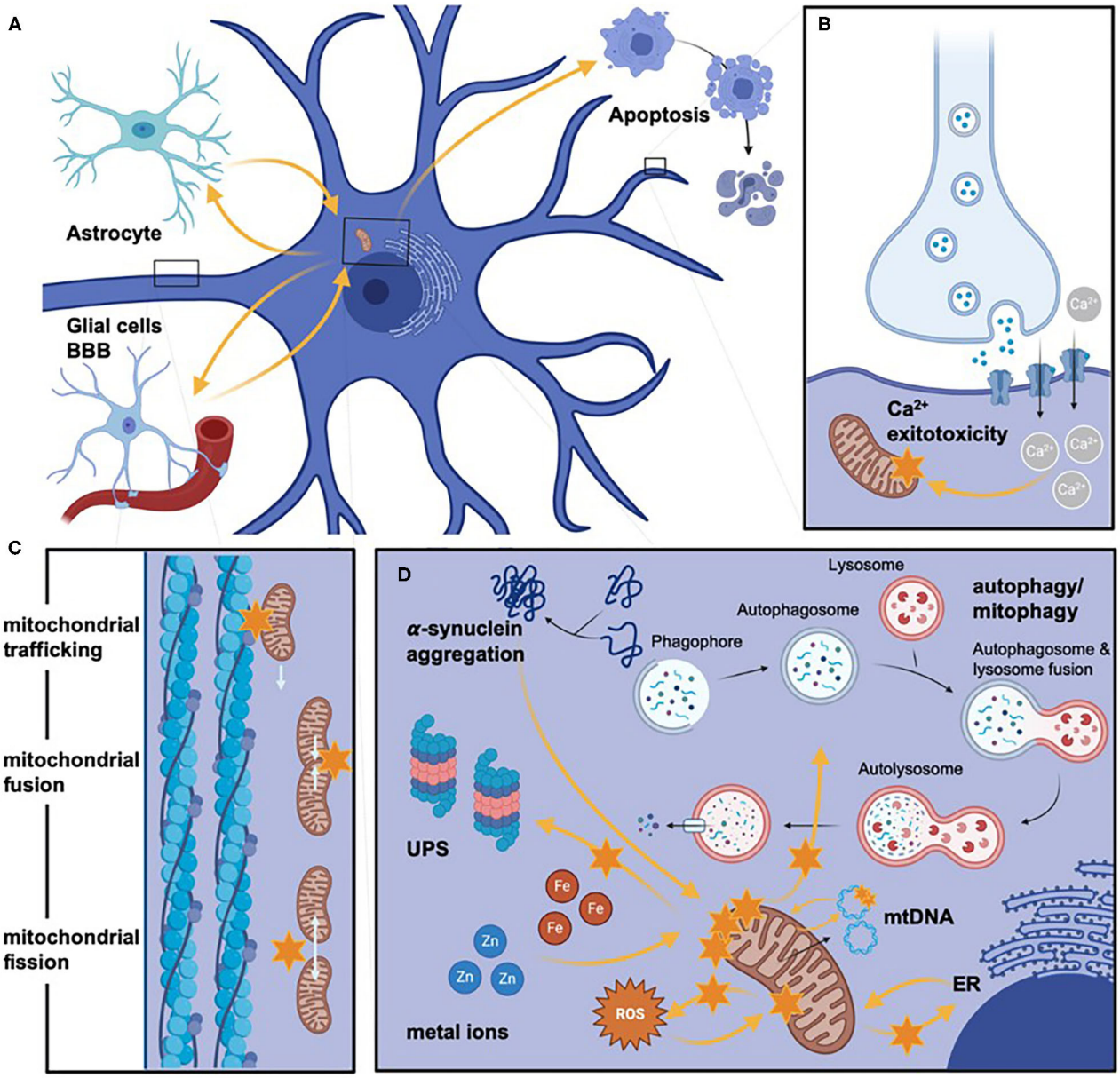

FIGURE 3 | Mitochondrial pathways involved in PD pathogenesis. Different impaired pathways in mitochondrial dysfunction are schematically highlighted but not exhaustively listed. **(A)** Provides an overview of neurons' histopathological orientation and otherwise active cell types (left upper corner: glial cells, left lower corner: pericytes, which form -mong others- the blood-brain barrier, right upper corner: schematic representation of neuronal cell death via apoptosis). Orange arrows are indicating the direct and indirect influence on various factors on mitochondrial dysfunction, which are additionally highlighted by orange stars to stress severe impairment (e.g., the bioenergetic relevance of glial cells for neurons). **(B–D)** Representing more in-depth insights on molecular aspects of mitochondrial dysfunction. **(B)** Illustrates the increased Ca^{2+} influx, which leads to mitochondrial impairment mediated by glutamate-excitotoxicity. **(C)** Displays (represented by a magnified axonal section) alterations of mitochondrial dynamics by impaired mitochondrial trafficking, fusion, or fission. **(D)** Depicts schematically (especially concerning the size of involved cellular components) the commonly shared pathomechanisms of mitochondrial dysfunction. Again, we highlighted the direct and indirect influence on the mitochondria with orange arrows. Even though not all pathomechanisms are named here, the lysosome/autophagosome pathway's involvement and the ubiquitin-proteasome clearance of dysfunctional mitochondria are stated. The influence of toxic cellular compounds is stressed by protein aggregation, Zn and Fe dyshomeostasis, and ROS. In addition, alterations of mtDNA are highlighted. Even though many altered pathways are interconnectedly leading to mitochondrial dysfunction, we only listed the respective influence on mitochondria and, for clarity, not on each other. BBB, blood brain barrier; Ca^{2+}, calcium; ER, endoplasmic reticulum; Fe, iron; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; UPS, ubiquitin-proteasome system; Zn, zinc. *Created with https://biorender.com/.*

of action of CoQ10 is to bypass dysfunctional C.I by electron transfer via the Q-cycle (Yang et al., 2016). Also, CoQ10 could directly decrease the extent of oxidative stress (Yang et al., 2016). Although studies in animal models yielded promising results, most clinical trials failed to reach relevant clinical endpoints (**Supplementary Table 2**), possibly because

of the unselected nature of the PD patients enrolled (Negida et al., 2016; Zhu et al., 2017; Attia and Maklad, 2018). To overcome additional pharmacodynamic and kinetic challenges, different galenic formulations (e.g., nano-emulsified ubiquinol) or derivatives of CoQ10 (e.g., EPI 589) were developed (Kumar et al., 2016; NCT02462603). Vitamin K2 (long-chain menaquinone 7; MK-7) may also act like CoQ10 based on their structural similarity, as demonstrated by studies in *Drosophila* flies carrying a homozygous *PINK1* knockout (Vos et al., 2012). However, these findings need to be validated in human trials.

Nicotinamide (vitamin B3, NAM) and its derivatives are also currently under investigation, where the underlying rationale is to normalise redox levels (NAM may also affect sirtuins, as discussed later). NAM, particularly NAD+ and NADH, are also highly relevant for C.I and could be beneficial for ETC disturbances (Lehmann et al., 2017; REPAIR-PD: NCT03815916). The innovative REPAIR-PD study (phase 2) assesses the cerebral metabolic effects, safety, pharmacokinetics, and pharmacodynamics of an oral, gold nanocrystal liquid suspension (CNM-Au8, **Supplementary Table 2**). Proposed antioxidative properties will be investigated by measuring the *in-vivo* NAD^+/NADH ratio via ^{31}P-MRSI.

N-acetyl-cysteine (NAC) has also been investigated in a small proof-of-concept study. It showed antioxidative properties measured by increased blood and brain glutathione levels after single-time point administration (Monti et al., 2019). The natural compound apocynin (Apo) has mitigating properties that are involved in inflammatory responses (Cheng et al., 2018). Apo has been adjusted to target mitochondria (Mito-Apo) (Ghosh et al., 2016; Langley et al., 2017), with preclinical PD models showing that it could prevent MPTP-induced nigral cell loss, indicating its potential use for mitochondrial dysfunction in PD (Ghosh et al., 2016; Langley et al., 2017). Another investigational drug candidate that has been assessed in animal models is N-Methyl, N-propynyl-2-phenylethylamine (MPPE). MPPE serves as an MAO-B inhibitor that prevents MPTP-induced nigral cell loss, upregulates mitochondrial superoxide dismutase to alleviate oxidative stress, and improves C.I function (Shin et al., 2016). Additionally, the S(-) enantiomer of pramipexole, an often used dopamine agonist in routine clinical care, has also been shown to have antioxidative properties (Izumi et al., 2007). Unfortunately, it seems to lack disease-modifying efficacy in humans.

The most recent clinical trial candidate showing promise is ursodeoxycholic acid (UDCA), a drug often used in chronic inflammatory liver disease with an extensive safety profile (Sathe et al., 2020). UDCA has been shown to prevent mitochondrial membrane depolarisation and stabilises cytochrome c in the mitochondrial membrane (Abdelkader et al., 2016; Bell et al., 2018). There is also convincing *in vivo* evidence that UDCA can be especially beneficial for treating mitochondrial impairment in $LRRK2^{G2019S}$ mutation carriers (Mortiboys et al., 2015). An upcoming oral UDCA study is also coupled with the study of brain energy metabolism via ^{1}H-MRSI (NCT02967250).

Exercise

The effect of physical exercise has been assessed in both animal models and PD patients and is shown to promote mitochondrial biogenesis and function.

The long-term effects of voluntary exercise were investigated in a transgenic MitoPark mouse PD model (Lai et al., 2019). Voluntary exercise was found to improve behavioural parameters and nigrostriatal dopamine input. Additionally, exercise increased oxygen consumption, in keeping with increased ATP production via oxidative phosphorylation (Lai et al., 2019).

In a unilateral PD rat model, rats were subjected to either 1 or 4 weeks intermittent moderate treadmill exercise (Fernandes Ferreira et al., 2020). The investigators showed that 1 week of exercise prevented a decrease in peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) and NRF-1 expression. Furthermore, 4 weeks of exercise prevented a reduction in transcription factor A, mitochondrial (TFAM) and C.I protein levels and augmented C.I activity. This suggests that intermittent exercise improves mitochondrial biogenesis signalling and respiratory chain modulation of the dopaminergic system in PD (Fernandes Ferreira et al., 2020).

Another study using a rodent model showed that exposure to 6-hydroxydopamine (6-OHDA) led to a reduction of mitochondrial factors adenosine monophosphate (AMP)-activated protein kinase, PGC-1α, and tyrosine hydroxylase (TH), and increased expression of silent information regulator T1, TFAM, and *p53* (Rezaee et al., 2019). Notably, gene and protein expressions upon exercise were elevated and the *p53* protein levels were lower in an exercise and 6-OHDA group compared with a no exercise and 6-OHDA group.

Furthermore, endurance exercise restored motor function and reduced apoptosis in a MPTP mouse model (Jang et al., 2018). These benefits were associated with mitochondrial phenotypic changes such as upregulated anti-apoptotic proteins, reduced pro-apoptotic proteins and improved mitochondrial biogenesis and fusion (Jang et al., 2018).

In addition to rodent PD models, *Drosophila Parkin* mutants were used to demonstrate that dietary management along with physical activity has the potential to improve mitochondrial biogenesis and delay the progression of PD (Bajracharya and Ballard, 2018).

A recent study in humans showed that exercise-induced improvements in the PD clinical state were associated with specific adaptive changes in muscle functional, metabolic, and molecular characteristics, although some parameters, such as muscle mitochondrial DNA content, improved with exercise in controls and not in PD patients (Krumpolec et al., 2017).

There are many advantages to exercise; it is inexpensive, practical, sustainable, and has additional health benefits (Lai et al., 2019). Overall, the evidence supports an unambiguous benefit of exercise in PD, which is at least partly explained by a beneficial effect upon mitochondrial function.

Enhancing the Clearance of Dysfunctional Mitochondria via Mitophagy or Other Mitochondrial Stress Response Pathways

Multiple lines of evidence point to the importance of mitophagy in the pathophysiology of PD. For example, the *PRKN* and *PINK1* genes mediate mitophagy (Pickrell and Youle, 2015) and are the major causes of autosomal recessive early onset mPD (**Supplementary Table 1**). Therefore, enhancing mitophagy is a key therapeutic strategy in PD (Aman et al., 2020). In keeping with this, investigators used a rodent model of PD to study the effect of kinetin, the precursor of kinetin triphosphate, an activator of both wild-type and mutant forms of *PINK1* (Orr et al., 2017). However, in *PINK1* null rodents, no degeneration of midbrain dopamine neurons was identified. Additionally, in rodent models of α-synuclein induced toxicity, boosting PINK1 activity with oral kinetin provided no protective effects, thus showing no evidence of a beneficial effect in a preclinical model of IPD (Orr et al., 2017). Another agent, celastrol, was shown to exert neuroprotective effects through activating mitophagy and inhibiting dopaminergic neuronal loss in PD cell and mouse models (Lin et al., 2019). Recently, a study used a high-throughput phenotype detection system for drug screening in dopaminergic neurons from induced-pluripotent stem cells (iPSCs) derived from patients with PD due to *PRKN* or *PINK1* mutations (Yamaguchi et al., 2020). After screening 320 compounds, they identified 4 candidate drugs that were effective for ameliorating impaired mitochondrial clearance, showing the utility of this method for identifying candidate PD drugs (Yamaguchi et al., 2020).

Improving Mitochondrial Biogenesis

Mitochondrial biogenesis is a complex process involving coordination of transcription, translation, import of nuclear-encoded components, as well as the expression of mitochondrial genes (Chandra et al., 2019). A recent study of Parkin-deficient human dopaminergic neurons demonstrated that while there was defective mitophagy in human dopaminergic neurons lacking Parkin, the mitochondrial dysfunction is chiefly a consequence of defects in mitochondrial biogenesis (Kumar et al., 2020). The defective mitochondrial biogenesis is driven by the upregulation of the Parkin substrate PARIS and the subsequent downregulation of PGC-1α (Ge et al., 2020; Kumar et al., 2020). Thus, strategies aimed at enhancing mitochondrial biogenesis should be a focus for the development of new therapeutic approaches to treat PD (Kumar et al., 2020).

Targeting of the AMP-activated protein kinase (AMPK)-SIRT1-PGC-1α axis may be the most promising approach to enhancing mitochondrial biogenesis, and could involve PGC-1α activating drugs targeting PPAR, AMPK, and SIRT1 (Chandra et al., 2019). A study using a 6-OHDA lesioned rat model of PD showed that the polyphenolic phytochemical ferulic acid can modulate PGC1α with beneficial effects on mitochondrial dynamics, supporting the concept of targeting PGC1α, a master regulator of mitochondrial biogenesis, as a therapeutic strategy (Anis et al., 2020; Bennett and Keeney, 2020). A recent study demonstrated a novel approach to increasing mitochondrial biogenesis in neuronal cells via RNS60 (0.9% saline solution containing oxygenated nanobubbles), through phosphatidylinositol 3-kinase-mediated upregulation of PGC1α (Chandra et al., 2018, 2019). Moreover, the drug exenatide has been shown to improve motor scores in PD and may have beneficial effects on mitochondrial biogenesis (Fan et al., 2010; Athauda et al., 2017). Additionally, dopamine D1 receptor agonism has been found to improve mitochondrial biogenesis and dopaminergic neurogenesis in a 6-OHDA rat model of PD (Mishra et al., 2020). Finally, baicalein, a bioactive flavone of *Scutellaria baicalensis Georgi*, has been shown to enhance mitochondrial biogenesis in a rotenone-induced PD rat model (Zhang et al., 2017).

Gene Therapies Targeting Mitochondrial Dysfunction in Parkinson's Disease

Gene therapy entails the treatment of disease via delivery of a transgene that either replaces or corrects a defective gene or is generally supportive of cells in the disease environment (O'Connor and Boulis, 2015). Gene therapy vectors can be either viral [commonly adeno-associated viruses (AAVs) and lentiviruses (LVs)] or non-viral (typically naked plasmid DNA or in complex with cationic lipids or polymers) (O'Connor and Boulis, 2015).

Autosomal recessive mutations in the *PRKN* gene cause loss of function (truncating variants) or inactivation (missense variants) of the parkin protein (Bruggemann and Klein, 1993). Overexpression of *PRKN* has been shown to have a protective effect against numerous cellular insults (Choong and Mochizuki, 2017). For example, overexpression of wild type parkin in a transgenic mouse model was shown to alleviate MPTP-induced dopaminergic neurodegeneration via protection of mitochondria and decreased striatal alpha-synuclein (Bian et al., 2012). Furthermore, recombinant AAV vector-mediated intranigral delivery of parkin prevented motor deficits and dopaminergic cell loss in a chronic MPTP-minipump mouse model of PD (Yasuda et al., 2011).

Loss of function mutations in *PINK1* can also lead to autosomal recessive PD (Valente et al., 2004). Removal of *Drosophila PINK1* homologue function leads to male sterility, apoptotic muscle degeneration, defects in mitochondrial morphology and increased sensitivity to multiple stressors including oxidative stress. In a portion of PINK1 mutants, expression of human PINK1 in the Drosophila testes restores male fertility and normal mitochondrial morphology (Clark et al., 2006).

Numerous studies indicate that mitochondrial pathology and muscle and dopaminergic degeneration due to *Drosophila* PINK1 inactivation and *PINK1* mutant can be rescued by *PRKN* overexpression and downregulation of Miro (Yang et al., 2006; Liu and Lu, 2010; Liu et al., 2012; Choong and Mochizuki, 2017). Furthermore, overexpression of *PINK1* results in the rescue of the alpha-synuclein-induced phenotype in a *Drosophila* model of PD (Todd and Staveley, 2008). Depletion of PINK1 by RNA interference increased neuronal toxicity induced by 1-Methyl-4-phenylpyridinium ion (MPP^+) (Haque et al., 2008). Moreover,

wild-type *PINK1*, but not the mutant form, protects neurons against MPTP/MPP^+ both *in vitro* and *in vivo* (Haque et al., 2008). Furthermore, viral-mediated expression of *PRKN* and *DJ-1* genes can protect dopaminergic neurons, even in the absence of PINK1, suggesting that DJ-1 and Parkin act in parallel or downstream of endogenous PINK1 to mediate survival (Haque et al., 2012).

In general, studies of autosomal recessive genes, such as *PRKN* and *PINK1*, support a loss of function mechanism ameliorated by replacement, thus suggesting gene therapy as a promising treatment strategy in PD. In addition, gene therapy based on transfection of mtDNA-complexed *TFAM* or recombinant *TFAM* to PD cybrid cells shows potential to restore mitochondrial bioenergetics of severely impaired nigral neurons, as TFAM is important for mtDNA maintenance (Keeney et al., 2009; Golpich et al., 2017).

Somatic mtDNA point mutations may be elevated in the substantia nigra of patients with PD (Lin et al., 2012). A recent study used bacterial cytidine deaminase toxin for CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-free mitochondrial base editing, allowing for the prospect of correcting mitochondrial mutations (Mok et al., 2020), a technique which may find utility for PD in the future.

Protein-Based Therapies Targeting Mitochondrial Dysfunction in Parkinson's Disease

A protein-based approach may be utilised to address mitochondrial dysfunction in PD. Cell-permeable Parkin protein (iCP-Parkin) was recently investigated as a protein-based therapy in cellular and animal-based models (Chung et al., 2020). iCP-Parkin promoted mitophagy and mitochondrial biogenesis, thereby recovering damaged mitochondria, suppressing toxic accumulation of alpha-synuclein, and preventing and reversing declines in TH and dopamine expression and improving motor function. These findings support iCP-Parkin as a potential PD-modifying agent (Chung et al., 2020).

Nrf2/ARE as a Drug Target for Parkinson's Disease

The nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) signalling cascade plays a key role in several aspects of mitochondrial homeostasis, such as mitochondrial biogenesis, mitophagy, ROS production and scavenging (Gureev and Popov, 2019). This makes the Nrf/ARE pathway a highly appealing target for a novel drug therapy for PD, which may be used in tandem with antioxidant protection to slow disease progression (Gureev and Popov, 2019). Very recently, an activator of the Nrf2-ARE pathway, TPNA10168, was investigated in a PD rodent model (Inose et al., 2020). TPNA10168 was found to inhibit dopaminergic neuronal death, and it was thought that heme oxygenase-1, an antioxidant enzyme expressed downstream of the Nrf2-ARE signalling pathway, might participate in this effect.

Restoring Mitochondrial Dynamics and Trafficking

Mitochondria form a complex network, the cohesiveness and shape of which is of direct functional relevance (Wai and Langer, 2016). Mitochondria are highly dynamic, constantly breaking off, spatially relocating, and rejoining the network. This is critical for neurons [particularly dopaminergic neurons, with ∼4.5 metres of total linear axonal length and ∼2.5 million synapses each (Bolam and Pissadaki, 2012)] as their complex architecture requires mitochondrial relocation from the soma to dendrites, axons and synapses to meet regional metabolic demands (Parrado-Fernandez et al., 2018). The majority of genetic PD proteins locating to mitochondria and mitochondria-ER contact sites are associated with processes influencing or influenced by mitochondrial dynamics and trafficking (Gao et al., 2017; Cutillo et al., 2020). A comprehensive summary of mitochondrial dynamics and trafficking in neuronal function can be found elsewhere (Seager et al., 2020).

Mitochondrial dynamics are highly complex but strictly balanced processes controlled by a diverse array of established and emerging molecular mediators, in addition to mitochondria-ER contact sites (Chan, 2020; Sabouny and Shutt, 2020). Fragmentation of the mitochondrial network is a salient observation in PD, highlighting mitochondrial dynamics as a key therapeutic target. To this end, mdivi-1, an inhibitor of the mitochondrial fission GTPase Drp1, has been used to inhibit mitochondrial fragmentation in an α-synuclein rat model of PD, reducing neurodegeneration, α-synuclein aggregation, mitochondrial dysfunction and oxidative stress (Bido et al., 2017). While therapeutic manipulation of mitochondrial dynamics shows great promise, it will require greater understanding to be effectively exploited.

Mitochondrial motility issues are also common in PD (Smith and Gallo, 2018). Simplistically, mitochondrial trafficking is subject to pernicious cause or consequence cycles, such as (1) reduced energy from mitochondrial dysfunction causing disorganisation of microtubule networks and α-synuclein protein aggregation, or (2) α-synuclein deposition impairing mitochondrial dynamics and axonal trafficking leading to chaotic organellar distribution, synaptic accumulation of autophagosomes and mitochondrial dysfunction (Esteves et al., 2014; Pozo Devoto and Falzone, 2017). The peptide Davenutide has been shown to be neuroprotective in sporadic PD cybrids by stabilising microtubule structure, thus restoring microtubule trafficking, organellar distribution, autophagic flux, mitochondrial membrane potential, as well as reducing α-synuclein accumulation and mitochondrial ubiquitination (Esteves et al., 2014). More recently it has been identified that mitochondria can be transferred between cells, which may be a mechanism for reducing cellular stress, either by enhancing energy production through supplementation of functional mitochondrial to a dysfunctional network or by removing defective organelles from cells with compromised quality control pathways (Shanmughapriya et al., 2020). As such, intercellular mitochondrial transfer may be impaired in PD and could be a worthwhile therapeutic approach. However, this will need careful

consideration as it has also been proposed as a potential route by which the neuronal propagation of α-synuclein deposition could occur (Valdinocci et al., 2019).

Pharmacological and genetic targeting of mitochondrial dynamics pathways has been investigated in a number of different disease models (Whitley et al., 2019), yet attempts in PD are limited. Given the complex architecture and minimal metabolic plasticity of neurons, as well as the predominance of mitochondrial dynamics and trafficking defects in genetic and sporadic PD, the preservation, restoration or optimisation of these functions is an important therapeutic consideration for PD and requires further intensive research.

Addressing Mitochondrial Calcium and Metal Ion Dyshomeostasis

Mitochondrial dysfunction can lead to excitotoxicity through a reduction in cellular ATP levels, an increase in cellular Ca^{2+}, or both (Ludtmann and Abramov, 2018). Inhibition of C.I, and consequently ATP generation, lowers intracellular ATP, leading to partial neuronal depolarization due to a reduction in the activity of Na^{+}/K^{+}-ATPase (Ludtmann and Abramov, 2018). Mitochondria can take up Ca^{2+} from the cytosol via a uniporter transporter, which relies on the mitochondrial membrane potential. The ROS generated by mitochondrial respiratory chain dysfunction can damage the mitochondrial membranes and disrupt this mechanism of Ca^{2+} uptake and storage, thereby raising intracellular Ca^{2+} levels and exacerbating the excitotoxicity (Carbone et al., 2017). Disruption in the mitochondrial membrane potential leads to an increased susceptibility to Ca^{2+} overload. This suggests that mitochondrial-driven excitotoxicity is a major contributory factor in PD (Carbone et al., 2017). Currently, there are no mechanistic treatment approaches available. Based on the ubiquitous role of Ca^{2+} for neuronal signalling, it is unlikely that this pathway will be currently considered as a viable treatment target. However, this might change as we gain a more in-depth insight into the crosslink of mitochondria-related Ca^{2+}-dyshomeostasis and the specific vulnerability of dopaminergic neurons.

Mitochondria are responsible for regulating intracellular metal ion [iron (Fe), copper (Cu), and zinc (Zn) levels], their macromolecular organisation (e. g. by forming Fe-Sulphur cluster or heme groups) and their respective distribution in cells (Mezzaroba et al., 2019). Preclinical and human models imply that impaired mitochondrial homeostasis leads to Fe accumulation and Cu deficiency *in vivo* (Tarohda et al., 2005). The SN of PD brains contain increased Fe and decreased Cu levels, which might link the pathology to mitochondrial dysfunction (Tarohda et al., 2005). The increased Fe levels in PD are mainly restricted to the mitochondria (Munoz et al., 2016). A hemiparkinsonian model in monkeys treated with MPTP showed an increase in the nigral iron deposition (Mochizuki et al., 1994), suggesting a functional link between metal ion dyshomeostasis and mitochondrial dysfunction. Also, in these models the Cu level decreased accordingly. One theory assumes that the inhibition of C.I increases the ROS level, which damages the Fe-Sulphur cluster, hinders their proper assembly, and forces the transport of Fe to the mitochondria (Liang and Patel, 2004; Mena et al., 2011). This mechanism results in a vicious circle as the mismatch of divalent and trivalent Fe promotes the formation of oxidative stress. Increased nigral Fe deposition also leads to decreased glutathione levels, which resembles another level of the interconnectedness of mitochondrial dysfunction and Fe-dyshomeostasis (Lee et al., 2009). Chelating agents that can cross the blood-brain barrier (such as deferiprone or deferoxamine) can redistribute Fe ions also on a cellular level (Kakhlon et al., 2010). Deferiprone leads to decreased nigral iron deposition, however, whether decreased Fe deposition also leads to improved mitochondrial function (e. g, by improving mitochondrial bioenergetics) in humans is currently unknown.

The pathophysiological role of Cu in the pathogenesis of PD is only poorly understood. The supplementation of Cu (by Cu-sulphate) seems to be protective against MPTP-induced toxicity in animal models (Rubio-Osornio et al., 2017). Paradoxically, administration of the sequestering Cu-chelator D-penicillamine protects mice from MPTP-related mitochondrial toxicity. In summary, the evaluation of Cu-targeted treatment approaches is limited due to conflicting experimental data and the unknown pathophysiological function.

Zn is likewise disturbed in PD (Park et al., 2015). *ATP13A2*-related parkinsonism may be caused by mitochondrial Zn-aggregation (Park et al., 2014). Besides, mitochondrial toxins have been shown to lead to increased Zn-aggregation in *in-vitro* models (Park and Sue, 2017). Whether treatment regimens targeting Zn provide a fruitful approach for future studies in mitochondrial PD is currently unknown.

In conclusion, preliminary evidence suggests targeting the redistribution of metal ions may be beneficial in PD, with potential neuroprotective effects involving mitochondrial function.

Targeting the Intersection of Neuroinflammation, Innate Immunity, and Mitochondrial Dysfunction

There is evidence that *LRRK2* mutations contribute to immune alterations both in peripheral organs and the brain, although our understanding of the underlying mechanism is incomplete (Wallings et al., 2020). For example, numerous studies have established that mutations in *LRRK2* confer susceptibility to mycobacterial infection, implying that LRRK2 plays a role in regulating immunity (Zhang et al., 2009; Wang et al., 2015, 2018; Fava et al., 2016).

A recent study demonstrated that loss of LRRK2 in macrophages led to elevated basal levels of type I interferon and interferon stimulated genes, resulting in an attenuated interferon response to mycobacterial pathogens and cytosolic nucleic acid agonists (Weindel et al., 2020). Altered innate immune gene expression in *LRRK2* knockout macrophages is driven by mitochondrial stresses, such as oxidative stress from low levels of purine metabolites and Drp1-dependent mitochondrial fragmentation. This subsequently promotes mtDNA leakage into the cytosol and chronic cyclic GMP-AMP synthase engagement.

Although *LRRK2* knockout mice can control Mycobacterium tuberculosis replication, they have exacerbated inflammation and lower interferon stimulated gene expression in the lungs. Thus, while LRRK2 inhibitors are candidate therapies in PD, they may have deleterious outcomes on immune responses. Specifically, they may affect the function of microglia, which are essential for healthy neurons (Weindel et al., 2020).

Sirtuins

Sirtuins are a family of NAD+ dependent protein deacetylases, with several other enzymatic capabilities (Wang Y. et al., 2019). Sirtuins are associated in general with metabolic regulation and longevity, but are also important in various disease states, including cancer, diabetes, and neurodegeneration (Wang Y. et al., 2019). PD is hallmarked by protein misfolding, mitochondrial dysfunction, oxidative stress and neuroinflammation, all of which are mediated by sirtuins (Zhang et al., 2020). It is therefore reasonable to assume that PD could be modulated by differentially targeting the three predominant sirtuins (SIRT1, 2, and 3), which are of primary relevance to mitochondrial dysfunction in PD (Lin et al., 2018).

In general, SIRT1 and SIRT3 activity is considered neuroprotective in PD, whereas SIRT2 activity appears to be contradictory. In this regard, the focus of therapy has been on enhancing SIRT1 and SIRT3 and inhibiting SIRT2 (Tang, 2017).

Despite the clear involvement of sirtuins in PD, there is a distinct lack of clinical trials investigating their modulation for treatment of neurodegeneration (Bonkowski and Sinclair, 2016; Mautone et al., 2020). This is largely due to a lack of isoform specific inhibitors and activators and the need to target specific cells to avoid unintended effects. Resolution of current controversies and a greater understanding of the tissue-specific functions, as well as the complexity and interconnectedness of sirtuin interaction and activity networks is required to optimally target these molecules (Dang, 2014; Wang Y. et al., 2019; Mautone et al., 2020; Wang et al., 2020).

miRNAs

Micro RNAs (miRNAs) are non-coding RNA fragments approximately 22 nucleotides in length that modify gene expression. There is considerable complexity in miRNA mediated gene expression given that one gene can be regulated by many miRNAs and one miRNA can influence many genes (Titze-de-Almeida et al., 2020). There is a plethora of literature demonstrating dysregulation of numerous miRNAs in association with PD (Martinez and Peplow, 2017), with consistencies emerging between studies (Goh et al., 2019; Schulz et al., 2019). In fact, most miRNAs associated with PD appear to be linked to mitochondria (Sun et al., 2019; John et al., 2020).

As an example, miRNA181a/b have the capacity to influence mitochondrial biogenesis and quality control, respiratory chain assembly and mitochondrial antioxidants, with downregulation of miRNA181a/b found to be neuroprotective in mitochondrial disorders with neurodegeneration, suggesting a broad gene-independent therapeutic potential for diseases with mitochondrial dysfunction, such as PD (Indrieri et al., 2019).

Nevertheless, a better understanding of the complex positive and negative interplay between different miRNAs and their associated genes is essential. There is therapeutic potential in controlling miRNA expression to modify various mitochondrial pathways, including bioenergetics, biogenesis, oxidative stress, mitophagy, and even cell death (Indrieri et al., 2020). There is also the prospect of developing diagnostic tests, particularly with miRNAs such as miR34b/c, which is altered in the brains of PD patients, even in early pre-motor stages (Minones-Moyano et al., 2011; Cressatti et al., 2020; Ravanidis et al., 2020).

ER-mitochondrial Links

Mitochondria and the endoplasmic reticulum (ER) are intimately linked by specialised mitochondria-ER contact sites (MERCs), which act as foci of enzymatic activity (Flis and Daum, 2013; Friedman et al., 2018), communication and signalling portals (Takeda and Yanagi, 2019; Reane et al., 2020), and initiation sites (Gelmetti et al., 2017; Elliott et al., 2018). Interactions via MERCs mediate diverse functions, including lipid biosynthesis, mitochondrial dynamics, Ca^{2+} homeostasis, bioenergetics, mitochondrial trafficking, protein folding, ER stress response, autophagy/mitophagy, apoptosis, and inflammation (Gomez-Suaga et al., 2018).

Disruption of MERCs is associated with several neurodegenerative diseases, including PD (Paillusson et al., 2016; Erpapazoglou et al., 2017). A number of proteins associated with mPD (α-synuclein, DJ-1, PINK1, Parkin, LRRK2) are concentrated at MERCs under normal conditions where they mediate diverse, often non-standard, functions (Ottolini et al., 2013; Guardia-Laguarta et al., 2014; Gautier et al., 2016; Gelmetti et al., 2017; Basso et al., 2018; Parrado-Fernandez et al., 2018; Toyofuku et al., 2020), and PD-causing mutations in these proteins are associated with disrupted mitochondria-ER contact and communication.

Although incompletely understood, the contribution of mitochondria-ER dysfunction in PD pathogenesis is evident and targeting MERCs could be of therapeutic benefit. As MERC disruption seems to be ubiquitous across neurodegenerative diseases, any therapies may come from or have utility in several diseases.

NIX

A putative protective mechanism against PD was identified in an asymptomatic compound heterozygous *PRKN* mutation carrier (Koentjoro et al., 2012). The compound heterozygous (c.8_171del/c.535_871del, p.V3EfsX3/p.G179LfsX7) mutation carrier had not developed PD by her seventh decade despite a complete loss of functional Parkin (Koentjoro et al., 2012). Cells from the asymptomatic carrier showed intact mitochondrial function and mitophagy-mediated by mitochondrial receptor Nip3-like protein X (Nix) (Koentjoro et al., 2017). Furthermore, PINK1 knockdown did not affect Nix-mediated mitophagy. Genetic and pharmacological induction of Nix was able to reestablish mitophagy in *PINK1*- and *PRKN*-related PD patient cell lines, and *Nix* over-expression resulted in an improvement in mitochondrial ATP production. Therefore, Nix could be an alternative mediator of mitophagy and could serve as a

neuroprotective therapy in PD due to *PINK1* or *PRKN* mutations (Koentjoro et al., 2017; Naeem et al., 2020).

Mitochondrial Transplantation in PD

Improving mitochondrial function by supplementing exogenous mitochondria is a promising strategy in PD (Shanmughapriya et al., 2020). A recent study compared the functionality of mitochondrial transfer with or without Pep-1 conjugation in 6-OHDA-induced PC12 cells and PD rat models (Chang et al., 2016). The investigators injected mitochondria into the medial forebrain bundle of PD rats following a unilateral 6-OHDA lesion. They showed that only peptide-mediated allogeneic mitochondrial delivery with allogeneic and xenogeneic sources preserved mitochondrial function against neurotoxin-induced oxidative stress and apoptotic death in the rat PC12 cells. Additionally, allogeneic and xenogeneic transplantation of peptide-labelled mitochondria improved the locomotive activity in the PD rats (Chang et al., 2016). Another study used a MPTP-induced PD rat model to demonstrate that mitochondria injected intravenously can prevent progression of PD by increasing the activity of the electron transport chain, reducing reactive oxygen species level, and restricting cell apoptosis and necrosis (Shi et al., 2017). These studies support the therapeutic strategy of mitochondrial supplementation injected either directly into the brain or intravenously. The success of mitochondrial transplantation is likely dependent upon the source and quality of the isolated mitochondria, the delivery protocol, as well as the cellular uptake of supplemental mitochondria to ensure adequate neuronal uptake within the brain (Chang et al., 2019).

Stem Cell Therapies in PD Addressing Mitochondrial Dysfunction

Astrocytes derived from human iPSCs may be therapeutic in PD via the provision of a continuous supply of healthy mitochondria as a form of mitochondrial donation (Cheng et al., 2020). In favour of this concept, a recent study used a rotenone-induced *in vitro* PD model to demonstrate that iPSCs-derived astrocytes or astrocytic condition media can rescue dopaminergic neurons through intercellular mitochondrial transfer (Cheng et al., 2020).

Soluble Epoxide Hydrolase Inhibition

There are multiple lines of evidence suggesting that soluble epoxide hydrolase (sEH) deficiency or inhibition can attenuate parkinsonism in MPTP-treated mice (Qin et al., 2015; Pallas et al., 2020). For example, deficiency and inhibition of sEH attenuates the loss of TH-positive cells and improves rotarod performance (Qin et al., 2015). The substrate of sEH, 14,15-epoxyeicosatrienoic acid (14,15-EET), protected TH-positive cells and alleviated the rotarod performance deficits of wild-type mice, but not sEH-knockout mice. Furthermore, the 14,15-EET antagonist [14,15-epoxyeicosa-5(Z)-enoic acid] abolished the neuronal protective effects of sEH deficiency. In primary cultured cortical neurons, MPP^+ induces Akt inactivation in neurons from sEH wild-type mice, but not in neurons from knockout mice. This indicates that sEH deficiency and inhibition can increase 14,15-EET in MPTP-treated mice, thereby activating the Akt-mediated protection of TH-positive neurons and behavioural functioning. Thus, sEH inhibition might be a powerful tool to protect dopaminergic neurons in PD (Qin et al., 2015). In addition to having anti-inflammatory properties, EETs may have numerous benefits such as antioxidant effects, a reduction in mitochondrial dysfunction and apoptosis and improved cerebral blood flow (Pallas et al., 2020).

Photobiomodulation

Photobiomodulation (PBM) refers to the use of light in the red to infrared wavelength range to enhance mitochondrial function by displacing nitric oxide, which competitively binds the molecular oxygen site on C.IV of the respiratory chain (Hamblin, 2018; Quirk and Whelan, 2020). Nevertheless, when applied to stressed cortical neurons with reduced mitochondrial membrane potential, PBM returned the membrane potential to normal, thereby reducing ROS production and excitotoxic Ca^{2+} levels (Huang et al., 2013, 2014), where the effects of PBM on mitochondrial function have been found to be both dose and time dependent (Silveira et al., 2019). In addition to enhancing neurometabolism, PBM has also been found to initiate anti-inflammatory, antioxidant, and anti-apoptotic pathways (Salehpour et al., 2018).

To date over 30 *in vitro* and *in vivo* studies of PBM in PD models have explored variations on the theme (Hong, 2019; Salehpour and Hamblin, 2020). The most comprehensive and compelling research on the neuroprotective effect of PBM in PD has come from studies of transcranial and intracranial PBM in chemically-induced rodents (Peoples et al., 2012; Shaw et al., 2012; Moro et al., 2013, 2014; Purushothuman et al., 2013; O'Callaghan et al., 2015; Reinhart et al., 2015, 2016a,b; Reinhart et al., 2017) and non-human primate PD models (Moro et al., 2016, 2017; El Massri et al., 2017). Evidence suggests that PBM need not be directly applied to neurons, instead showing that non-invasive remote PBM treatment (i.e., to extremities or the abdomen) has a so-called "abscopal" neuroprotective effect (Johnstone et al., 2014; Kim et al., 2018), although the precise mechanisms are still uncertain.

One human pilot trial and one clinical trial of transcranial and intranasal PBM have been conducted to date, which have shown improvements in speech, cognition, gait, and freezing episodes in patients with established PD (Hamilton et al., 2019; Santos et al., 2019). However, the utility of PBM neuroprotection relies on there being neurons to protect in the first instance (Yang et al., 2020), meaning this approach would likely be useful in the early stages of PD with sustained use to moderate disease progression (Foo et al., 2020).

DISCUSSION

The current clinical diagnosis of Parkinson's disease (PD) is primarily based on the appearance of motor symptoms (Postuma et al., 2015). However, phenotypic changes often manifest several years earlier, driven by early pathogenic processes in a so-called "prodromal" phase (Berg et al., 2015). Symptoms presenting in the prodromal stage are becoming more widely appreciated and highlight a period that may offer a unique window of opportunity for neuroprotective treatment regimens to be administered to

avoid or mitigate severe pathology (Postuma and Berg, 2019). However, the underlying aetiology and pathophysiology of PD is complex, hindering the translation of research insights into improved clinical outcomes (Grunewald et al., 2019).

Mitochondrial dysfunction plays a central, multifaceted role in the pathogenesis of PD (**Figure 3**), whether cause or consequence. It therefore represents an ideal target for candidate drug therapies as its modulation will undoubtedly have an impact on disease progression. However, to date, there has been limited success for clinical trials targeting mitochondrial pathways in PD (**Supplementary Table 2**). This may be due to a failure to address the multiple interconnected systems and pathways in the highly sensitive homeostatic cellular systems of neurons. Furthermore, it may be difficult to ensure adequate delivery of the drugs to their site of action. Moreover, disease progression in these trials may be too advanced to regain sufficient mitochondrial and therefore target cellular function.

The future of neuroprotective trials will likely rely on combinations of drugs acting directly and indirectly on affected pathways using personalised precision medicine (Titova and Chaudhuri, 2017). Furthermore, it will be necessary to enrich study cohorts for participants that display remarkable dysfunction in these respective pathways and who might benefit the most from targeted treatment approaches (Redensek et al., 2017). It would be helpful to identify these participants early in the prodromal phase to be able to treat prior to advanced stages with neuronal loss. Within this scope, targeting the right patients, with the right treatments, at the right time is the optimal paradigm for clinical PD research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.615461/full#supplementary-material

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REVIEW
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Mitochondrial Dysfunction in Astrocytes: A Role in Parkinson's Disease?

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Mitochondrial dysfunction is a hallmark of Parkinson's disease (PD). Astrocytes are the most abundant glial cell type in the brain and are thought to play a pivotal role in the progression of PD. Emerging evidence suggests that many astrocytic functions, including glutamate metabolism, Ca^{2+} signaling, fatty acid metabolism, antioxidant production, and inflammation are dependent on healthy mitochondria. Here, we review how mitochondrial dysfunction impacts astrocytes, highlighting translational gaps and opening new questions for therapeutic development.

Keywords: mitochondria, astrocyte, Parkinson's disease, inflammation, cGAS/STING pathway, NLRP3, PINK1/Parkin pathway

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (AD) and the most common movement disorder worldwide (Dorsey et al., 2018). PD affects approximately 1 million Americans alive today, and the US National Institute of Neurological Disorders and Stroke (NINDS) predicts that 50,000 new cases of Parkinson's disease are diagnosed in the US each year (Marras et al., 2018). With the aging of the Western World, the burden of this disease is set to rise tremendously over the next decade. Clinically, PD is primarily characterized by tremor, bradykinesia, rigidity, and postural instability. The main pathological hallmarks are degeneration of dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc) and presence of Lewy bodies and neurites that consist mainly of α-synuclein (α-syn) aggregates. In addition to dysfunctional α-syn proteostasis, neuroinflammatory glial activation, mitochondrial dysfunction, and oxidative stress have also been implicated in PD pathogenesis.

PD is mostly an idiopathic disorder with an age-related increase in incidence. Historically, exposure to pesticides and viruses have been linked to increased incidence of disease; however, lack of geographic clusters with epidemiological studies goes against environmental toxins or viral infections as being the primary cause of sporadic PD (Pang et al., 2019). Identification of rare autosomal dominant and recessive forms of PD in the 1990s suggested a broad contribution of genetics to PD etiology. Recent genome-wide association studies (GWAS) have provided new genetic insights into the disease etiology, strengthening the possibility of specific gene variants playing a role in PD pathogenesis (Nalls et al., 2019). To date, there are 90 significant known independent genome-wide risk signals that explain 16–36% of PD heritability, leaving a large portion of cases unexplained (Nalls et al., 2019). Research is just beginning to elucidate coalescing molecular pathways and mechanisms among different forms of PD, and accumulating

evidence suggests that PD is linked to combinatorial interactions between genetic risk factors, pathogens, exposure to environmental toxins, and aging. Thus, aging, genetics, and environmental stressors each alone are unlikely to initiate PD but together may be able to induce disease (Johnson et al., 2019).

Importantly, several lines of evidence converge on mitochondrial dysfunction as a common central pathway that could integrate the pathobiological processes of sporadic and genetic PD (Cabezas et al., 2018; De Miranda et al., 2018; Gegg and Schapira, 2018; Sliter et al., 2018; Sun et al., 2018; Pang et al., 2019; Russo et al., 2019). Toxins like paraquat and rotenone have been linked to PD and act on the mitochondrial respiratory chain. Genetic studies have shown that loss of function mutations in the gene PINK1, a mitochondrial kinase, and Parkin, a cytoplasmic E3 ubiquitin ligase, cause autosomal recessive PD. PINK1 and Parkin work together in a pathway to remove damaged mitochondria by mitophagy (Pickrell and Youle, 2015). Mitophagy is the selective degradation and clearance of defective mitochondria by autophagy following mitochondrial damage or stress (Pickles et al., 2018). Mitophagy can eliminate dysfunctional mitochondria to maintain mitochondrial homeostasis and protect against neuroinflammatory activation induced by ROS and pathogen-/damage-associated molecular patterns (PAMPs/DAMPs) (Youle, 2019). Moreover, among the 90 risk alleles identified in previous GWAS studies, many of the risk variants seem to directly and indirectly impact cellular degrading pathways and other pathways related to mitochondrial functions. Finally, aging is a major risk factor, and mitochondrial dysfunction is a hallmark of aging. Thus, one hypothesis is that excessive mitochondrial damage, as observed in PINK1 and Parkin mutants, likely contributes to the degeneration of the nigrostriatal system.

Increasing evidence suggests that astrocytes play a significant role in the progression of PD (Liddelow and Barres, 2017; Clarke et al., 2018; Bantle et al., 2019; Caggiu et al., 2019; di Domenico et al., 2019; Filippini et al., 2019; Harischandra et al., 2019). Within the central nervous system (CNS), astrocytes represent over 30% of all cells and are the most abundant cell type in the brain. While early descriptions of these cells labeled them as the "glue of the brain" with a primarily passive structural role, contemporary research is shedding light to many more functions of glia in the developing and adult brain. Multiple risk alleles that have been identified in the most recent GWAS studies and previous candidate gene studies show cellular penetrance in astrocytes (Booth et al., 2017). Astrocytes function to support neuronal homeostasis, participate in the maintenance of the blood–brain barrier (BBB), and are dynamic regulators of the neuronal synaptic communication and cerebral blood flow. They also provide continuous trophic support and energy metabolism to neurons by secreting glial-derived neurotrophic factor (GDNF), regulating extracellular ion balance in the CNS, and shuttling lactate and glutamine to neurons (Sofroniew and Vinters, 2010). Additionally, although microglia have been previously thought of as the primary inflammatory cell in the CNS, inflammatory activation of astrocytes is often more persistent than microglia and is believed to be important in chronic inflammatory activation associated with PD (Saijo et al., 2009).

Recent research indicates that mitochondria regulate essential astrocyte functions, including glutamate regulation, Ca^{2+} signaling, fatty acid metabolism, transmitophagy, antioxidant production, and neuroinflammatory activation (De Miranda et al., 2018; Mouton-Liger et al., 2018; Sliter et al., 2018; Ho et al., 2019; Pang et al., 2019; Russo et al., 2019). Therefore, one hypothesis is that mitochondrial dysfunction in astrocytes impacts dopaminergic neuronal health through a gain of inflammatory function but also via loss of supportive functions, including trophic and antioxidant support. Preserving astrocytic mitochondrial function therefore may represent a disease-modifying a to slow the progression of PD. In this review, we discuss precisely how mitochondrial dysfunction in astrocytes may contribute to Parkinson's disease and suggest new avenues for therapeutic development.

ASTROCYTE MITOCHONDRIAL FUNCTION AND DYSFUNCTION IN PD

Mitochondria are highly dynamic organelles with a plethora of functions, including regulation of calcium homeostasis, energy metabolism, and inflammatory activation (Winklhofer and Haass, 2010). While the majority of research investigating mitochondrial dysfunction in PD has been focused on neurons, recent studies suggest that mitochondrial dysfunction in astrocytes likely play a role in PD as well. Key astrocyte functions such as glutamate–glutamine cycle, regulation of Ca^{2+} metabolism, fatty acid metabolism, and regulation of innate immunity are dependent on functional mitochondria. Genes implicated in autosomal-recessive PD such as DJ-1 are highly expressed in astrocytes. Moreover, astrocytes can play a role in mitochondrial quality control of striatal axons via transmitophagy. While the field is still emerging, collective evidence presented here suggests that dysfunctional mitochondria in astrocytes can play a pivotal role in the progression of PD. The following sections of this review highlight the current literature supporting mitochondrial dysfunction in astrocytes as a contributing factor in PD pathophysiology.

Astrocyte Mitochondria and Glutamate Metabolism

Magnetic resonance spectroscopy has shown disbalances in GABA-ergic as well as glutamatergic signaling in the thalamus, pons, basal ganglia, substantia nigra, and cortical regions in PD patients. Cortical glutamatergic and substantia nigra dopaminergic afferents converge onto the dendrites of medium spiny neurons in the striatum/caudate putamen where they act to modulate motor and cognitive functions (Mahmoud et al., 2019), and many so-called "axial" motor symptoms appear as a consequence of dysregulations in GABA/glutamatergic neurotransmitter systems (O'Gorman Tuura et al., 2018). One of the fundamental tasks of all astrocytes, including those in the striatum, is glutamate reuptake via glutamate uptake

transporters, such as excitatory amino acid transporter (EAAT). Exacerbated activation of glutamate receptors can lead to excitotoxicity, and the balance between physiological and toxic levels of glutamate are largely controlled by astrocytes at the level of the synaptic cleft (Armada-Moreira et al., 2020). After synaptic release of glutamate, it is estimated that only 20% is absorbed by postsynaptic neurons, while the majority diffuses out of the synaptic cleft for uptake by EAAT-1 and EAAT-2 transporters in astrocytes. Upon entry in the astrocyte, glutamate is metabolized into α-ketoglutarate (α-KG) and glutamine (Schousboe et al., 2013). The glutamate dehydrogenase enzyme [alpha-ketoglutarate-dehydrogenase complex (KGDHC)], which resides in the mitochondria, catalyzes the conversion of glutamate into α-KG (**Figure 1.1a**). KGDHC is inhibited by the mitochondrial toxin MPP+, known to induce Parkinson's-like phenotypes and also acts a major source of reactive oxygen species (Starkov et al., 2004). The levels of KGDHC, but not much the levels of complex II or IV of the mitochondrial respiratory chain, have been reported to be decreased in basal ganglia of PD patients (Mizuno et al., 1994), raising the possibility that the metabolism of glutamate via KGDHC plays a role in the progression of the disease.

Glutamate can also be converted by astrocytes into glutamine via the glutamine synthetase enzyme. Glutamine is transported to presynaptic terminals via glutamine transporters to be converted back to glutamate by the mitochondrial enzyme glutaminase (Ortinski et al., 2010; Schousboe et al., 2013). The dysregulation of glutamine cycle is caused by reactive astrocytosis. Experimentally induced astrocytosis, by high-titer AAV2/5-GFP transduction, induced deficits in inhibitory signaling in the mouse hippocampus and enhanced excitability as a consequence to the downregulation of astrocytic glutamine synthetase (Ortinski et al., 2010). Importantly, mitochondrial dysfunction in astrocytes induces alterations in glutamate metabolism and excitotoxicity. In an elegant study, Murru and colleagues recently showed that an astrocyte-specific deletion of mAAA protease, an enzyme involved in mitochondrial quality control and proteostasis, resulted in aberrant astrocyte morphology, altered expression of EAAT-2, and a reactive inflammatory signature (Murru et al., 2019; **Figure 1.1b**), pointing to a common mechanism. A recent study has suggested an additional link of glutamate dyshomeostasis to PD (Vallerga et al., 2020), where hypermethylation in PD is associated with downregulation of the SLC7A11 gene. SLC7A11 codes for a cysteine-glutamate antiporter, which is predominantly expressed by astrocytes in the brain, and regulates levels of the antioxidant glutathione. This study focused on genome-wide blood-based DNA methylation data, and further experiments are required to determine if there is similar epigenetic control in the brain. However, reduced levels of glutathione have been reported in the substantia nigra in PD patients compared with aged-matched controls (Sian et al., 1994), which could be attributed to a pathological downregulation of the cysteine–glutamate antiporter, potentially via this mechanism. Therefore, alterations in the levels of glutamate and its metabolic intermediaries, as seen in PD, can be one of the pathological outcomes of astrocytic mitochondrial dysfunction.

Astrocyte Mitochondria and Intracellular Ca^{2+} Regulation

One of the key features of mitochondria is their ability to regulate cellular Ca^{2+} concentrations (Bagur and Hajnoczky, 2017). A major source of mitochondrial Ca^{2+} is the endoplasmic reticulum (ER), where Ca^{2+} efflux is regulated by canonical G-coupled receptor/inositol triphosphate (IP3) signaling pathway. The IP3-gated Ca^{2+} efflux in astrocytes can be negatively regulated by the inositol triphosphate kinase ITPKB—a kinase that phosphorylates the 3′ position of inositol-1,4,5-triphosphate (IP_3) to generate inositol 1,3,4,5 tetrakisphosphate (IP_4) (Communi et al., 1999; Miller et al., 2015). The mitochondrial transmembrane protein voltage-dependent anion-selective channel (VDAC) mediates the transfer of Ca^{2+} to the mitochondrial intermembrane space. Once Ca^{2+} enters the mitochondria, the ion is channeled to the matrix via the mitochondrial calcium uniporter (MCU) complex. MCU resides in the mitochondrial inner membrane and consists of the MCU transmembrane channel and two regulatory subunits, MICU1 and MICU2. A brain/specific isoform (MICU3) has also been described (Kamer et al., 2018). Notably, recent GWAS studies have identified a signal in the ITPKB locus as well as the MICU3 locus as risk factors for Parkinson's disease (Chang et al., 2017; Nalls et al., 2019). Given that both ITPKB and MICU3 are expressed in astrocytes, it is plausible that dysregulated mitochondrial calcium uptake in astrocytes can play a role in the progression of PD (**Figure 1.2**). The precise contribution of intracellular Ca^{2+} stores to overall Ca^{2+} signaling in astrocytes, especially in astrocyte processes, is nevertheless controversial. Ca^{2+}-dependent release of gliotransmitters have been implicated in learning and memory, but knockout of IP3R2, the main isoform found in astrocytes, produced no changes in anxiety or motor behavior, and no changes in learning and memory were observed (Petravicz et al., 2014). Extracellular Ca^{2+} in the astrocyte processes can arrive via transient receptor potential A1 channels (Shigetomi et al., 2011), reverse sodium-calcium exchange (Gerkau et al., 2018), and perhaps N-methyl-D-aspartate receptors (NMDARs) (Stephen et al., 2015).

Miro1/RhoT1 is a component of the mitochondrial motor/adaptor complex with Ca^{2+} EF sensing hands and has been shown to participate in the localization of mitochondria close to sources of extracellular Ca^{2+} (Macaskill et al., 2009; Wang and Schwarz, 2009). In an elegant study, Stephen et al. showed that Miro1 also localizes mitochondria adjacent to sources of extracellular Ca^{2+} in astrocytic processes (Stephen et al., 2015). Interestingly, Miro1/RhoT1 is a target of the PINK1/Parkin pathway for mitochondrial quality control (Wang et al., 2011; Shlevkov et al., 2016), and defective proteostasis of Miro1 has been observed in human-induced pluripotent stem cell (hIPSC) lines derived from PD patients (Hsieh et al., 2019). It is possible that defective Miro1 turnover in PD astrocytes can lead to disbalanced Ca^{2+} signaling in astrocytic processes (**Figure 1.3**).

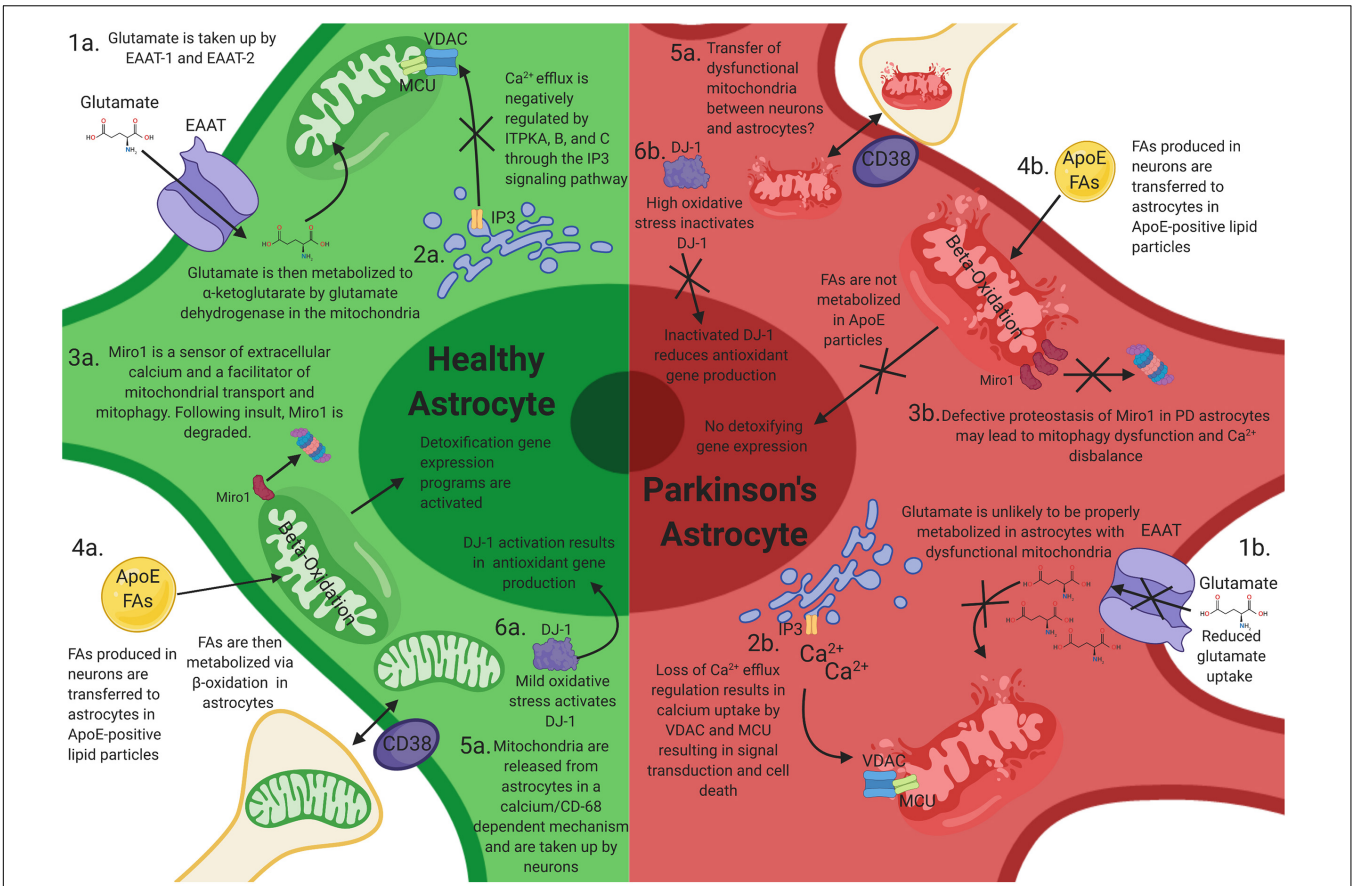

FIGURE 1 | How mitochondrial dysfunction in astrocytes can contribute to Parkinson's disease (PD) progression. Mitochondrial dysfunction in astrocytes may elicit neuronal toxicity through multiple mechanisms. **(1a)** Functional astrocyte mitochondria are needed for glutamate regulation and metabolism. **(1b)** Dysfunctional astrocyte mitochondria likely have reduced glutamate uptake and metabolism, resulting in excitatory neurotoxicity in neurons. **(2a)** Astrocytes house large stores of intracellular calcium and other ions, which is negatively regulated by ITPKA, B, and C. **(2b)** Perturbations of these calcium reservoirs results in an increase in mitochondrial intracellular calcium through VDAC1 and MCU, resulting in signal transduction and astrocytic cell death. **(3a)** Miro1 is a sensor of extracellular calcium and a facilitator of mitochondrial transport and mitophagy. Following mitochondrial stress, Miro1 is degraded, which facilitates the clearance of damaged mitochondria. **(3b)** Defective proteostasis of Miro1 in PD astrocytes may lead to mitophagy dysfunction and Ca^{2+} disbalance. **(4a)** Toxic fatty acids (FAs) are produced in neurons and are transferred to astrocytic lipid droplets by ApoE-positive lipid particles. Astrocytes then consume the FAs stored in lipid droplets via mitochondrial β-oxidation in response to neuronal activity and turn on a detoxification gene expression program. **(4b)** Loss of mitochondrial function in astrocytes prevents proper metabolism of these FAs and results in FA induced toxicity **(4b)**. **(5a,b)** Astrocytes serve as the primary cell type responsible for the clearance and transfer of damaged and healthy mitochondria to and from neurons. The transfer is completed in a calcium-dependent manner, regulated by CD38. During transmitophagy, mitophagy is thought to begin in neurons and be completed in astrocytes. **(6a)** Mild mtROS induces DJ-1 activation, which results in antioxidant gene transcription. **(6b)** High levels of mtROS inactives DJ-1 and prevents antioxidant gene transcription.

Astrocytes *in vivo* typically show cellular structures called lamellar sheets as well as peripheral astrocytic processes, both of which contain mitochondria (Jackson and Robinson, 2018). Spontaneous, cell-autonomous Ca^{2+} spikes have been recently observed in these microdomains (Khakh, 2019). It is plausible that Ca^{2+} transients can regulate glucose mobilization (Howarth, 2014) and influence the activity of neighboring neurons and glia by the release of ATP, D-serine, and glutamate (Haydon, 2001). Astrocyte Ca^{2+} transients have been shown to occur more frequently following CNS injury (Kuchibhotla et al., 2009). Importantly, mitochondria are the key mediator of spontaneous Ca^{2+} increases in astrocytes *in vivo* independently of Ca^{2+} release from ER stores in a mechanism that involves the transient opening of the permeability transition pore (mPTP) (Agarwal et al., 2017). Mitochondrial Ca^{2+} uptake in astrocytes is not only involved in homeostatic regulation of astrocytic functions but also plays a role in the astrocyte response to acute injury. Gbel at el. recently found that acute injury and blood–brain barrier disruption trigger the formation of a prominent mitochondria-enriched compartment in astrocytic endfeet, enabling vascular remodeling (Gbel et al., 2020). Vascular remodeling in this model was dependent on mitofusin 2 and mitochondria–ER contact sites. These structural changes were mirrored by impaired mitochondrial Ca^{2+} uptake leading to abnormal cytosolic transients within endfeet. Since mitochondrial remodeling happens as a general response to injury in astrocytes (Motori et al., 2013), the precise cellular mechanisms of astrocyte reaction to injury via mitochondria may also be relevant for PD.

Finally, Ca^{2+} transients in astrocytic processes have also been implicated directly in synaptic transmission (Di Castro et al., 2011; Panatier et al., 2011); however, whether mitochondrial Ca^{2+} uptake can directly regulate synaptic transmission has yet to be established. Taken together, these studies suggest that mitochondrial dysfunction in astrocytes can lead to perturbations of Ca^{2+}-mediated astrocyte functions as well as reinforce a maladaptive response to injury. Emerging evidence suggests that specific alterations of ITPKB, MICU3 and Miro1 functions are predicted to result in altered mitochondrial Ca^{2+} handling in astrocytes and thereby contribute to PD progression.

Astrocyte Mitochondria and Fatty Acid Metabolism

Mitochondria also serve as a metabolic hub in the cells, and metabolic coordination between neurons and astrocytes is critical for the health of the brain. Recent research from Jie Liu's laboratory has shown that toxic fatty acids (FAs) produced in hyperactive neurons are transferred to astrocytic lipid droplets by ApoE-positive lipid particles (Ioannou et al., 2019). Astrocytes then consume the FAs stored in lipid droplets via mitochondrial β-oxidation in response to neuronal activity and turn on a detoxification gene expression program (**Figure 1.3a**). Therefore, FA metabolism is coupled in neurons and astrocytes to protect neurons from FA toxicity during periods of enhanced activity. Mitochondrial dysfunction in astrocytes likely decreases FAs metabolism (**Figure 1.3b**). Interestingly, fatty acids, as well as lipid droplets, have been associated to α-syn toxicity in cellular and animal models of PD (Vincent et al., 2018; Fanning et al., 2019). Using genetic screens for suppressors of α-syn toxicity in yeast, two laboratories independently identified stereoacyl-CoA-desaturase 1 (SCD1) as a mediator of α-syn toxicity. α-syn elevation increased the levels of oleic acid, which accumulated in lipid droplets in yeast and in neurons. SCD1 mediates the conversion of stearic acid to oleic acid—and its inhibition reduced the levels of oleic acid and, concomitantly, the toxicity of α-syn in a variety of models. Given that break down, via beta-oxidation, of oleic acid happens in astrocytic mitochondria and beta-oxidation in astrocytes is activated in model systems with elevated fatty acids (Ioannou et al., 2019), it is plausible that astrocytic mitochondria play a key role in mitigating the toxicity of α-syn in PD brains.

Astrocyte Mitochondria Transfer and Transmitophagy

Advances in imaging techniques have enabled demonstration of mitochondrial transfer between neurons and astrocytes in the context of injury. The phenomenon of transcellular mitophagy (transmitophagy) was first demonstrated in the mouse optic nerve tract (Davis et al., 2014), where basal mitophagy of axonal mitochondria was shown to occur primarily in neighboring astrocytes. Similar processes are likely to happen elsewhere in the brain (Davis et al., 2014). A recent paper has presented elegant evidence suggesting that transneuronal mitophagy occurs *in vivo* in PD models (Morales et al., 2020). Here, astrocytes serve as the primary cell type responsible for the clearance of damaged mitochondria—a concept highly relevant in the context of PD associated to Parkin and PINK1 loss of function mutations. Notably, PINK1 activity was recently predominately found in astrocytes while almost absent in neurons (Barodia et al., 2019). The phenomenon of transcellular mitophagy may also point at novel therapeutic avenues. For example, enhancing PINK1/Parkin-mediated mitophagy specifically in striatal astrocytes may help alleviate the burden of damaged mitochondria in dopaminergic neurons.

Moreover, the converse has also been observed: astrocytes can transfer healthy mitochondria to axons in the context of injury (Hayakawa et al., 2016; Joshi et al., 2019; **Figures 1.4a,b**). In this model, astrocytes release mitochondria in a calcium-dependent mechanism involving CD38 and cyclic ADP ribose signaling and are then taken up by neurons. Recently, Cheng et al. used human-induced pluripotent stem cells to show that iPSC-derived astrocytes can act as donors of mitochondria and rescue dopaminergic neuronal toxicity in coculture systems (Cheng et al., 2020). Others have also demonstrated how astrocytic mitochondria can alleviate neuronal toxicity. For example, coculture of cisplatin-treated neurons with astrocytes increased neuronal survival, restored neuronal mitochondrial membrane potential, and normalized neuronal calcium dynamics especially in neurons that had received mitochondria from astrocytes, which underlines the importance of mitochondrial transfer (English et al., 2020). These beneficial effects of astrocytes were associated with transfer of mitochondria from astrocytes to cisplatin-treated neurons. In this model, small interfering RNA (siRNA)-mediated knockdown of the Rho-GTPase Miro-1 in astrocytes reduced mitochondrial transfer from astrocytes to neurons and prevented the normalization of neuronal calcium dynamics (Fu et al., 2020). Whether similar processes occur *in vivo* in the striatum/caudate putamen is an exciting avenue of research and can point to novel therapeutic interventions.

Astrocyte Mitochondria, Reactive Oxygen Species, and DJ-1

Oxidative stress is an important pathogenic factor in PD. Despite neurons being highly dependent on oxidative metabolism, they display limited defense mechanisms against oxidative stress compared to astrocytes. Astrocytes play a key role in controlling redox homeostasis in the brain (Fernandez-Fernandez et al., 2012), and the adaptive response of astrocytes to oxidative stress seems indispensable to maintain redox homeostasis in the brain (Liddell, 2017).

Mitochondria are a major source of reactive oxygen species (ROS) in the cell as a byproduct of the electron transport chain activity. Damaged mitochondria-induced oxidative stress is a well-known contributor to neurodegeneration. ROS occur mainly at complexes I and III of the respiratory chain, and ROS production increases when the electron transport chain is compromised, leading to a leakage of electrons, which react with oxygen to form superoxide. ROS can change mitochondrial metabolism (Nemoto et al., 2000), and the production of excess superoxide can cause oxidative DNA damage and genomic instability (Samper et al., 2003). Mitochondrial DNA (mtDNA) is

highly susceptible to damage because it is very close to the source of ROS, is not protected by histones, and DNA repair capacity in mitochondria is low. Mutations in mtDNA can, in turn, make mitochondria produce more ROS, initiating a self-perpetuating vicious cycle (Hahn and Zuryn, 2019). Importantly, mutations in mtDNA have been observed in PD patients (Simon et al., 2004; Lin et al., 2012), although they seem to preferentially accumulate in neurons (Cantuti-Castelvetri et al., 2005).

Furthermore, DJ-1 point mutations and gene deletions are one of the causes of autosomal-recessive PD (PARK7) (Bonifati et al., 2003; Honbou et al., 2003; Ariga et al., 2013). In brain tissue obtained from sporadic PD patients, DJ-1 is strongly upregulated in reactive astrocytes but not in neurons (Bandopadhyay et al., 2004). DJ-1 protects against metal-induced neurotoxicity and regulates intracellular antioxidant stress responses through the transcription factor Nrf2 (nuclear factor-like 2) (Dolgacheva et al., 2019), and mitochondrial localized DJ-1 is thought to be cytoprotectant against oxidative-stress-induced cell death (Bjorkblom et al., 2013; **Figure 1.6**).

During oxidative stress, DJ-1 can be oxidized at position 106 (Cys106) (Repici and Giorgini, 2019). DJ-1 oxidation has been reported in patients with Parkinson's disease (PD), and oxidized DJ-1 is consistently observed in astrocytes (Repici and Giorgini, 2019). Although the relationship between DJ-1 oxidation and PD is still unclear, some have attempted to use astrocytic oxidized DJ-1 as a biomarker of PD (Repici and Giorgini, 2019). In mice, DJ-1 deficiency induces enhanced sensitivity of dopaminergic neurons to oxidative stress, and DJ-1 KO mice also suffer deficient glutamate uptake, which can induce excitoneurotoxicity and neurodegeneration (Kim et al., 2005, 2013, 2016; Booth et al., 2017). A more recent study found that deficiency in DJ-1 in mice delays neuronal repair due to a decrease in the astrocytes specific chemokine CCL2/MCP-1 (Choi et al., 2020), and that astrocytic DJ-1 may regulate inflammatory activation in astrocytes (Waak et al., 2009). Together, these studies indicate that mitochondrial ROS impacts DJ-1 function in astrocytes, which in turn can contribute to PD progression.

ROLE OF ASTROCYTIC MITOCHONDRIA AS INFLAMMATORY MEDIATORS IN PD

Inflammatory activation of astrocytes contributes to the neuropathology induced by mitochondrial toxins rotenone, paraquat, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Cabezas et al., 2018; De Miranda et al., 2018; Kirkley et al., 2019). ROS, mitochondrial DNA (mtDNA), and ATP, all produced primarily by mitochondria, are noxious and inflammatory stimuli to astrocytes. Recent evidence indicates that mitochondria are at the center of innate immunity pathways with relevance to neurodegeneration such as NLR family, pyrin domain containing 3 (NLRP3)-inflammasome pathway and cyclic guanosine monophosphate–adenosine monophosphate cGAMP synthase (cGAS)/stimulator of interferon gene (STING) pathway. In this section, we highlight the evidence supporting the notion that damaged mitochondria are a major source of neuroinflammatory signals.

Mitochondrial Dysfunction and NLRP3 Activation

The NLRP3 inflammasome is a pattern recognition receptor activated in response to a variety of pathogen-derived and endogenous stimuli. Upon activation, NLRP3 forms a heptameric ring that binds ASC and procaspase 1, cleaving and activating caspase-1, which results in the maturation and secretion of the proinflammatory cytokines interleukin (IL)-1β and IL-18. The NLRP3 inflammasome is present in microglia and astrocytes in the CNS (Song et al., 2017). Mitochondria are closely connected to the activation of the inflammasome. Mitochondrial disruption caused by NLRP3 stimuli leads to the generation of mitochondrial ROS and release of mtDNA to the cytoplasm (Nakahira et al., 2011; Zhou et al., 2020). Oxidized mitochondrial DNA can reinforce NLRP3 activation and enhance IL-1β secretion (Shimada et al., 2012). Zhou et al. have shown that, upon a Toll-like receptor 4 (TLR4) lipopolysaccharide (LPS) stimulation, nuclear factor kappa B (NF-κB) induces the expression of NLRP3 and pro-IL-1β during inflammasome priming, which activates interferon regulatory factor 1 (IRF1) and induces the expression of the nucleoside monophosphate kinase cytidine/uridine monophosphate kinase 2 (CMPK2) (Zhou et al., 2020). CMPK2 then locates to the outer mitochondrial membrane and increases mtDNA synthesis. Oxidized mtDNA can interact with the NLRP3 to induce IL-β. Subsequent escape of mtDNA from the cell is a potent extracellular inflammatory stimulus to astrocytes. Upon mtDNA binding to TLR9 on astrocytes, NF-κB translocates to the nucleus and drives astrocytic-specific inflammatory cytokine and chemokine transcription of CCL2, Cxcl10, IL-6, and IL-1β (Choi et al., 2014).

PINK1/Parkin-mediated mitophagy is a key mechanism to mitigate mitochondrial damage, and incomplete mitophagy can trigger NLRP3 and other inflammatory pathways (**Figures 2.2a,b**; Gkikas et al., 2018). Deficiency in LC3B-, ATG5-, ATG16L1, and Beclin in macrophages results in increased levels of cytosolic levels of mtDNA and mtROS, which triggers NLRP3-dependent IL1-β secretion (Saitoh et al., 2008; van Bruggen et al., 2010). NLRP3 can also be activated by the mitochondria-specific lipid cardiolipin. Mitochondrial depolarization translocates cardiolipin from the inner mitochondrial membrane (IMM) to the outer mitochondrial membrane (OMM) where it associates to NLRP3 (Iyer et al., 2013). An attractive hypothesis is that, in PINK1 and Parkin mutant astrocytes, elevated levels of mtROS and cardiolipin result in sustained activity of the NLRP3 inflammasome (**Figure 2.1b**). Notably, there are also reports indicating that, *in vitro*, PINK1/Parkin mitophagy can be inhibited upon inflammasome activation, since Parkin can be cleaved by caspase-1, and caspase-8, possibly to facilitate maximal activation of the mitochondria-associated NLRP3 activity (Kahns et al., 2003; Yu et al., 2014). Collectively, these studies demonstrate the crucial role that mitochondria play in the activation of NLRP3 and underline PINK1/Parkin pathway of

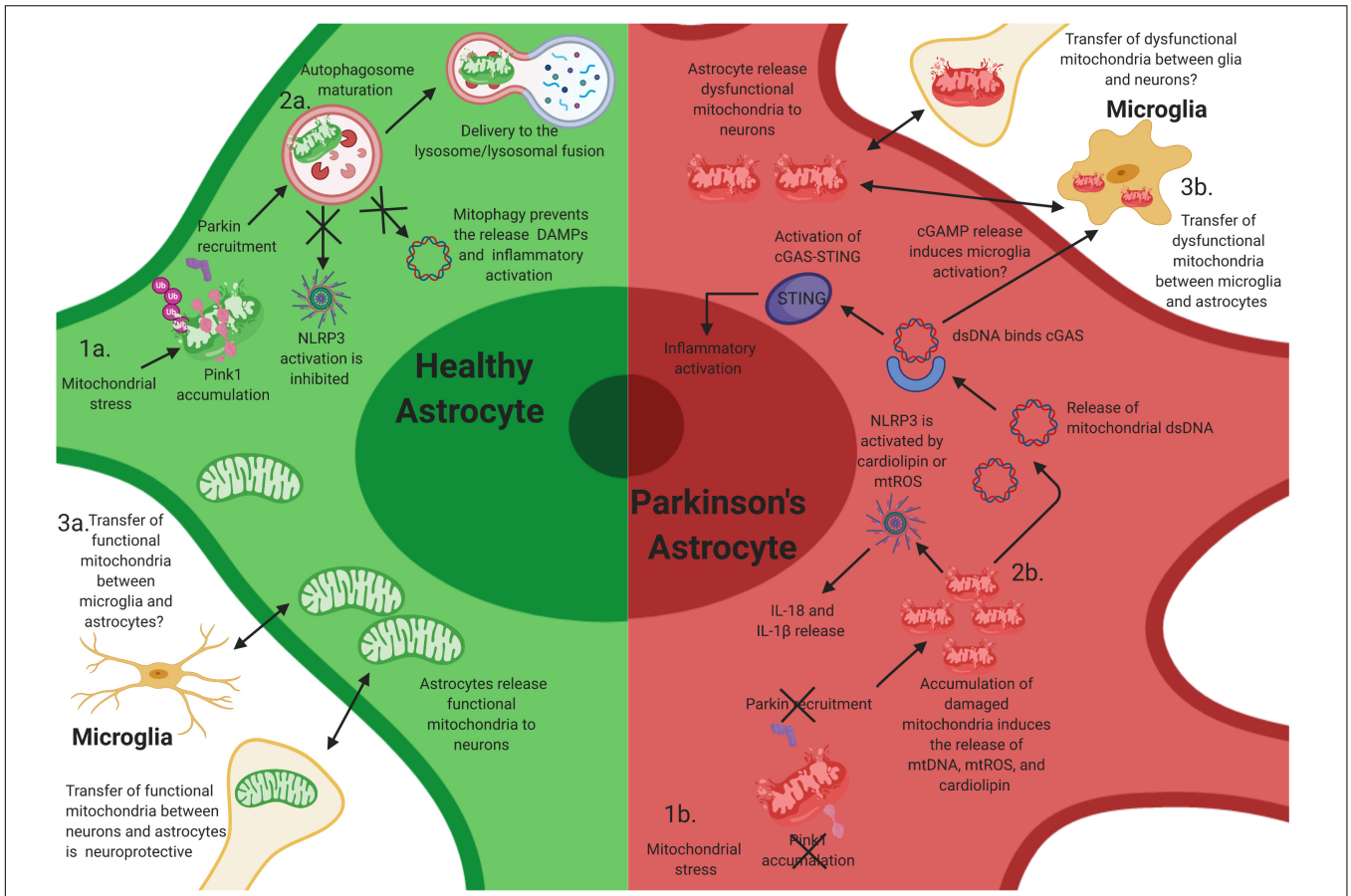

FIGURE 2 | Mitochondrial dysfunction in astrocytes contributes to neuroinflammatory activation of astrocytes. Mutations in PINK1 and Parkin likely elicit neuroinflammatory activation in astrocytes. **(1a)** After mitochondrial stress, PINK1 protein accumulates on the outer mitochondrial membrane. Phosphorylation of polyubiquitin and Parkin induces autophagosome maturation and eventually delivery to the lysosome. **(1b)** Loss-of-function (LOF) mutations in PINK1 and Parkin prevent PINK1 accumulation on the mitochondrial membrane and the activation of Parkin during mitochondrial stress, resulting in the accumulation of damaged mitochondria within astrocytes. This likely facilitates chronic inflammatory activation through multiple inflammatory pathways, including NLRP3 activation. **(2a)** Functional mitophagy prevents the release of damage-associated molecular patterns (DAMPs)/pathogen-associated molecular patterns (PAMPs) and activation of the NLR family, pyrin domain containing 3 (NLRP3) inflammasome in astrocytes. **(2b)** The accumulation of damaged mitochondria likely results in the release of intracellular DAMPs/PAMPs, cardiolipin, and mtROS, which activates NLRP3 and the cyclic guanosine monophosphate–adenosine monophosphate cGAMP synthase (cGAS)/stimulator of interferon genes (STING) pathway, resulting in inflammatory activation and release of interleukin (IL)-1β and IL-18, and potentially inducing the activation of microglia via extracellular release of cGAMP. **(3a)** Transfer of functional mitochondria from astrocytes to neurons is thought to be neuroprotective; however, whether microglia release functional mitochondria to astrocytes is elusive. **(3b)** Joshi et al. have shown that activated microglia can release dysfunctional and fragmented mitochondria to astrocytes. Astrocytes then transfer dysfunctional mitochondria to neurons, resulting in neuronal death. However, whether this occurs *in vivo* is unknown.

mitophagy as a key mechanism limiting excessive inflammation and preserving CNS homeostasis.

Mitochondrial Damage and cGAS/STING Pathway

Cytosolic double-stranded DNA (dsDNA) is recognized by the cGAS/STING pathway DNA sensing system (Ablasser and Chen, 2019). Binding of double-stranded polynucleotide to cGAS activates STING to induce interferon-β (IFNβ). The cGAS/STING pathway is not expressed in neurons but is highly expressed in astrocytes and in microglia. Probably as an evolutionary consequence of its role in the detection of viral dsDNA, cGAS also recognizes mtDNA. When the mtDNA nucleoid is disrupted, mtDNA is released to the cytosol where it activates cGAS and STING-induced pathway of inflammation (**Figures 2.2a,b**; West et al., 2015). In addition, proapoptotic Bcl-2 family members Bax and Bak can permeabilize the outer membrane, releasing cytosolic mtDNA. Low levels of Bax and Bak activity without caspase activation can induce cytokine expression (Brokatzky et al., 2019), but upon stronger stimulus, apoptotic caspases eliminate cGAS signaling (McArthur et al., 2018; Riley et al., 2018).

Recent data from the Youle lab has shown that the PINK1/Parkin pathway of mitophagy also mitigates unwanted cGAS/STING activation (Sliter et al., 2018; **Figure 2.1**). Mice that lack either PINK1 or Parkin do not display parkinsonism-related phenotypes. However, if Parkin KO mice are crossed

to mitochondrial DNA mutator mice (a mouse model of stress resulting from mtDNA mutation accumulation), these mice show loss of nigrostriatal neurons as well as L-DOPA rescuable motor deficits (Pickrell et al., 2015). Notably, these mice have increased serum levels of mtDNA as well as IL-6 and IFNβ signaling mediated by the cGAS/STING pathway (Sliter et al., 2018). Moreover, preventing inflammation in this model by removal of STING prevented neurodegeneration. However, a caveat is that it has not yet been shown if mtDNA release is controlled by mitophagy or if it is a mitophagy-independent function of PINK1/Parkin pathway. Further, it is still unclear which cells in this model release mtDNA and promote cGAS activation and which cells express STING and secrete cytokines. One possibility is that dysfunctional mitochondria in neurons and in astrocytes can act as a source of cGAMP, which can travel between cells (Ablasser and Chen, 2019) and activate STING in other astrocytes as well as in microglia. In this context, the recent identification of cGAMP receptor SLC19A1 is posed to advance the mechanistic understanding of neuroinflammation induced by mitochondrial damage (Luteijn et al., 2019; Ritchie et al., 2019).

Crosstalk in Astrocyte Inflammatory Activation

These previous studies highlight the immunological roles of astrocytes in PD and hint at the importance of glial–glial and glial–neuronal crosstalk. Similar to microglia, astrocytes can be transformed into A1 neurotoxic astrocytes after activation by IL-1α, TNF, and complement component 1q (C1q) from microglia (Liddelow and Barres, 2017; Liddelow et al., 2017). Joshi et al. recently demonstrated that fragmented and dysfunctional mitochondria released from microglia can also induce A1 neurotoxic astrocytes and subsequent neuronal loss (**Figure 2.3b**; Joshi et al., 2019). In addition, they show that activated astrocytes also secrete dysfunctional mitochondria to neurons and that transferring conditioned media from the astrocyte cultures to primary neurons induced neuronal damage. Importantly, filtering out the mitochondria from the astrocyte conditioned media reduced neuronal death (Joshi et al., 2019). Thus, the propagation of the inflammatory response from microglia to astrocytes may be, in part, mediated by mitochondria fragments. Whether microglia have the capacity to release functional mitochondria to astrocytes, and if this mechanism translates to *in vivo* models is still elusive (**Figure 2.3a**).

This study, together with other work, also highlights the possible existence of a positive feedback loop between mitochondria perturbations and astrocyte inflammatory activation. For example, incomplete mitophagy from PINK1 or Parkin mutations in neurons and glia may promote mitochondrial fragmentation, enhance inflammatory responses, and mtROS in astrocytes, which then further potentiates inflammatory activation and dysfunction of neighboring cells. Considering the minimal turnover of astrocytes coupled with their long and persistent inflammatory activation, repairing dysfunctional mitochondria or removing damaged mitochondria from astrocytes may be broadly neuroprotective and beneficial against the undesired chronic neuroinflammation in PD (Gkikas et al., 2018).

ASTROCYTES AND THERAPEUTIC CONSIDERATIONS IN PD

Striatal astrocytes play a key role in supporting the extensive branches of the SNpc axons. Mitochondria in striatal astrocytes regulate local levels of ions, glutamate, and fatty acids and regulate inflammatory signals. Moreover, astrocytes facilitate mitophagy of axonal damaged mitochondria via transmitophagy (**Figure 3**; Morales et al., 2020). We hypothesize that, perhaps due to the extensive branching of SNpc axons, it is the caudate and putamen regions of the striatum where the need for astrocyte support can be the highest and where loss of astrocytic function may have the most detrimental effect in PD. Thus, modulation of mitochondrial function in striatal astrocytes may represent a disease modifying strategy for PD.

It is becoming clear that astrocytes represent a diverse population of cells with brain-area and disease-specific functions (Khakh, 2019). New techniques such as single nucleus RNA (snRNA) seq are revealing cellular diversity in many brain areas, including the basal ganglia and the striatum (Saunders et al., 2018; Zeisel et al., 2018). There are likely several distinct populations of astrocytes within the nigrostriatal pathway, and further research is required to better understand their respective physiologies, including any differential reactivity (Gokce et al., 2016). Batiuk et al. have recently identified regional specific astrocyte markers in the mouse hippocampus and cortex with variable functions and phenotypes (Batiuk et al., 2020), supporting concepts that were proposed 30 years ago (Wilkin et al., 1990). Although regional specific midbrain astrocytes have not yet been identified, studies like this one highlight the possibility of region-specific astrocyte phenotypes in PD-relevant brain regions. Future studies using single-cell and single-nuclear RNA sequencing of different neuroanatomical brain regions will likely address this possibility. Additionally, considering the cellular penetrance of PD-related gene variants in astrocytes, whether the cellular penetrance of PD-related gene mutations in astrocytes are regionally specific could readily be elucidated with single-cellular omics studies. This represents a knowledge gap and cellular "omics" studies in diseased compared to control (both young and aged) will be critical in advancing this field and increasing our understanding of the underlying pathophysiology of PD.

The rapidly evolving field of gene therapy is providing promising therapies and targeting directly astrocytes is an attractive idea. One can conceive a therapeutic approach where genes are expressed specifically in astrocytes or capsids are targeted specifically to this population. Given that astrocytes are relatively spared in PD, understanding precisely how manipulating astrocyte function represents a window of opportunity. Based on the literature reviewed above, we propose that modulating mitochondrial function in astrocytes is predicted to affect a myriad of astrocyte functions and potentially diminish their inflammatory state. Targeting these cells could promote a beneficial environment in the

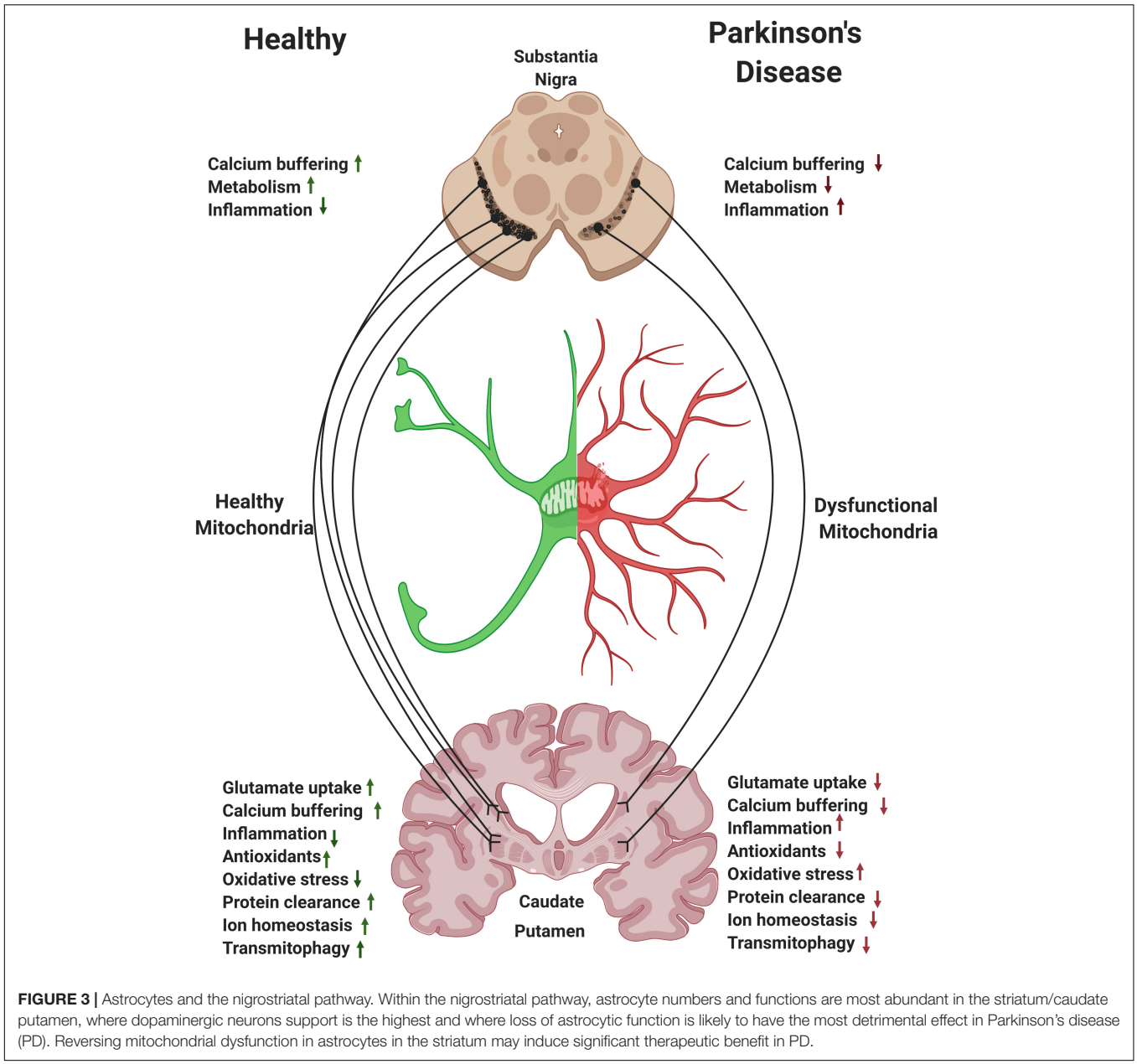

FIGURE 3 | Astrocytes and the nigrostriatal pathway. Within the nigrostriatal pathway, astrocyte numbers and functions are most abundant in the striatum/caudate putamen, where dopaminergic neurons support is the highest and where loss of astrocytic function is likely to have the most detrimental effect in Parkinson's disease (PD). Reversing mitochondrial dysfunction in astrocytes in the striatum may induce significant therapeutic benefit in PD.

striatum and adjacent regions and slow the progression of the disease. With the ever-increasing amount of data supporting mitochondrial dysfunction in PD, it is reasonable to believe that preserving and replenishing mitochondrial function in astrocytes may slow the progression of PD by broadly regulating glutamate metabolism, modulating calcium signaling, regulating fatty acid metabolism, increasing transmitophagy, and reducing oxidative stress and chronic neuroinflammation. As technologies advance, astrocytes become an attractive target for precision therapeutics.

However, future studies will need to explicitly address the therapeutic potential of manipulating mitochondrial function in astrocytes. Many of the links to PD drawn in the literature are indirect, and mechanistic details are mostly obscure. Direct proof for mitochondrial dysfunction in astrocytes in human PD is incomplete, as is the involvement of this cell type in neurodegeneration in animal models. Nevertheless, we believe that further exploration of these questions are key for a comprehensive understanding of the disease and that focusing on mitochondrial dysfunction in astrocytes will pave the way for novel therapeutic avenues to disease modifying treatments.

AUTHOR CONTRIBUTIONS

ES conceived the manuscript and CMB created figures. All authors contributed to the article and approved the submitted version.

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Mitochondrial Dysfunction, Macrophage, and Microglia in Brain Cancer

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Glioblastoma (GBM) is the most common malignant brain cancer. Increasing evidence suggests that mitochondrial dysfunction plays a key role in GBM progression as mitochondria is essential in regulating cell metabolism, oxidative stress, and cell death. Meanwhile, the immune microenvironment in GBM is predominated by tumor-associated macrophages and microglia (TAM), which is a heterogenous population of myeloid cells that, in general, create an immunosuppressive milieu to support tumor growth. However, subsets of TAMs can be pro-inflammatory and thereby antitumor. Therapeutic strategies targeting TAMs are increasingly explored as novel treatment strategies for GBM. The connection between mitochondrial dysfunction and TAMs phenotype in the tumor microenvironment is unclear. This review aims to provide perspectives and discuss possible molecular mechanisms mediating the interplay between glioma mitochondrial dysfunction and TAMs phenotype in shaping tumor immune microenvironment.

Keywords: tumor associated macrophages and microglia, mitochondrial dysfunction, mitochondrial DNA, brain cancer, inflammatory response

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INTRODUCTION

Brain cancer is the leading cause of cancer-related deaths in patients younger than 35 (Wen and Kesari, 2008). Glioblastoma (GBM) accounts for 70% of malignant primary brain tumors, taking more than 13,000 lives in the United States each year (Wen and Kesari, 2008). GBM remains an incurable cancer with a 5 year survival <5% and median survival <15 months (Arrigo et al., 2012; Koshy et al., 2012). The current standard of care is a combination of surgery, chemotherapy, and radiation, which are of limited efficacy and often cause devastating neurological side effects. Therefore, safer and more effective therapeutic modalities are urgently needed for GBM.

GBM tumor microenvironment is predominated by a heterogenous population of myeloid cells composed of brain-resident microglia and bone-marrow-derived macrophages, which are collectively referred to as tumor-associated macrophages and microglia (TAMs). TAMs represent about 40% of tumor mass in GBM (Kennedy et al., 2013). Tumor cells has been shown to dynamically interact with TAMs, a phenotypically plastic population, to induce an immunosuppressive microenvironment that facilitates tumor growth and evasion of immunosurveillance. Meanwhile, mitochondrial dysfunction is a hallmark of GBM. Current studies mostly focus on the impact of mitochondrial dysfunction on intrinsic tumor function. How tumor mitochondrial dysfunction influences the function of non-tumor cells such as TAMs is not well-studied. In this review, we will discuss the key features of mitochondrial dysfunction in

glioma and provide our perspective on how GBM mitochondrial dysfunction can regulate immune response in the tumor microenvironment.

HETEROGENOUS MACROPHAGES AND MICROGLIA IN GLIOMA

The prevalence of TAMs drives the immunosuppressive tumor microenvironment in GBM. They are associated with poor prognosis in GBM patients. Interestingly, depletion of TAMs failed to demonstrate any clinical benefit (Butowski et al., 2016), which suggests that a subset of TAMs is necessary for effective antitumor immunity. In classic *in vitro* macrophage polarization experiments, macrophages can be polarized into M1-like (antitumor) macrophage, which produces pro-inflammatory cytokines [interleukin (IL)-12, tumor necrosis factor alpha (TNF-α), IL-1ß] or M2-like (protumor) macrophage, which produces immunosuppressive cytokines such as transforming growth factor beta (TGF-β) and IL-10. However, recent evidence suggests that this M1 and M2 classification is an oversimplification *in vivo*. Single cell sequencing of TAMs in human GBM samples showed a highly heterogenous population that frequently coexpress pro-inflammatory (M1) cytokines, such as TNF-α, IL1ß, and immunosuppressive (M2) cytokines, such as IL10, Arg1, vascular endothelial growth factors (VEGFs), in individual cells at the transcription level (Müller et al., 2017; Takenaka et al., 2019). Flow cytometry of patient samples also confirm coexpression of both M1 marker CD86 and M2 marker CD206 on the protein level (Müller et al., 2017). *In vitro*, glioma-conditioned medium also induced upregulation of both M1-like (*Stat1*, *Cd274*, *Il1b*, *Tnfa*, and *Il27*) and M2-like (*Arg1*, *Vegfa*, *Il10*, *Klf4*, and *Pparg*) markers in macrophages, consistent with TAMs phenotype in patient samples (Takenaka et al., 2019).

In addition to their functionally heterogenous phenotypes, TAMs have two distinct cells of origin. Blood-derived macrophages originate peripherally from bone barrow monocytes precursors. These cells normally are excluded from the brain with an intact blood–brain barrier (BBB). In glioma, the BBB is partially compromised, allowing for peripheral monocytes to infiltrate into tumors and differentiate into macrophages. This process is dependent on enhanced expression of the monocyte chemoattractant family of proteins (MCPs) from tumors and their receptors on monocytes (such as CX3CR1 and CCR2). Peripheral monocytes are $CX3CR1^{Lo}CCR2^{Hi}$, but once they are recruited to the tumor, they transition into $CX3CR1^{Hi}CCR2^{Lo}$ macrophage or $CX3CR1^{Hi}CCR2^{-}$ cells (Chen et al., 2017). Brain-resident microglia is the other major cell type of TAMs. Microglia are unique resident macrophages that are essential for normal brain function. Fate-mapping and lineage-tracing studies have shown that immature yolk sac progenitor cells are the predominant source of brain microglia.

Recent studies of TAMs have demonstrated significant spatial and functional heterogeneity within these two major subsets of TAMs. Single-cell RNA sequencing (scRNAseq) showed that blood-derived macrophages are enriched in perivascular and necrotic regions, with upregulations of immunosuppressive genes and altered metabolic gene signatures (Müller et al., 2017). Importantly, infiltration of blood-derived macrophages instead of microglia correlates with poor survival in low-grade glioma (Müller et al., 2017). ScRNAseq of mouse glioma shows that there are eight to nine clusters of microglia and three clusters of blood-derived macrophages (Ochocka et al., 2019). Among the microglia subsets, there are three major subgroups: one with high expression of homeostatic microglia signature genes, one with high transcriptional activity of gene that inhibit nuclear factor kappa B (NFκB) signaling, and one with increased expression of genes for antigen presentation (Ochocka et al., 2019). For blood-derived macrophages, three clusters were also identified: one characterized by an inflammatory monocyte signature (Ly6c2, Ccr2, Tgfbi), one with intermediate state of mixed monocytes and macrophage signature (Ly6c2, Tgfbi), and one with differentiated macrophage signature (Ly6c2, Ifitm2, Ifitm3, S100a6) (Ochocka et al., 2019). These findings demonstrate the dynamic plasticity of both microglia and macrophages in the tumor microenvironment.

Recently, another type of tissue-resident macrophage was identified: border-associated macrophages (BAMs) with tissue-specific transcriptional signatures that reside in the dura mater, subdural meninges, and choroid plexus (Mrdjen et al., 2018; Van Hove et al., 2019). BAM is also heterogenous, which can be characterized into subsets based on CD38, major histocompatibility complex II (MHCII) expression, and their specific location in the central nervous system (CNS) compartment (Mrdjen et al., 2018; Van Hove et al., 2019). In addition, transcriptional factor IRF8 has been identified to regulate maturation and diversity of BAM (Van Hove et al., 2019).

Given the vast heterogeneity of TAMs, it is critical that any novel strategies targeting TAMs need to take into account the effect on specific TAMs subpopulations and the impact on cancer immunity.

INNATE IMMUNE RESPONSE IN TAMS AND CROSS TALK WITH GLIOMA CELLS

Macrophages and microglia are critical innate immune cells that sense signals through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). In the tumor microenvironment, TAMs are highly plastic and can be educated by cancer cells. To identify molecular mechanisms regulating immune response mediated by TAMs, it is critical to develop TAMs-based clinical strategies. A previous study has shown that, although TAMs in glioma express TLRs and surface MHC-II, they are not sufficient to mediate inflammatory/antitumor response when stimulated with TLR agonist (Hussain et al., 2006). In addition, TAMs are unable to activate CD4 T cells *in vitro* culture (Hussain et al., 2006). TLR2 along with TLR1 or TLR6 on tumor-associated microglia mediates an immunosuppressive role by enhancing production of matrix metalloprotease (MMP) to facilitate tumor invasion (Vinnakota et al., 2013; Hu et al., 2014). NFκB signaling pathway plays a significant role in macrophages and microglia-mediated inflammatory response. From transcriptional gene analysis, it has been shown that TLR signaling pathway genes was

reduced in high-grade gliomas compared to low-grade gliomas. In particular, IKKβ, a key protein leading to NFκB activation, is downregulated in high-grade glioma, and downregulation of IKKβ is correlated with immunosuppressive gene signatures in tumors (Mieczkowski et al., 2015). A recent study support these findings by identifying a subset of microglia that highly express genes that inhibiting NFκB signaling (Ochocka et al., 2019).

Type I interferon (IFN) plays a critical role in antitumor immunity by promoting antigen presentation in dendritic cells, enhancing CD8 T cell proliferation, and inhibiting regulatory T cells (Fujita et al., 2010; Ohkuri et al., 2014). Loss of type I IFN signaling has been linked to tumorigenesis in glioma. Intratumoral administration of stimulator of interferon gene (STING) agonist to enhance type I IFN production improved mouse survival in glioma models. Mechanistically, type I IFN suppresses FOXP3 regulatory T cells and therefore increases IFNγ-producing CD8 T cells (Ohkuri et al., 2014). In addition, type I IFN has an inhibitory effect on proliferation of glioma stem cell (GSCs) and inhibits GSC stemness (Du et al., 2017).

Previous studies suggest that DCs are the major type I IFN-producing cells by sensing dying tumor cells (Deng et al., 2014). However, in glioma, $CD11b^{+}$ myeloid cells express higher transcriptional level of type I IFN than $CD11c^{+}$ DCs (Ohkuri et al., 2014). In addition, more recent studies demonstrated macrophage phagocytes double-stranded DNA (dsDNA) from dying tumor cells to elicit downstream STING-type I IFN signaling pathways, resulting in strong tumor immunogenicity (Ahn et al., 2018; Zhou et al., 2020b). Considering the prevalence of TAMs in glioma, we speculate that TAMs play a critical role in sensing dying tumor cells and thereby producing type I IFN, particularly in the context of radiation-treated glioma. This is an area worth investigating more efforts to delineate the cross talk between dying tumor cells and TAMs response. Furthermore, few studies have demonstrated the role of cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS)–STING pathway in microglia. In stroke model, cGAS–STING signaling polarized microglia into pro-inflammatory phenotypes by increasing TNFα production (Jiang et al., 2020). A neuroinflammation model showed activation of STING-dependent type I IFN in microglia reduced microglia activity and attenuate neuroinflammation (Mathur et al., 2017). These studies suggest that the role of cGAS–STING type I IFN signaling in microglia is highly dependent on the disease models, and it is highly interesting to investigate the role of this pathway in microglia-mediated antitumor immunity.

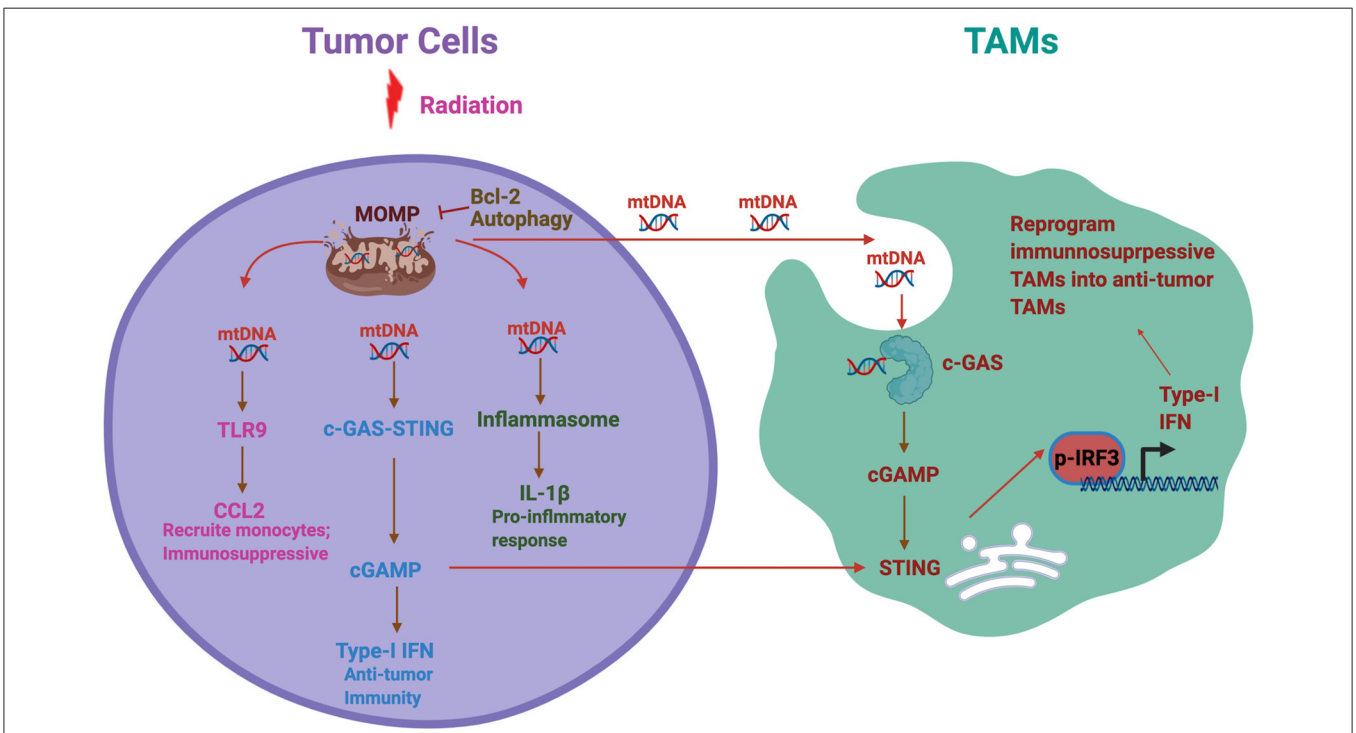

FIGURE 1 | Mitochondrial DNA (mtDNA)-induced inflammatory response in cancer. A schematic summary of the potential cross talk between tumor mitochondrial dysfunction, hence mtDNA release, and tumor-associated macrophages and microglia (TAMs) functional phenotype. Radiation and other mitochondrial stress induce mitochondrial outer membrane permeabilization (MOMP) and thereby mtDNA release to the cytosol. Antiapoptotic factor Bcl-2 and autophagy inhibit the MOMP and prevent mtDNA release. mtDNA further activates multiple inflammatory signaling pathways including Toll-like receptor 9 (TLR9), stimulator of interferon gene (STING), and inflammasome, each of which plays different roles in immune response. On the other side, we hypothesize that tumor-derived mtDNA also stimulates TAMs to activate cGAS–STING pathways, resulting in type-I interferon (IFN) production in TAMs. In addition, tumor derived cGAMP can be secreted as an immunotrasmitter that enter TAMs to stimulate STING and thereby Type 1 IFN production. This process could reprogram the TAMs in the tumor microenvironment from immunosuppressive to antitumor phenotypes.

In summary, innate immune response of TAMs is educated by tumor cells and significantly different from other inflammatory models. Therefore, to identify cancer intrinsic factors that influence the change of TAMs phenotypes in mediating immune response could be critical for understanding the cross talk between cancer cells and TAMs and thereby the development of TAMs-based cancer treatment (**Figure 1**). Mitochondrial dysfunction is a hallmark of glioma and impact multiple functions of glioma. How mitochondrial dysfunction in tumor influences innate immune responses is unclear in glioma. We will provide several perspectives and hypothesis based on previous findings.

MITOCHONDRIAL DYSFUNCTIONS IN GLIOMA

Mitochondria play a significant role in the number of essential cellular processes including metabolism, management of oxidative stress, and apoptosis. The catabolic engine of mitochondria produces redox reactions to create flux of electrons across the inner mitochondrial membrane to produce ATP. In addition, mitochondria also mediate programmed cell death, which is controlled by mitochondrial outer membrane permeabilization (MOMP) (Lopez and Tait, 2015). Following MOMP, mitochondrial inner space protein cytochrome c is released into cytosol to activate caspases, resulting in apoptotic cell death (Lopez and Tait, 2015). Furthermore, mitochondria are also a main source of reactive oxygen species (ROS), which is produced by the electron transport chain of the inner mitochondrial membrane. ROS could cause oxidative damage to mitochondrial and genomic DNA, affecting mitochondrial metabolic ability to generate ATP and widely impact many cellular functions (Murphy, 2009).

Mitochondrial dysfunction is a hallmark of cancer (Pavlova and Thompson, 2016; Vander Heiden and Deberardinis, 2017). In glioma, mitochondrial function is impaired due to significant alteration in mitochondrial genome, leading to altered morphology and abnormal bioenergetics including enhanced generation of ROS (Guntuku et al., 2016; Strickland and Stoll, 2017). This reprograming causes a shift in metabolism in glioma cells, creating a decoupling event in the metabolic pathway, leading to enhanced utilization of glycolytic metabolism instead of oxidative phosphorylation pathway (Guntuku et al., 2016; Strickland and Stoll, 2017). As a result of this switch to glycolytic metabolism, known as the Warburg effect, abnormal mitochondria phenotypes arise (Guntuku et al., 2016; Strickland and Stoll, 2017). These abnormal mitochondria phenotypes include swelling and osmophilic granules, which is thought to play a role in the invasive nature or pathobiology of gliomas (Guntuku et al., 2016; Strickland and Stoll, 2017).

Elevated mtROS from dysfunctional mitochondria induces oxidative stress, which lead to apoptosis through activation of p53 and downstream of bcl-2 family protein. However, glioma cells can survive this oxidative stress through multiple mechanisms. Oncogenic activation of PI3K/AKT pathway in glioma can induce p53 degradation to avoid mitochondrial-stress-induced apoptosis. Consistently, glioma cells with p53 mutations are also resistant to mtROS-induced apoptosis (Guntuku et al., 2016). In addition, ROS generation facilitates protumorigenesis transcription factors HIF1a and nuclear factor erythroid 2-related factor 2 (NRF2) inevitably driving proliferation and promotion of cell viability (Pavlova and Thompson, 2016). In summary, mitochondrial dysfunction in glioma regulates numerous cancer intrinsic pathways involving tumor metabolism, survival, proliferation, and cell death. In glioma tumor microenvironment, glioma cells dynamically interact with other cells such as macrophage/microglia, astrocytes, and neurons (Antunes et al., 2020). How mitochondrial dysfunction in glioma affects the cross talk between glioma cells and other cells are not clear. Below, we will discuss the possible link between mitochondrial dysfunction in glioma with TAMs-mediated cancer immunity.

MITOCHONDRIAL DYSFUNCTION AND IMMUNE RESPONSE

Mitochondria evolutionarily originate from an endocytic event of a proteobacteria (Zhang et al., 2010; McArthur et al., 2018). Therefore, mitochondrial DNA (mtDNA), similar to bacteria DNA, has damage-associated molecular patterns (DAMPs), which are conserved motifs that potently bind PRRs expressed by innate immune cells such as TAMs. The release of mtDNA to cytosol or extracellular environment is a process tightly controlled by cells and can potently activate innate immune cells. Mitochondrial dysfunctions and pathological leakage of mtDNA has been associated with many diseases such as infections, inflammatory diseases, or stress induced by irradiation or trauma (Patrushev et al., 2004, 2006; García et al., 2005; Zhang et al., 2010; Shimada et al., 2012; West et al., 2015).

Mitochondrial-mediated apoptosis is an immunologically silent event that does not trigger downstream innate immunity, as the presence of apoptotic caspases suppresses cGAS–STING-mediated type I IFN production (Rongvaux et al., 2014; White et al., 2014). However, BAK/BAX-mediated apoptosis is able to trigger mtDNA-dependent type I production by forming pores on mitochondrial outer membrane, allowing the release of mtDNA (McArthur et al., 2018). In addition to activating STING–type I IFN pathway, mtDNA induces IL-1ß production during apoptosis by activating NLRP3 inflammasome (Shimada et al., 2012).

In cancer models, emerging evidence suggest that mtDNA plays a critical role in antitumor immunity, particularly in the setting of radiation therapy. mtDNA from tumor receiving radiation or anti-CD47 antibody, which blocks the "do not eat me" signal, promotes dendritic cells' ability to cross present antigen to CD8 T cells (Xu et al., 2017; Fang et al., 2020). Mechanistically, radiation induces MOMP and release of mtDNA into the cytosol, thereby potently activating type I IFN production, which is required for effective abscopal response to radiation therapy (Yamazaki et al., 2020). Evidence using high-resolution confocal and conventional microscopy demonstrates that, following radiation of tumors, cytosolic dsDNA colocalizes

with mitochondrial elements rather than nuclear envelope markers. This suggests that mtDNA rather than nuclear DNA is the primary driver of type I IFN production, highlighting the importance and potency of mtDNA in mediating RT-induced innate immune response (McArthur et al., 2018; Yamazaki et al., 2020). The production of type I IFN is severely compromised in the presence of autophagy and the antiapoptotic protein Bcl2, both of which inhibit MOMP and its immunological response (Yamazaki and Galluzzi, 2020; Yamazaki et al., 2020). Therefore, a potential strategy for brain tumor treatment is to combine radiation therapy with Bcl2 or autophagy inhibitor in order to achieve long-lasting immune-mediated antitumor immunity and abscopal effect. On the other hand, there is evidence that mtDNA in certain context could also promote immunosuppression in the tumor microenvironment. mtDNA has been reported to activate TLR9 in hepatocellular carcinoma to induce CCL2 production, which in turns promotes macrophage infiltration and sustain the immunosuppressive phenotype of macrophages (Bao et al., 2019). In this context, the release of mtDNA to cytosol is facilitated by hypoxia tumor microenvironment (Liu et al., 2015).

In glioma, the connection between tumor-derived mtDNA and the immune response is not clear. As we discussed above, mitochondrial dysfunction is the hallmark of glioma and has been associated with release of mtDNA to cytosol and extracellular space in many diseases' models. Particularly, hypoxia, which is also a hallmark of glioma, has been shown to facilitate the release of mtDNA to cytosol. Therefore, we postulate that the release of tumor-derived mtDNA to cytosol occurs commonly in glioma. Considering the abundance of TAMs in the glioma microenvironment and the mtDNA-dependent inflammatory response TAMs demonstrate in other diseases models and cancer types (Collins et al., 2004; Bao et al., 2019), we hypothesize that mtDNA is an important driver of innate immunity in glioma. Importantly, considering radiation is the standard of care in GBM, we also posit that that tumor-derived mtDNA is critical in mediating response to radiation therapy through STING–type I IFN pathway. Previous findings of mtDNA-mediated type I IFN production is focused on cancer intrinsic signaling (Yamazaki et al., 2020); it is therefore critical in future studies to investigate the effect of tumor-derived mtDNA on TAMs.

The source of cGAS-mediated cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) generation in the tumor microenvironment is not completely elucidated. Some evidence suggest that tumor cells are phagocytosed by DCs or TAMs; then, tumor DNA (nuclear or mtDNA) subsequently enter the cytoplasm from the phagosomes to activate the cGAS–STING axis. Several recent studies, however, demonstrated that type1 IFN production is dependent on tumor cGAS to generate cGAMP, which can then be secreted as an immunotrasmitter that enter other cells such as DC/TAMs to stimulate STING and thereby type 1 IFN production (Marcus et al., 2018; Carozza et al., 2020; Zhou et al., 2020a). This mechanism is supported by recent discoveries of multiple cGAMP transporters such as SLC19A1 and LRRC8 (Luteijn et al., 2019; Zhou et al., 2020a). In addition, a cGAMP hydrolase, ENPP1, was identified as a tumor-expressed surface and secreted enzyme to clear extracellular cGAMP as a means of preventing activation of innate immune cells. ENPP1 inhibitor could synergize with radiation to enhance immune-mediated tumor rejection by increasing cGAMP availability in the tumor microenvironment (Carozza et al., 2020). Therefore, it goes to reason that a rational strategy in glioma treatment is to (1) enhance production of cytosolic mtDNA in order to increase tumor-derived cGAMP and (2) to maximize availability of cGAMP in the tumor microenvironment. Increasing mtDNA damage in glioma to enhance its immunogenicity would be a novel approach to treat GBM. A recent study showed that monoamine oxidase B (MAOB) is highly expressed in GBM mitochondria, and targeting MAOB resulted in mitochondrial-specific DNA damage and efficacy against GBM in mouse xenograft models (Sharpe et al., 2015). Whether this approach can augment cGAMP–STING activation and thereby tumor immunogenicity has not been explored. It is also important to note that, given the heterogeneity of TAMs, which subset of TAMs has the greatest sensitivity to cGAMP or has the greatest capacity to produce type I IFN has not been well-studied. Any treatment strategies to enhance TAMs sensitivity to cGAMP could therefore also be beneficial. Recently, blockade of a phagocytic receptor, MerTK, in macrophage has been shown to enhance cGAMP uptake by enhancing opening of an ATP-gated channel, P2X7R, that mediate cGAMP uptake (Zhou et al., 2020b). Whether merTK is expressed differentially in different subsets of TAMs is not clear. In conclusion, further study of the relationship between mitochondrial dysfunction and antitumor immune response could uncover novel immunotherapeutic strategies against GBM.

LESSONS FROM NEURODEGENERATIVE DISEASE

It is well-recognized that inflammation plays a critical role in the pathogenesis of neurodegenerative disorder such as Parkinson's (PD) and Huntington's (HD) disease. Mitochondrial dysfunction is a common feature, and the molecular mechanisms are increasingly being elucidated. For example, mutations in Pink1 or Prkn, which function within the same biochemical pathway, were identified in familial PDs (Kitada et al., 1998; Valente et al., 2004). It is essential for mitochondrial quality control and is responsible for removing damaged mitochondria through mitophagy (Pickrell and Youle, 2015). A recent study showed that in Pink1−/− or Prkn−/− mice, acute-exercise-induced or chronic mtDNA mutation-induced mitochondrial stress leads to STING-mediated type I IFN response resulting in systemic inflammation. This effect was completely abolished in the absence of STING in $STING^{gt/gt}$ mice crossed with Pink1−/− or Prkn−/− mice. Pink1/Prkn deletion in *mutator* mice with a proofreading-defective mtDNA polymerase showed enhanced circulating mtDNA and higher mtDNA to nuclear DNA ratio (Sliter et al., 2018). While chronic inflammation may contribute to pathogenesis of PDs, in the setting of the immunosuppressive tumor microenvironment, Pink1/Prkn deficiency may be beneficial to enhance STING-mediated type I

IFN production especially in the setting of mitochondrial stress such as radiation treatment. Targeting Pink1/Prkn pathway may therefore be a novel strategy against GBM. Another potential insight could be drawn from HD. Mutations of the HD causative gene huntingtin result in impaired mitochondrial protein import and thereby increase in mitochondrial oxidative stress resulting in enhanced mtDNA damage (Yano et al., 2014). Using a mouse model of HD, a study showed that deficiency in melatonin, a potent free radical scavenger, leads to increased cytosolic mtDNA release and potent activation of the cGAS/STING/IRF3 pathway, resulting in a pathological inflammatory response causing synaptic loss and neurodegeneration (Jauhari et al., 2020). Similar to PINK1/PRKN deficiency in PD, while defect in melatonin may be detrimental in the setting of chronic neuroinflammation, downregulating melatonin may be beneficial in activating innate immune response in the context of tumor-mediated immune suppression.

CONCLUSION

In GBM, TAMs are increasingly recognized to play a critical role in shaping the tumor microenvironment that could affect prognosis and response to therapy. Understanding the plasticity and functional heterogeneity of TAMs is crucial in developing therapeutics targeting TAMs. Mitochondrial dysfunctional in GBM is a well-known phenomenon, but how it affects TAMs functionally and thereby the immune microenvironment has been largely unexplored. A growing number of studies, in other disease and cancer models, suggest that mtDNA release is a significant byproduct of mitochondrial dysfunction and that mtDNA can potently activate STING-dependent type I IFN production. Whether this dynamic holds true in the unique GBM microenvironment with an abundance of blood-derived macrophages and resident microglia has not been explored. Understanding this cross talk between GBM mitochondrial dysfunction and phenotypical subsets of TAMs may be crucial in developing novel therapeutic strategies against GBM.

AUTHOR CONTRIBUTIONS

RL provided ideas for the project and wrote the initial draft. WH completed final revision. All authors read and approved the final manuscript.

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Mitochondrial Redox Signaling Is Critical to the Normal Functioning of the Neuronal System

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Mitochondrial dysfunction often leads to neurodegeneration and is considered one of the main causes of neurological disorders, such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and other age-related diseases. Mitochondrial dysfunction is tightly linked to oxidative stress and accumulating evidence suggests the association between oxidative stress and neurological disorders. However, there is insufficient knowledge about the role of pro-oxidative shift in cellular redox and impairment of redox-sensitive signaling in the development of neurodegenerative pathological conditions. To gain a more complete understanding of the relationship between mitochondria, redox status, and neurodegenerative disorders, we investigated the effect of mitochondrial thiol-dependent peroxidases, peroxiredoxins (Prxs), on the physiological characteristics of flies, which change with pathologies such as PD, ALS and during aging. We previously found that through their ability to sense changes in redox and regulate redox-sensitive signaling, Prxs play a critical role in maintaining global thiol homeostasis, preventing age-related apoptosis and chronic activation of the immune response. We also found that the phenotype of flies under-expressing Prxs in mitochondria shares many characteristics with the phenotype of *Drosophila* models of neurological disorders such as ALS, including impaired locomotor activity and compromised redox balance. Here, we expanded the study and found that under-expression of mitochondrial Prxs leads to behavioral changes associated with neural function, including locomotor ability, sleep-wake behavior, and temperature-sensitive paralysis. We also found that under-expression of mitochondrial Prxs with a motor-neuron-specific driver, D42-GAL4, was a determining factor in the development of the phenotype of shortened lifespan and impaired motor activity in flies. The results of the study suggest a causal link between mitochondrial Prx activity and the development of neurological disorders and pre-mature aging.

Keywords: mitochondria, redox state, peroxiredoxin, neuronal function, aging, *Drosophila*

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INTRODUCTION

The mitochondrion is an organelle that plays a key role in the control of many cellular processes. Given the ability of mitochondria to act as the primary generator of reactive oxygen species (ROS), maintaining a balanced redox status in this organelle is of paramount importance for normal cell function. It is known that mitochondria and redox signaling play an important role in maintaining

normal functioning of different organs and tissues in a wide range of species (Amigo et al., 2016). Studies in model organisms have shown that dysregulation of signaling pathways in a single tissue can significantly affect organismal longevity (Grotewiel et al., 2005). Altered or dysregulated mitochondrial redox state and increased oxidative stress (OS) underlies pre-mature aging and many pathological conditions, including neurodegenerative disorders such as Parkinson's disease (PD) (Xia et al., 2020) and amyotrophic lateral sclerosis (ALS) (Wang et al., 2018).

The redox state in mitochondria is maintained by many factors, among which are thiol-dependent peroxidases or peroxiredoxins (Prxs). Prxs are able to sense and regulate cellular concentrations of hydrogen peroxide and other peroxides, thereby acting as antioxidants and regulators of cellular redox and redox-sensitive signaling pathways. Prxs are found in virtually all phyla (Rhee et al., 1999), and their representatives are documented in invertebrates, including *Drosophila*, which possess all of the Prx mammalian homologs. There are two subtypes of Prxs in mitochondria, Prx3 and Prx5. These Prxs are implicated in the development of various neurological disorders, as shown experimentally using transgenics and mutants (Chen et al., 2012, 2014; Davey and Bolanos, 2013; Angeles et al., 2014; Kim et al., 2016; Park et al., 2017; Agrawal and Fox, 2019; Pharaoh et al., 2019; Wang et al., 2019; Lee et al., 2020).

We have previously investigated the functions of mitochondria-localized Prxs (Prx3 and Prx5) in *Drosophila* and found broad effects on the redox environment, tissue-specific apoptosis, life span, resistance to OS, geotaxis, and the immune response (Klichko et al., 2019). The most dramatic effects were observed in flies called double mutants (DM) that under-express both Prxs (Radyuk et al., 2010). We also found that these changes in cellular function and physiology in the DM were largely similar to those seen in normal physiological aging, but at an accelerated pace, suggesting that these Prxs interact with longevity pathways.

Here, we have expanded our research to investigate the potential links between mitochondrial Prxs and neuronal function and to determine the role of mitochondrial Prxs in the development and progression of neurodegenerative disorders. It is established that OS and impaired redox status, as well as dysfunctional mitochondria, correlate with neuroinflammation and the development of many age-related neurodegenerative disorders (De Rose et al., 2017; Kumar et al., 2017; Xia et al., 2020). However, while the connection between OS and inflammation is established, the mechanistic underpinnings have not been sufficiently delineated.

We set two goals: (a) to further characterize Prx mutants, focusing on behavioral characteristics associated with neural function; (b) to identify the critical tissues responsible for the DM phenotype.

MATERIALS AND METHODS

Fly Strains and Procedures

All mutant, transgenic, and enhancer fly lines were backcrossed into the *y w* reference strain background a minimum of 8 times. The *daughterless* Da-GAL4, Appl-GAL4, and D42-GAL4 driver lines were supplied by Dr. Blanka Rogina (University of Connecticut Health Science Center). Properties of the drivers are described in FlyBase and in publications (Parkes et al., 1998; Taghert et al., 2001; Orr et al., 2005; Legan et al., 2008). The *dprx5* mutant allele is described in Michalak et al. (2008). Under-expression of dPrx3 was achieved using UAS-RNAi-*dprx3* transgenic fly lines described in Radyuk et al. (2010). Under-expression of dPrx3 by RNAi globally and in neuronal tissues was achieved by crossing the UAS-RNAi-*dprx3* transgene to Da-GAL4 or Appl-GAL4 and D42-GAL4 drivers correspondingly. The genotypes of the flies and abbreviations are shown in **Table 1**.

In all experimental studies, flies were cultured on standard sucrose-cornmeal fly food at 25°C. Age-synchronized cohorts of flies were generated by collecting newly-enclosed flies over a period of 48 h. Approximately 25 flies were placed in each vial and transferred to fresh food on a daily basis. Survivorship studies were conducted as described in our previous publications (Radyuk et al., 2010; Odnokoz et al., 2016). Fly deaths were recorded approximately every 24 h.

Negative Geotaxis Assay

The negative geotaxis (climbing assay) was performed according to Pendleton et al. (2002) and Ali et al. (2011) with some modifications. Briefly, flies were placed in an empty glass vial. After 10 min of acclimation the flies were gently tapped down to the bottom of the vial and allowed to climb for 30 s. A number of flies that are able to climb or jump ∼4 cm distance and to reach the top of a vial was counted. The assay was repeated for the same group in triplicate, allowing for 10 min rest period between each trial. The geotaxis was expressed as a number of climbers/jumpers to the total number of flies. Studies were performed at 25°C under standard lighting conditions.

Phototaxis Assay

Phototaxis was evaluated essentially as described in Vang et al. (2012). The vial containing 25 flies was left for 30 min in the dark

TABLE 1 | Genotypes of control and experimental flies and fly line names.

Line name	Genotype
dprx5	*dprx5*/ Da-GAL4, *dprx5*
dprx3	RNAi-*dprx3*/ Da-GAL4, *dprx5*
DM	RNAi-*dprx3*, *dprx5*/ Da-GAL4, *dprx5*
Control	+/ Da-GAL4, *dprx5*
Appl DM	Appl-GAL4/ +; RNAi-*dprx3*, *dprx5*/ *dprx5*
Appl Control	Appl-GAL4/ +; *dprx5*/ +
D42 DM	D42-GAL4/ +; RNAi-*dprx3*, *dprx5*/ *dprx5*
D42 Control	D42-GAL4/ +; *dprx5*/+

DM – flies underexpressing dPrx3 with Da-GAL4 driver in dprx5 mutant background; Appl DM – flies underexpressing dPrx3 with Appl-GAL4 driver in dprx5 mutant background; D42 DM – flies underexpressing dPrx3 with D42-GAL4 driver in dprx5 mutant background. Controls were heterozygous flies carrying driver and transgene alleles. Since there was no significant difference in effects on lifespan and other fly characteristics between driver and transgene controls (17), we only present here the data for one of them, +/Da-GAL4, dprx5 obtained in the current experiments. Appl Control and D42 Control–corresponding driver controls for fly lines under-expressing Prxs with these drivers.

room to allow adaptation of the flies to darkness. Dim yellow illumination was turned on so the flies could be seen in absence of the white light. The vial then was attached to the 20 cm test tube and place horizontal and perpendicular to the light source 15 cm away. The light source was turned on and number of flies was counted after 2 min in the last quarter of the apparatus.

Sleep-Wake Behavior

TriKinetics Locomotor Activity Monitoring System (TriKinetics Inc.), Data Acquisition (DAMSystem308X) and File Scan (DAMFileScan110X) Software were used to measure sleep-wake behavior. Studies were done under a 24 h light/dark cycle regimen (12 h:12 h LD) at 25°C, 50% humidity. For single fly sleep-wake behavior study, we used Trikinetic Activity Monitor 1 (http://www.trikinetics.com/). Flies were placed into separate 10 × 0.5 cm tubes with fly food from one side and closed with cotton from other side. As a fly walks from end to end back and forth, its passage is detected and counted by an infrared beam, which is located in the middle of the tube.

Single fly activity data was analyzed using R version 3.1.2, RStudio Version 0.98.1091, Microsoft Excel Version 14.6.1, and Prism 5.0c (GraphPad Software, CA) to calculate total activity, total sleep and night activity over time. R script was written and used to organize the data in the format represented in **Figures 3**, **4** to calculate the various parameters (available upon request). Flies were transferred to clean tubes with fresh food every 2–3 days. Day of fly death was identified from activity measurements. Data was recorded as number of crossings per 5-min bin. Sleep was counted if the fly was inactive for a 5-min period (Shaw et al., 2000). Night activity offset, an index of circadian rhythmicity, was measured as a time between lights off and the proceeding end of an activity bout.

Acquired Temperature-Sensitive Paralysis

The assay developed by Dr. Rogina's research group (30 2538) has been largely adapted to assess neuronal function. Since we were unable to accurately replicate the analysis because very few flies became paralyzed when held at 30–40°C, presumably due to differences in the genetic backgrounds, a modified version of the procedure has been conducted using the following approach. Flies were collected at several time points (young, middle, and old) predicted to match percentage of lifespan in control flies and short-lived mutants. Flies were exposed to 45.5°C for 30 s, which led to paralysis in 100% of flies at all ages. Then the time that it took for 50% of the flies in each trial to recover from paralysis was measured. This modified approach allowed for more precise and replicable measurements. This modified method demonstrates the same scaling with age across multiple genotypes as reported by Reenan and Rogina (2008), and is coherent with the age-associated decline in neuronal excitability hypothesized to underlie the temperature-sensitive paralysis phenotype.

Statistical Methods

Statistical analysis was performed using GraphPad Prism 5.0c and Microsoft Excel. The mean survivorship time and statistical significance of differences between survival curves were assessed using the log rank test. Differences in the behavior activity levels were compared between groups by analysis of variance. For multiple comparisons, Bonferroni correction has been used. Statistical significance of the age-specific variations in the activity levels between fly lines was determined by comparing the slopes and intercepts among regression lines. Differences were considered statistically significant at $p < 0.05$. Sample size and statistical methods are listed in details in Figure and Table Legends.

RESULTS

Life Span Was Shortened in Flies Underexpressing Mitochondrial Prxs in Neuronal Tissues

It has previously been noted that under-expression of mitochondrial Prxs is particularly damaging to certain tissues, leading to apoptosis in the cardia, intestinal epithelium, oenocytes and thoracic muscles, while no significant pro-apoptotic changes were found in the brain (Radyuk et al., 2009, 2010). Similar characteristics have also been reported in normal flies during aging (Zheng et al., 2005). These observations suggest that changes in mitochondrial Prx levels in certain tissues or cells may be particularly important in the development of the "rapid death" phenotype of the double mutant.

To uncover the potential Achilles' heel responsible for the aberrant neurological behavior of the DM, we investigated the tissue-specific effects of dPrx under-expression using pan-neuronal (Appl-GAL4) and motor neuron-specific (D42-GAL4) drivers. To achieve under-expression of both mitochondrial Prxs together, we targeted the expression of UAS-RNAi-*dprx3* transgene to motor neurons using the D42-GAL4 driver and also to a broad range of the brain neurons using the Appl-GAL4 pan-neuronal driver in a *prx5* null background.

The under-expression of mitochondrial Prxs with Appl-GAL4 pan-neuronal driver showed very little to no effect on longevity. In contrast, the under-expression of dPrx3 specifically in the motor neurons in a *prx5* null background had dramatic effects on longevity in both males and females (**Figure 1**, **Table 2**). The shortening of life span in the motor neuron-specific DM flies was comparable to that in the flies under-expressing mitochondrial Prxs globally, suggesting that the mitochondrial Prxs in motor neurons are critical for survivorship and maintenance of normal life span.

The Effects on Behavior: Geotaxis and Phototaxis

Sleep-wake behavior, climbing behavior (negative geotaxis) and reaction to a light stimulus (phototaxis) are parameters commonly used to study the age-related changes in reflex locomotor behavior in *Drosophila* (Gargano et al., 2005).

Negative geotaxis and phototaxis were measured in both sexes in the motor neuronal double mutant (D42 DM) with corresponding driver control (D42 Control) and in the

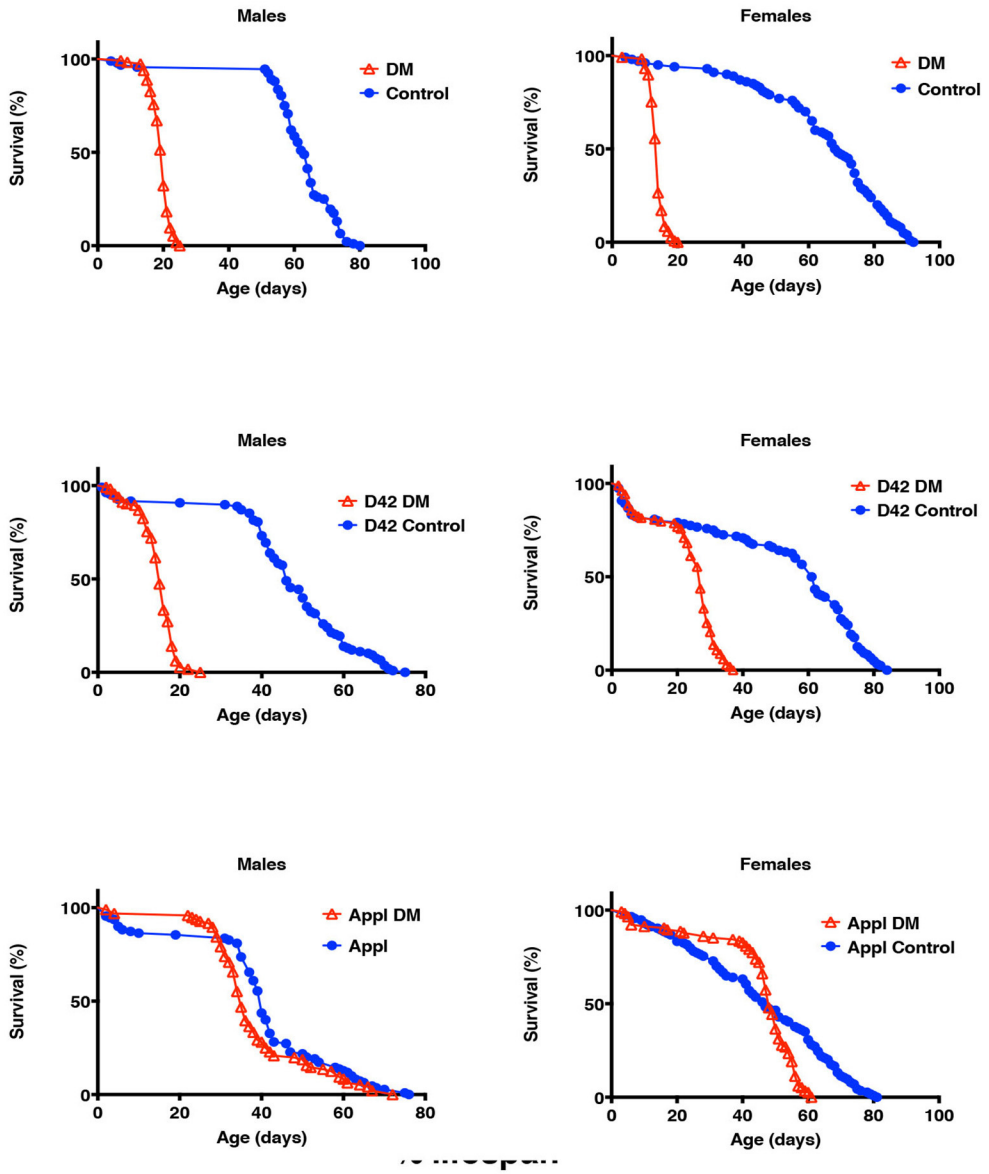

FIGURE 1 | Effects of under-expression of mitochondrial Prxs on fly life spans. dPrx3 was under-expressed in *dprx5* null background globally with Da-GAL4 driver (DM), pan-neuronally with Appl-GAL4 driver (Appl DM) and in motor neurons with D42-GAL4 driver (D42 DM). Approximately 100–125 flies were used for each fly line. Shown are representative data of two independent biological experiments. Similar results were obtained in the biological replicate experiment. The data are summarized in **Table 2**. The names of fly lines and genotypes of flies are described in **Table 1**.

pan-neuronal double mutant (APPL DM) with corresponding driver control (APPL Control) at multiple time points as indicated (**Supplementary Table 1**). Since differences in lifespans that lasted for a shorter period in D42 DM, the data were also plotted as a function of physiological age. Both Control and the DM flies showed age-dependent decline in the ability to climb and to walk toward the light source (**Figure 2**).

There was little difference in negative geotaxis and phototaxis between flies underexpressing Prxs with APPL driver and their corresponding APPL driver control. Control and mutant flies showed similar age-dependent decreases in both sexes (**Figures 2A,C**).

In contrast, both males and females underexpressing Prxs with the D42 driver showed a steep decrease in negative geotaxis and phototaxis in 10 day-old D42 DM flies relative to the D42 Control (**Figures 2B,D**). Analysis of the trajectories of these changes during physiological aging also showed significant differences in the slopes (**Table 3**) due to the greater ability of younger flies to climb and to walk toward a light source.

There were no significant differences in phototaxis and negative geotaxis between the D42 DM and Control when scaled to physiological age (approximately at 60% of their respective life span, which corresponded to ∼10 and ∼50 days of chronological age of D42 DM and D42 Control, respectively

TABLE 2 | Mean life span of the double-mutant flies under-expressing dPrx3 in *dprx5* null mutant background shown in **Figure 1**.

Males				Females			
Line	**Mean, days 1**	**% vs. control 2**	***p*-value 3**	**Line**	**Mean, days 4**	**% vs. control 5**	***p*-value 6**
Da	63			Da	69		
Control	63			Control	72		
DM	20	**31.7**	**<0.0001**	DM	14	**20.3**	**<0.0001**
	19	**30.2**	**<0.0001**		15	**20.8**	**<0.0001**
APPL	36			APPL	47		
Control	40			Control	35		
APPL DM	36	0.0	0.5035	APPL DM	48	102.1	0.1293
	35	**87.5**	**0.0112**		46	**76.1**	**0.0002**
D42	46			D42	61		
Control	44			Control	51		
D42	15	**32.6**	**<0.0001**	D42	27	**43.9**	**<0.0001**
DM	14	**31.8**	**<0.0001**	DM	25	**48.0**	**<0.0001**

Columns 1 and 4 indicate mean life spans of two independent biological experiments; columns 2 and 5 indicate the percent changes in experimental groups vs. corresponding controls; columns 3 and 6 indicate the significance probabilities of comparisons of survival curves obtained by the log rank tests. Statistically significant differences are shown in bold.

– **Supplementary Table 1**). Thus, the depletion of mitochondrial Prxs in motor neurons accelerates the decline in locomotor behavior, and this decline follows similar changes as control when scaled to corresponding % of lifespan, or physiological age.

Age-Dependent Changes in Sleep-Wake Behavior in Prx Mutants

During aging, flies, like other organisms, including humans, experience changes in behavioral characteristics. In particular, characteristics associated with neural function, such as locomotion and circadian rhythm, gradually decrease while sleep fragmentation and sleep duration increase (Jones and Grotewiel, 2011; Koudounas et al., 2012).

Previously, we have found that underexpression of mitochondrial Prxs results in changes in biochemical and physiological characteristics, similar to those observed in flies during aging (Klichko et al., 2019). Here, we expanded the study and examined the effects of underexpression of mitochondrial Prxs on locomotor activity and sleep-wake behavior as an indicator of neuronal health in flies of different ages. Locomotor activity in the Prxs mutants and control flies was continuously monitored across their entire life span by using the locomotor activity monitoring devices from Trikinetics Inc (see Material and Methods).

To investigate changes scaled to the corresponding life spans, flies were collected at different chronological, but at equivalent physiological ages denoted as % of life span (**Supplementary Table 2**).

The study of the activity patterns during a daily light-dark cycle showed similar age-dependent changes in the double and single mutants and control when they were normalized to percentage of life span (**Figure 3**). The total activity, which represents the number of beam crosses over 24 h significantly decreases with age (**Figure 3**). In contrast, the total sleep or inactivity of flies significantly increased with age (**Figure 3**). The night activity offset, which represents how long the fly was active after lights go off (ZT12), was significantly decreased during aging and the decrease was more prominent in the DM (**Figure 3**). Thus, in the double mutant flies with depletion of both Prx3 and Prx5, changes in sleep-wake behavior were similar to those during normal aging, but occurred over a shorter period of time. Moreover, in contrast to control and single mutants, the DM already exhibited a reduction in night activity in chronologically and physiologically young flies.

We also measured different sleep-wake parameters, including daily total activity, total sleep intervals and night activity offset, all known to change with age (Koudounas et al., 2012), in flies under-expressing Prxs pan-neuronally and in motor neurons. There were no significant differences between APPL DM and control at either chronological or physiological ages (**Figure 4A**, **Table 4**). In both control and APPL DM mutant, activity slightly declined during aging while sleep duration increased. The data indicate that in APPL mutant sleep-wake behavior was not affected by Prx underexpression.

In flies under-expressing Prxs in motor neurons, sleep patterns were age-dependent (**Figure 4B**). The pattern of changes in all sleep-wake parameters was similar in the D42 DM and Control flies, starting at 45% of life span when scaled to lifespan (**Figure 4B**, **Table 4**). There were no significant differences between 10 da old D42 DM and 10 da old Control flies (**Figure 4B**), suggesting that function of motor neurons affected in D42 DM does not influence sleep-wake behavior.

Age-Dependent Changes in Acquired Temperature-Sensitive Paralysis in the Double and Single Prx Mutants

It has been shown that temperature-sensitive paralysis, a marker of decreased neural transmission, is a biomarker of aging (Reenan and Rogina, 2008). As flies age they become progressively more susceptible to high temperatures displaying a paralysis phenotype that is presumably due to failure in neural signal transmission. Using a modified form of Reenan and Rogina's method, we observed this age-dependent increase in temperature sensitive

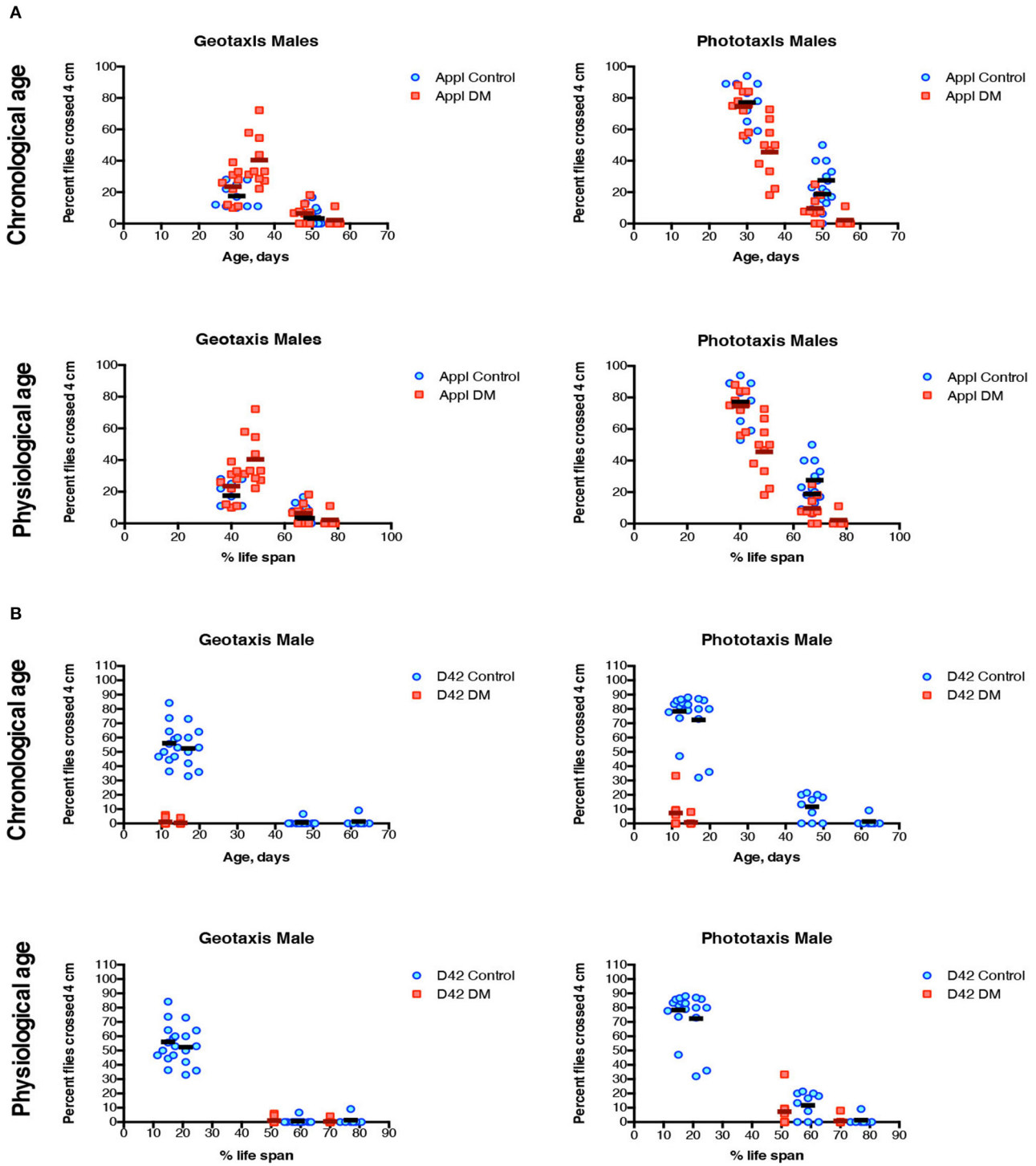

FIGURE 2 | Continued

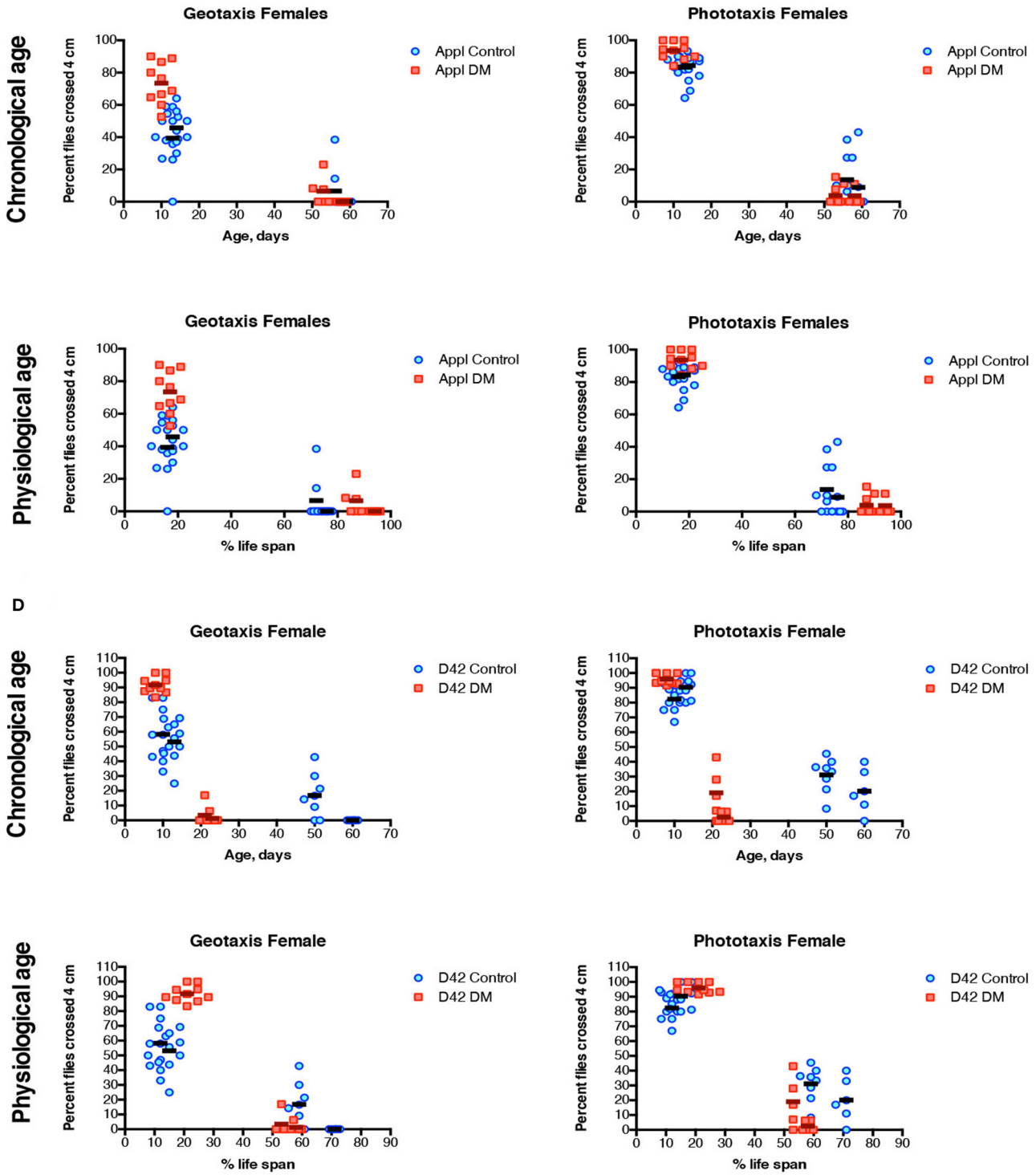

FIGURE 2 | Negative geotaxis and phototaxis of DM flies under-expressing dPrx3 pan-neuronally **(A,C)** and in motor neurons **(B,D)**. The measurements were performed at different ages as indicated in **Supplementary Table 1**. Results are represented for each single measurement of two experiments with independent cohorts with mean values. The abbreviations and genotypes of flies are described in **Table 1**. To evaluate the differences in age-dependent changes in sleep-wake behavior parameters between controls and the DM fly lines regression curve slopes and intercepts were compared (**Table 3**).

TABLE 3 | Statistical analysis of geo- and phototaxis scaled to percent life span, relied on comparison of the regression curve slopes and intercepts.

Parameters	Slope, *p*-value	Intercepts, *p*-value
Males, Appl Control vs. Appl DM		
Geotaxis	0.1433	**0.0008**
Phototaxis	0.8598	**0.0158**
Males, D42 Control vs. D42 DM*		
Geotaxis	0.4057	0.7682
Phototaxis	0.3775	**0.0232**
Females, Appl Control vs. Appl DM		
Geotaxis	**0.0095**	
Phototaxis	0.4921	**0.0001**
Females, D42 Control vs. D42 DM		
Geotaxis	**<0.0001**	
Phototaxis	**<0.0001**	

Statistical analysis of age-dependent changes in negative geo- and phototaxis between Appl DM, D42 DM and their corresponding controls. To compare the difference of trajectories of these changes during physiological aging, comparison of regression curve slopes and intercepts was performed using Prism GraphPad Software. Statistically significant differences are shown in bold.

paralysis in the driver and *y w* controls, and the DM flies (**Figure 5**). When time to recovery from paralysis at various time points in fly lifespan was scaled to percentage of lifespan, DM flies exhibited an increase in time to recovery that was comparable to control flies, suggesting that DM flies experience neuronal decline at a rate comparable to that of control flies when scaled with their shortened lifespan.

Brain Morphology

We also investigated the brain morphology for vacuoles that normally indicate neurodegenerative processes (Kretzschmar et al., 2005) and found no significant difference between DM and single *prx3* and *prx5* mutants at different ages. The relatively low number of vacuoles (one-three) observed in the short-lived DM flies and a single *prx3* and *prx5* mutants that live almost normal life span (**Supplementary Figure 1**), would suggest that these signatures of neurodegeneration were probably not a causal factor for the mortality observed in the DM flies.

DISCUSSION

Consistent with previous studies (Radyuk et al., 2010; Odnokoz et al., 2016), we found that the DM flies develop characteristics normally associated with aging but at a significantly earlier age than control flies. In this study we found that the neuronal system is affected by global under-expression of mitochondrial Prxs. The double mutants exhibit disruption of sleep-wake behavior, impaired geo- and phototaxis, and failure in neuronal transmission (**Figures 2–5**). Some of these parameters, such as total activity and sleep were determined by physiological aging of DM flies while changes in other characteristics, such as onset of night sleep were more pronounced even in physiologically young flies, suggesting cumulative effects of aging and toxicity due to under-expression of mitochondrial Prxs.

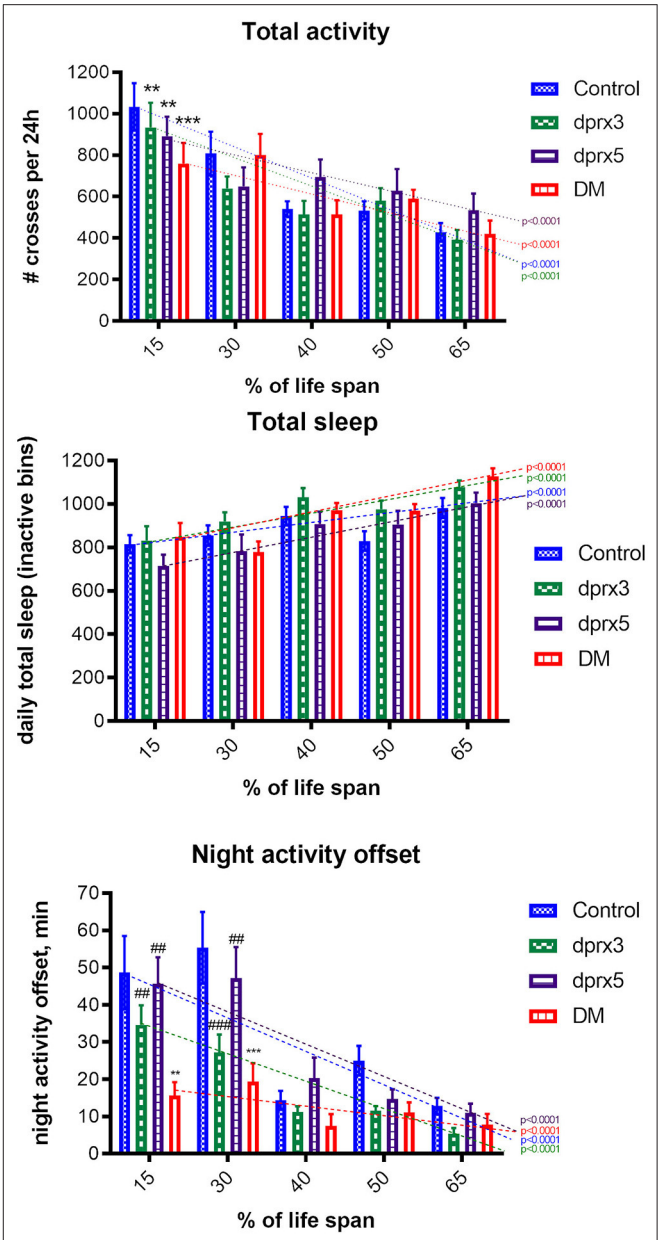

FIGURE 3 | Sleep-wake behavior of single fly. Analysis of daily total activity, daily total sleep and night activity offset in control (Control), single (*prx3* and *prx5*) and Prx double mutants was undertaken in male flies at different physiological ages (**Supplementary Table 2**). All measurements were performed from Zeitgeber time 0 (ZT0) to ZT24. Results are means ± SEM (n = 14–16) for each time point of control and single mutants. The dotted lines represent trend lines of changes during aging, and *P*-values are shown for each fly line. There was a significant decrease in 24-h total activity during aging in all four fly lines ($p < 0.0001$). Statistical analysis has been done by two-way ANOVA with Bonferroni post-tests: Control vs. *dprx3,* Control vs. *dprx5* at 15% life span ($p < 0.01$); Control vs. DM at 15% life span ($p < 0.001$). No statistically significant difference in the changes during aging was found between Control, the double and single mutant lines. The Bonferroni post-test showed a significant difference only between the mean values at 15% of life span. There was a gradual increase in 24-h total sleep during aging in all four lines resulting in statistically significant differences between flies at 15 and 65% of their life spans ($p < 0.0001$). There was no statistically significant difference

(Continued)

FIGURE 3 | in the changes during aging between Control, the double (DM) and single mutant lines. There was a significant decrease in night activity offset during aging in all four lines ($p < 0.0001$). Bonferroni post-tests: Control vs. *dprx3* at 30% life span ($p < 0.001$); control vs. DM at 15% ($p < 0.01$) and 30% life span ($p < 0.001$), *dprx3* vs. DM at 15% life span ($p < 0.01$), *dprx5* vs. DM at 15 and 30% life span ($p < 0.01$). To compare the differences between different ages in control and mutant lines, we used two-way ANOVA analysis. To compare differences between means at each % of life span, we used Bonferroni post-tests, Prism software. *compare to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); #compare to DM (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). *P* values marked by "*" indicate difference between mutants and control. *P* values marked by "#" indicate difference between single mutants and DM.

Some of these parameters, such as changes in total activity and sleep, progressed throughout lifespan according to the physiological age of DM flies while changes in other characteristics, such as onset of the night sleep and temperature-sensitive paralysis were more pronounced even in physiologically young flies, suggesting cumulative effects of aging and toxicity due to under-expression of Mitochondrial Prxs.

Many studies showed that impairment of behavior in many species is a hallmark of aging and neurodegenerative disorders (Shaw et al., 2000; Koh et al., 2006; Froy, 2011; Umezaki et al., 2012). The changes in sleep-wake behavior during aging in *Drosophila* have been well-described (Martin and Grotewiel, 2006; Serway et al., 2009; Jones and Grotewiel, 2011; Koudounas et al., 2012; Ismail et al., 2015). In old age flies show decreased daily activity and increased total sleep. Their sleep becomes more fragmented, and the time between lights off and the end of an activity bout (the night activity offset), a circadian rhythmicity parameter, decreases (Koudounas et al., 2012). Studies on transgenic flies with altered expression of genes encoding antioxidants, showed accelerated age-dependent decline in locomotor behavior. For instance, both Sod1 and Sod2 RNAi flies displayed accelerated decline in the ability to climb and fly during aging (Oka et al., 2015). *trx-2* mutant flies also showed an accelerated age-dependent decline in climbing activity (Tsuda et al., 2010). Koh et al. showed that flies exposed to low concentration of paraquat throughout their life span showed an increase in sleep fragmentation (Cirelli, 2006; Koh et al., 2006). Our studies and those of others suggest that oxidative stress and damage, and compromised redox in the DM can contribute to deterioration of sleep-wake behavior in these flies. Since the nervous and muscular system are responsible for these behaviors, the age-related behavior changes observed in response to under-expression of mitochondrial Prxs are likely to be signs of deterioration of one or possibly both of these two key systems.

Another parameter that was altered in the DM was locomotor activity, which is known to progressively decline in both flies and humans during age and also impaired in neurodegenerative diseases (Ostchega et al., 2000; Jones and Grotewiel, 2011). There are also well-documented links between oxidative stress and changes in behavior (Sakashita et al., 2010; Truong et al., 2015). Fly strains that have increased resistance to oxidative stress displayed delayed impairment in locomotor behavior (Arking and Wells, 1990; Kang et al., 2002), and over-expression of enzymes, which protect from oxidative damage, showed positive effects on fly locomotor behavior (Chavous et al., 2001; Ruan et al., 2002). In contrast, under-expression of antioxidants, such as Sod1 and Sod2, display an accelerated decline in locomotor ability (Martin et al., 2009). Thus, the observed changes in locomotor activity in the DM can be attributed to OS/changes in redox found in the DM.

Drosophila aging is also associated with increased acquired temperature sensitive paralysis (Reenan and Rogina, 2008), which has also been induced experimentally in young flies through targeted mutations in ion channels, in synaptic transmission proteins and in other genes that result in lower levels of Na+ channels (Vijayakrishnan and Broadie, 2006). These data suggest that aging is associated with a decline in the same proteins. Our experiments with temperature sensitive paralysis in mitochondrial Prxs mutants demonstrate a similar rate of acquired temperature sensitive paralysis, scaled across their shortened lifespan (**Figure 5**), suggesting that under-expression of mitochondrial Prxs may potentially affect Na+ channels and cause dysregulation of synaptic signal transmission. Interestingly, another study conducted by our laboratory in flies with enhanced pro-reducing capacity found changes in transcription of the genes that control ion transport (Radyuk et al., 2012).

In attempts to identify a particular tissue responsible for the life-shortening phenotype, we investigated the effects of under-expression of mitochondrial Prxs with different neuronal drivers. Despite the fact that these drivers share some overlap in driving the expression of the target genes (Legan et al., 2008), D42 is more effective in the expression of target genes in motor neurons while APPL driver is characterized by high-level expression of the target genes in the brain.

Previously, we found that global under-expression of dPrx3 and dPrx5 has a variety of effects on organ systems, although no pro-apoptotic changes were observed in the brain tissue of the DM (Radyuk et al., 2010) or the control flies undergoing normal aging (Zheng et al., 2005), and no obvious signs of neurodegeneration of the brain tissue due to vacuolization (**Supplementary Figure 1**). However, there were still indications that neuronal function is affected in the DM, including acquired temperature sensitive paralysis (**Figure 5**), an indicator of failure in neuronal transmission that can be associated with motor-neuronal pathology. Early onset in disruption of sleep-wake behavior (**Figures 3**, **4**) also suggests the effects on neurons that regulate motor activity and circadian behavior.

The effects of Prx under-expression in motor neurons, comparable to those observed in flies with global under-expression of mitochondrial Prxs, suggest that changes in redox specifically in this tissue can be a causal factor for rapid death and neurogenerative pathology. The *Drosophila* motor neurons are part of the giant fiber system, which mediates rapid escape behavior and has been used in studying behavior modifications such as seizures and paralysis (Pavlidis and Tanouye, 1995). The motor neurons innervate target muscles, such as indirect muscles and tergotrochantal muscles that are responsible for flying and jumping, respectively (Bravo-Ambrosio and Kaprielian, 2011). Since motor neurons transmit signals from CNS to target

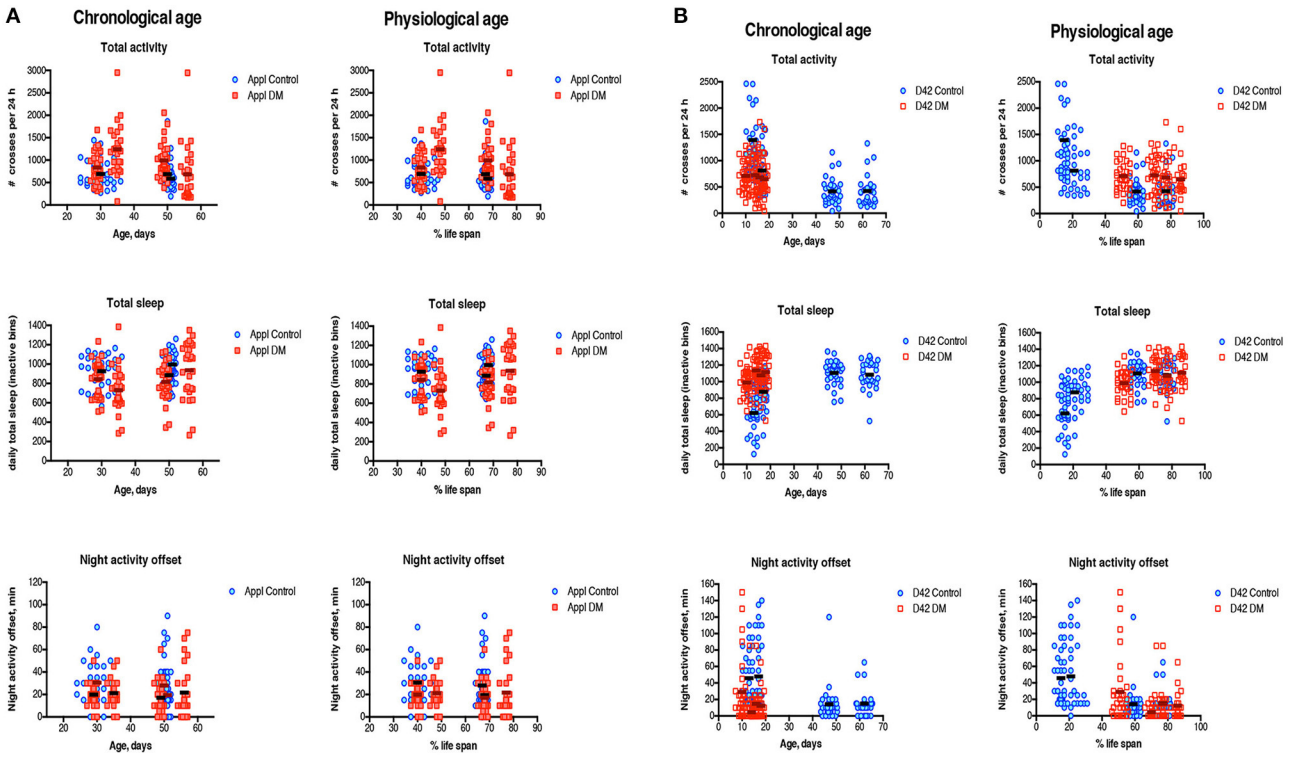

FIGURE 4 | Sleep-wake behavior of single fly. Measures of daily total activity, daily total sleep and night activity offset in the DM obtained with Appl-Gal4 driver **(A)** and D42-Gal4 driver **(B)** and corresponding Controls have been done in male flies at different ages as indicated in **Supplementary Table 3**. Data are scaled to both chronological age and physiological age. All measurements were performed at Zeitgeber time 0 (ZT0), the time when the lights go "on." Results are represented for each single measurement of two experiments with independent cohorts with mean values. The abbreviations and genotype of flies are described in **Table 1**. To evaluate the difference in age-dependent changes in sleep-wake behavior parameters between Control and DM lines, regression curve slopes and intercepts were compared (**Table 4**).

TABLE 4 | Statistical analysis of sleep-wake behavior parameters scaled to percent of life span, relied on comparison of regression curve slopes and intercepts.

Sleep-wake parameters	Appl Control vs. Appl DM		D42 Control vs. D42 DM*	
	Slope, *p*-value	Intercepts, *p*-value	Slope, *p*-value	Intercepts, *p*-value
Daily total activity	0.4232	**<0.0001**	0.7117	**<0.0001**
Daily total sleep	0.2635	**0.0004**	0.0910	0.3542
Night activity offset	0.1773	**0.0057**	0.1415	0.6584

*Statistical analysis of age-dependent changes in sleep-wake behavior parameters between DM and corresponding controls depicted in **Figure 4**. To compare the difference of trajectories of these changes during physiological aging, we performed comparison of regression curve slopes and intercepts using Prism GraphPad Software. Statistically significant differences are shown in bold.*

muscles, the changes in motor neurons caused by depletion of both Prxs may drive many of the age-related changes in behavior. Indeed, the deficit in climbing, movements toward a light source (**Figure 2**) and changes in multiple sleep-wake characteristics (**Figure 4**) may be due to a deterioration of motor neuron-connected muscles.

Previous studies have shown that in a wide range of species, the pathogenesis of many age-related diseases and mortality rate is associated with deterioration of muscles (Metter et al., 2002; Nair, 2005; Ruiz et al., 2008; Augustin and Partridge, 2009), suggesting the importance of normal skeletal muscle function in modulating systemic aging. The disruption in behavior and severe shortening of life span by removal of mitochondrial Prxs specifically in the motor neurons supports the common belief that maintenance of muscular function has a beneficial effect on organismal longevity (Chen et al., 2005; Boyle et al., 2009; Demontis and Perrimon, 2010).

Parkes et al. suggested that oxidative stress specifically in the motor neurons could be a critical causative factor in aging (Parkes et al., 1998). Besides, it is well-established that mutations in the gene encoding SOD1, an antioxidant enzyme catalyzing conversion of superoxide anion into hydrogen peroxide, are associated with loss of motor neurons in the spinal cord and in the brain (Rosen et al., 1993; Andersen, 2006). The failure of motor neurons is a causal factor in the development of ALS, the neurodegenerative disease affecting selectively both upper and lower motor neurons with consequent muscle atrophy, paralysis and eventually rapid death due to respiratory failure (Rosen et al., 1993; Robberecht and Philips, 2013; Chai and Pennetta, 2015).

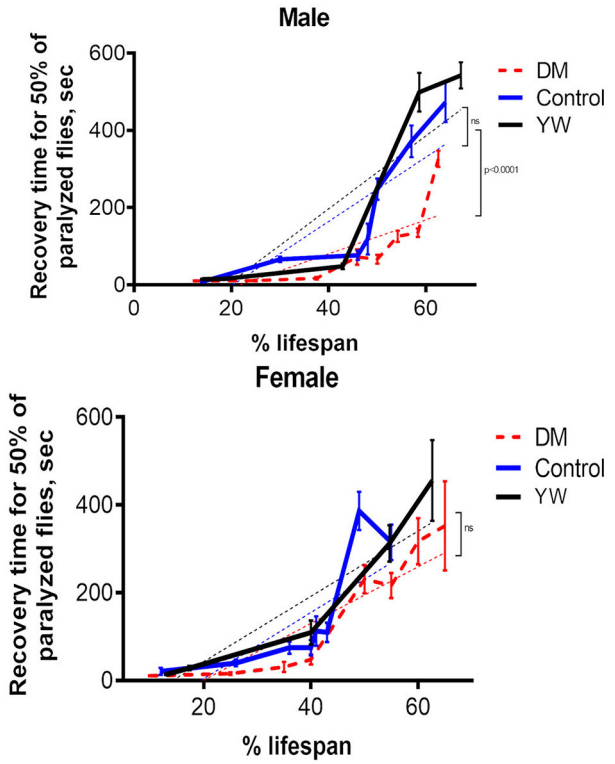

FIGURE 5 | Acquired temperature sensitive paralysis. Measurements were done in the DM, control (Da-GAL4, *dprx5*/+), and *y w* flies at different ages (**Supplementary Table 4**). Results are means ± SEM normalized to percent of life span. The dotted lines represent linear regression analysis curves. There were no statistically significant differences in age-specific changes in recovery time for 50% of paralyzed female flies between the DM and control lines, as determined by analysis of the slopes of corresponding regression lines. Statistically significant differences were observed between the DM and control male flies ($P < 0.0001$). There were no significant differences between driver control (+/Da-GAL4, *dprx5*) and *y w* control.

Experiments with the ALS model in *Drosophila* have shown that alleles with mutated SOD1 live shorter and are also characterized by change in redox, mainly in the ratio of GSH/GSSG (Mockett et al., 2003). It may be concluded that the motor neurons are very sensitive to changes in redox and are very sensitive to oxidative impairment, which is well-documented in both vertebrates and invertebrates (Simpson et al., 2003; Ferraiuolo et al., 2011; Smith et al., 2017).

Although we did not explore the effects of the DM specifically in the muscles, the data suggest that deterioration of thoracic muscles might also contribute to the shorter-lived phenotype. The muscles, which are under control of motor neurons, are rich in mitochondria and characterized by high metabolic rate. Since, mechanical, thermal, and oxidative stressors occur during muscle contraction (Arndt et al., 2010), a muscular tissue is particularly susceptible to damage compared to other tissues. Different stressors, such as paraquat and hypoxia, resulted in increased levels of apoptotic cells in the thorax, where motor neurons and muscles are located (Zheng et al., 2005). The removal of mitochondrial Prxs using the global driver also induced a strong apoptotic response in the thorax (Radyuk et al., 2010). Other studies have shown that changes in resistance to oxidative stress in muscle tissue can modulate life span (Vrailas-Mortimer et al., 2011). Thus, the increase in levels of mitochondrial SOD2 in *Drosophila* muscles delays age-related muscle dysfunction and extends life span (Vrailas-Mortimer et al., 2011). Future studies to investigate the role of mitochondrial Prxs in muscles are well-warranted.

The results of this and previous studies add new insights into the redox hypothesis of aging, specifically that dysregulation of redox signaling in a limited number of critical cell types may have a strong impact on longevity. This study provides evidence that redox changes in the motor neurons play a particularly important role in modulating longevity and determining the onset of age-dependent changes. It also suggests a critical role of redox balance in motor neurons in development of various pathologies. In this light, the changes in locomotor activity and dysregulation of sleep-wake behavior observed in the DM phenotype could signal a failure of neuromuscular control implicating neuronal and/or muscular dysfunction.

Surprisingly, pan-neuronal under-expression of mitochondrial Prxs resulted in little or no deficits in longevity or physical activity (**Figures 1**, **2**, **4**). Brain tissue is relatively deficient in antioxidant enzymes, rich in oxidizable substrates such as polyunsaturated fatty acids and catecholamines, and has a high level of ROS production (Chong et al., 2005; Lin and Beal, 2006). It is characterized by a higher rate of metabolism and lower capacity for regeneration as compared to other organs (Andersen, 2004) and therefore is highly susceptible to oxidative damage. Thus, this was unexpected, as studies conducted in our lab have shown that bolstered activity of other redox-affecting enzymes, GCLc and G6PD in the brain tissue had strong beneficial effects on life span while under-expression conferred the opposite effects (Orr et al., 2005; Legan et al., 2008). Over-expression of another Prx, the ER-localized dPrx4 in neuronal tissue also led to extension of life span (Klichko et al., 2016).

Overall the findings suggest that the impact on sleep-wake behavior is more likely due to motor neuron failure rather than brain degeneration. Why does depletion of dPrx3 and dPrx5 have such a minor effect on brain tissue? One possible reason is that sufficient dPrx3 suppression in critical tissues was not achieved with the APPL driver and there can be differences in tissue and cell specificity as well as efficiency between APPL and D42 drivers. Alternatively other Prxs may compensate for the depletion of Prx3 and Prx5 in this tissue. Among six distinct Prxs, *Drosophila* Jafrac1 is an ortholog and functional homolog of human PrxII (Lee et al., 2009). The neuronal over-expression of Jafrac1 prolonged, while the knockdown of Jafrac1 shortened, the *Drosophila* life span and affected mitochondrial function (Lee et al., 2009). Thus, Jafrac1 may play an important role in compensating the mitochondrial dysfunction found in flies under-expressing mitochondrial Prx3 and Prx5 (Radyuk et al., 2010; Odnokoz et al., 2016).

Another question that remains to be resolved is whether the under-expression of mitochondrial peroxiredoxins interferes with the neuronal transmission directly or acts indirectly through other signaling pathways that are affected by impaired mitochondrial function in these mutants. As was found in our

previous studies using the DM transcriptome analysis, under-expression of mitochondrial Prxs influenced many biological processes and signaling pathways (Odnokoz et al., 2016). For example, it affects the immune pathways, where the state of the immune system changes to overactive / pro-inflammatory. It also influences sodium ion transport, which is known to be related to neuronal function. It also leads to oxidative damage. Thus, it remains to be determined which particular process/pathway is influenced by under-expression of Prxs in motor neurons.

To conclude, our studies confirm the essential role of mitochondrial Prxs in maintaining normal physiology and in preventing an early onset of mortality. We showed that the molecular, cellular and phenotypic hallmarks of normal physiological aging are generally observed in the DM flies and these changes scale with life span. We also found that changes in neuronal function and aging followed the same trend in males and females, suggesting that mitochondrial Prxs target pathways common to both sexes. Thus, potential interventions to alleviate pathologies associated with physiological decline due to under-expression of mitochondrial Prxs would be applicable to both sexes. Another important finding is that under-expression of Prxs in motor neurons determines the short-lived phenotype of DM and is also responsible for changes in physiological parameters. It is likely that mitochondrial Prxs play a more important role in maintaining neuromuscular function than that of the brain tissue. Thus, motor neurons appear to be a culprit for the physiological decline in activity and lifespan observed in the DM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

OO and SR: conceived and designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the paper. KN and DK: conceived and designed the experiments, performed the experiments, and analyzed and interpreted the data. CW and JC: performed the experiments and analyzed the data. VK and WO: analyzed and interpreted the data and contributed to writing the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.613036/full#supplementary-material

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NADPH and Mito-Apocynin Treatment Protects Against KA-Induced Excitotoxic Injury Through Autophagy Pathway

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Aim: Previous research recognizes that NADPH can produce reduced glutathione (GSH) as a coenzyme and produce ROS as a substrate of NADPH oxidase (NOX). Besides, excessive activation of glutamate receptors results in mitochondrial impairment. The study aims at spelling out the effects of NADPH and Mito-apocynin, a NOX inhibitor which specifically targets the mitochondria, on the excitotoxicity induced by Kainic acid (KA) and its mechanism.

Methods: The *in vivo* neuronal excitotoxicity model was constructed by stereotypically injecting KA into the unilateral striatum of mice. Administrated NADPH (*i.v*, intravenous) 30 min prior and Mito-apocynin (*i.g*, intragastric) 1 day prior, respectively, then kept administrating daily until mice were sacrificed 14 days later. Nissl staining measured the lesion of striatum and survival status of neurons. Cylinder test of forelimb asymmetry and the adhesive removal test reflected the behavioral deficit caused by neural dysfunction. Determined Total superoxide dismutase (T-SOD), malondialdehyde (MDA), and GSH indicated oxidative stress. Western blot presented the expression levels of LC3-II/LC3-I, SQSTM1/p62, TIGAR, and NOX4. Assessed oxygen consumption rate using High-Resolution Respirometry. *In vitro*, the MitoSOX Indicator reflected superoxide released by neuron mitochondria. JC-1 and ATP assay Kit were used to detect mitochondrial membrane potential (MMP) and energy metabolism, respectively.

Results: In this study, we have successfully established excitotoxic model by KA *in vivo* and *in vitro*. KA induced decreased SOD activity and increased MDA concentration. KA cause the change of LC3-II/LC3-I, SQSTM1/p62, and TIGAR expression, indicating the autophagy activation. NADPH plays a protective role *in vivo* and *in vitro*. It reversed the KA-mediated changes in LC3, SQSTM1/p62, TIGAR, and NOX4 protein expression. Mito-apocynin inhibited KA-induced increases in mitochondrial NOX4 expression and activity. Compared with NADPH, the combination showed more significant neuroprotective effects, presenting more neurons survive and better motor function recovery. The combination also better inhibited the over-activated autophagy.

In vitro, combination of NADPH and Mito-apocynin performed better in restoring mitochondria membrane potential.

Conclusion: In summary, combined administration of NADPH and NOX inhibitors offers better neuroprotection by reducing NADPH as a NOX substrate to generate ROS. The combined use of NADPH and Mito-apocynin can better restore neurons and mitochondrial function through autophagy pathway.

Keywords: excitotoxicity, ROS, NADPH, Mito-apocynin, NOX, *m*itochondria, autophagy

INTRODUCTION

Glutamate is an important excitatory neurotransmitter in the central nervous system and plays a crucial role in a variety of neural processes including cognition, learning and memory (Barker-Haliski and White, 2015; Reiner and Levitz, 2018). However, under pathophysiology, excessive release of glutamate can lead to over-activation of glutamate receptors, leading to death of neurons (Wang and Qin, 2010). Excitotoxicity induced by overstimulation of glutamate receptors can be observed in varied brain diseases, such as ischemia, stroke, epilepsy, and neurodegenerative diseases (Dong et al., 2009; Lai et al., 2014). It is related to free radicals produced by activation of calcium-dependent enzymes, nitric oxide synthase, xanthine oxidase and mitochondrial oxidative dysfunction, and reduce brain antioxidant enzymes, such as glutathione (GSH) peroxidase, superoxide dismutase (SOD) and catalase, lead to the development of neurodegenerative diseases (Rueda et al., 2016). Therefore, if not balanced by endogenous antioxidant mechanisms, ROS increase may pose a potential threat to intracellular homeostasis and neuronal survival.

In our previous studies, NADPH supplementation significantly increased TIGAR expression, inhibited ROS levels and autophagy/lysosomal pathways, and thus protected neurons from KA-induced excitotoxicity *in vivo* and *in vitro* (Liu et al., 2020). However, the therapeutic dose window for NADPH is narrow, so thinking about strategies is necessary for its clinical application (Huang et al., 2018). NADPH has two biological roles in the regulation of redox: the first is as a pivotal component of cellular antioxidant system; the second is to act as a substrate for NADPH oxidase (NOX), which plays a key role in many biological and pathological processes by producing ROS. NOX1, NOX2, and NOX4 are the major subtypes of NOX in the central system that play a major role in brain injury and neurodegenerative diseases. They can produce superoxide anion or hydrogen peroxide from NADPH as substrate (Liu et al., 2020). Therefore, it is necessary to investigate whether NOX inhibitors such as apocynin can be used in combination with NADPH to better protect neurons from KA-induced excitotoxicity.

In addition, mitochondria in the central nervous system are often thought to be the source of ROS in brain aging and age-related neurodegenerative diseases (Wang et al., 2019). Recent evidence suggests that isotypes of enzymes such as isocitrate dehydrogenase (IDH), malic enzyme (ME), aldehyde dehydrogenase (ALDH), and methylene tetrahydrofolate dehydrogenase (MTHFD) catalyze similar reactions of NADPH regeneration in both of cytoplasm and mitochondria and may transfer reduction equivalents between mitochondria and cytoplasm (Lewis et al., 2014; Xu et al., 2018; Bradshaw, 2019). Data have shown that NADPH plays an important role in mitochondrial oxidative damage and the protection of mitochondrial DNA integrity (Ying, 2008). At the same time, NOX enzymes (especially NOX4) are expressed in mitochondria, and the mitochondrial respiratory chain may be the victim rather than the source of ROS production in the cell. NOX can interact with mitochondrial complex 1 and inhibit its activity, and meanwhile regulate mitochondrial biogenesis and energy generation through Nrf2 pathway (Dinkova-Kostova and Abramov, 2015). Therefore, whether exogenous NADPH supplementation and the use of inhibitors targeting mitochondrial NOX can affect mitochondrial redox and mitochondrial function in excitotoxicity remains to be investigated. In this study, Mito-apocynin (apocynin conjugated to the mitochondria-targeting triphenyl phosphonium cation moiety TPP^+) was applied *in vitro* to verify this hypothesis.

In this study, we investigated whether the combination of NADPH and Mito-apocynin had a better protective effect on neuronal survival and function in the KA-induced excitotoxicity, as well as the effect of exogenous NADPH supplementation and mitochondrial targeted NOX inhibitor on mitochondrial REDOX and function.

MATERIALS AND METHODS

Animal Treatment

SPF grade Institute of Cancer Research (ICR) mice, male, 25–30 *g*, were purchased from Zhaoyan (suzhou) new drug research center. Keep the mice at a constant temperature of 22°C and a humidity of 50–60%. Raise them in a well-ventilated environment and give artificial day and night (12 h/12 h) with freedom of drinking water. The animals were utilized in compliance with the institutional animal healthcare regulations. All animal protocols were approved by the Institutional Animal Care and Use Committee of Soochow University.

After anesthetizing mice with chloral hydrate (400 mg/kg), we infused 0.625 nmol KA into right striatum within 2 min at the following coordinates: 0.8 mm anterior to the bregma, 1.8 mm lateral to the sagittal suture, and 3.5 mm ventral to the pial surface. The volume of all intracranial injections was 1 μL. Administrated NADPH (BT04, BONTAC; *i.v*) and Mito-apocynin (HY-135869, MCE; *i.g*) 30 min and 1 day, respectively.

Nissl Staining

Coronal sections of the brain of mice were prepared. The brain slices were soaked in nissl staining solution for 20–30 min, and decolorized with 75, 95, and 100% ethanol for 2 min in turn. Permeate with paraformaldehyde for 10 min. The film was sealed and observed under a microscope. Count the number of normal striatum central morphology under 20x magnification. Nissl staining and its quantification was performed as described previously (Liu et al., 2020).

Measurement of Total SOD Activity and MDA Content

Isolated the striatum tissue after clearing the blood of the animals by cardiac perfusion with PBS. After adding 100 μL SOD sample preparation solution to every 10 mg of tissue, homogenize it on ice. Centrifuge for 3–5 min at 12,000 *g* at 4°C, and then extract the supernatant to be measured. 20 μL sample and 60 μL WST-8/enzyme working solution were added to the 96-well plate. After adding the reaction reagent, mix well. Incubate at 37°C for 30 min. The absorbance was determined at 450 nm. Inhibition percentage = $(A_{blankcontrol1} - A_{sample})/(A_{blankcontrol1} - A_{blankcontrol2}) \times 100\%$. SOD activity unit = inhibition percentage/(1-inhibition percentage) units. SOD activity unit was converted into U/mg protein according to the protein concentration and dilution ratio of the sample. All operations follow the instructions the test kit (wst-8 method; S0101, Beyotime).

Lyse the striatum tissues of animals with appropriate cell lysate and homogenized. Centrifuge at 12,000 *g* for 5 min at 4°C. Determined protein concentration for subsequent calculation. 0.1 mL sample and 0.2 ml MDA detection working fluid were added into the centrifuge tube. Heat at 100°C for 15 min. Centrifuge at 1,000 *g* room temperature for 10 min. 200 μL microliters of supernatant were added to a 96-well plate and the absorbance was then measured at 532 nm. The MDA content in the sample solution was calculated and converted to μmol/mg according to the protein concentration. All operations follow the instructions the test kit (S0131, Beyotime).

Separation of Mitochondrial and Cytoplasmic Fractions

After the animals are killed, the striatum tissue is immediately separated and placed on ice. Add about 10 times of mitochondrial separation reagent A solution (add 1% PMSF before use), and homogenate on ice for about 30 times. After homogenization, absorb approximately 50 μL as the total component. Centrifuge the supernatant at 4°C at 11,000 *g* for 10 min for extracting mitochondria. Absorb its supernatant as a cytoplasmic component. Added an appropriate amount of mitochondrial separation reagent to the precipitation, and blow the solution evenly, centrifuge at 4°C at 11,000 *g* for 10 min, remove the supernatant. Add solution A to resuspended, add the resuspended solution to the tube containing 22 and 50% Percoll solution, centrifuge at 4°C at 20,000 *g* for 20 min, and carefully transfer the fraction between the Percoll gradient into the new tube, namely the mitochondrial fraction.

Western Blot Analysis

Western blot analysis was performed on striatal tissues as described previously (Bradshaw, 2019). We used the following primary antibody: anti-NOX4 (ab109225, abcam), anti-LC3B (NB100-2220, Novus), anti-β-Actin (A5441, Sigma Aldrich), anti-SQSTM1/p62 (P0067, Sigma Aldrich), anti-TIGAR (sc-67273, Santa Cruz), anti-PINK1 (6946s, Cell Signaling), and anti-α-Tublin (NB100-690, Novus).

Behavioral Tests

Adhesive removal test: Before the experiment begins, the animals adapt to the environment in advance. The adhesive strip (0.2 in^2) was attached to the mouse's muzzle (dorsal position). Put the animals into the test cage to move freely, observe and record for 60 s or until strip is removed. The result is the waiting time for strip removal (Hernández-Espinosa et al., 2019).

Cylinder test of forelimb asymmetry: Place mice in cylinder with a height of 20 cm and a diameter of 10 cm. Record the frequency of unilateral and bilateral explorations for 3 min. The proportion of unilateral touch assessed limb asymmetry (Hernández-Espinosa et al., 2019).

Cell Cultures

On day 18, the cortex was dissected from ICR mouse embryos. Digested for 15 min with 2.5% trypsin at 37°C. Then add DNA enzyme and blow for 3 min. Filter with 400 mesh cell filter. Dilute the cell suspension to about 1 million/mL. Neurons were grown for 6–8 DIV in maintenance media (Neurobasal medium supplemented with 2% B-27, 0.5 μM glutamine, and 100 U/ml penicillin and streptomycin) at 37°C in a 5% CO2 incubator. 50% media was replaced in the fifth day. Incubate neurons for 4 h before KA treatment with neurobasal medium containing NADPH and/or Mito-apocynin (HY-135869, MCE). All animal protocols were approved by the Institutional Animal Care and Use Committee of Soochow University.

Cell Activity Detection

Primary cortical neurons were inoculated with 96-well plates. The medium was removed and 100 μL of medium containing 10% CCK8 reagent were added to each well. Incubate at 37°C in dark for 3 h. The absorbance at 450 nm was detected. Calculate the relative cellular activity.

Assessment of Oxygen Consumption Rate Using High-Resolution Respirometry

Mitochondria respiration activity was assessed in striatum by High Resolution Respirometry (OROBOROS Instruments, Innsbruck, Austria). Add 2 ml of striatum tissue homogenate to the chamber and wait for the oxygen flux to stabilize. We measured Oxygen Consumption Rate (OCR) during sequential addition of sample. After adding each substance, wait until the OCR stabilizes before adding the next substance. After a stable oxygen routine flux is achieved, add complex I substrates pyruvate 5 μL (final 5 mM), glutamate 10 μL (final 10 mM), and malate 10 μL (final 2 mM) to obtain the leak of complex I.

Then, add 10 μL ADP (final 2.5 mM) to obtain the maximum oxidative phosphorylation value of complex I. After stabilization, add 5 μL Cyt C (final 10 μM) to verify the integrity of the mitochondrial membrane. Add complex II substrate succinate 20 μL (10 mM) to get the maximum oxidative phosphorylation value of complex II. Add uncoupler CCCP 1 μl of per step (final 0.05 μM per step) to obtain the maximal uncoupled respiration. We should wait for the stabilization of oxygen flux between the additions and titrate CCCP until it starts inhibiting the tissue oxygen flux. Add 1 μL complex I inhibitor rotenone (final 0.5 μM) to interrupt the electron transfer system to obtain the maximum electron transfer capacity of complex II. Add 1 μL complex III inhibitor Antimycin A (final 2.5 μM) to obtain the residual non-mitochondrial respiration after stabilization of the oxygen flux. Finally, add complex IV substrates 5 μl ascorbate (final 2 mM) and 5 μL TMPD (final 0.5 mM) to detect the function of complex IV.

Measurement of Mitochondrial Superoxide, Membrane Potential and ATP Levels

Determine the levels of mitochondrial superoxide, membrane potential and ATP with a MitoSOX Red mitochondrial superoxide indicator (40778ES50, Yeasen), mitochondrial membrane potential (MMP) assay kit with JC-1 (C2006, Beyotime), or ATP assay Kit (S0026, Beyotime) following the manufacturer's instructions.

Primary cortical neurons were inoculated with 24-well plates. Remove the medium and wash twice with HBSS. Add 0.3 mL MitoSOX working fluid and incubated at 37°C in dark for 20 min. Remove supernatant and wash twice with HBSS. Add 0.3 mL Hoechst reagent and incubated at 37°C in dark for 10 min. Remove supernatant and wash twice with HBSS. Add 0.2 mL HBSS and observe with fluorescence microscope.

Primary cortical neurons were inoculated with 24-well plates. Remove the medium and wash twice with PBS. Add 0.3 mL JC-1 working fluid and incubated at 37°C in dark for 20 min. Remove supernatant and wash twice with pre-cooled JC-1 buffer. Add 0.2 mL medium and observe with fluorescence microscope. The presence of green fluorescence indicates that MMP decreases.

The tissue or cultures was mixed with cracking fluid and homogenized with a glass homogenizer. Centrifuge at 4°C for 5 min at 12,000 *g*. Supernatant was taken for subsequent determination. Add 100 μL ATP test working fluid into the test hole. React at room temperature for 3–5 min. Add 20 μL sample, mix quickly and measure RLU value with luminometer. The ATP concentration

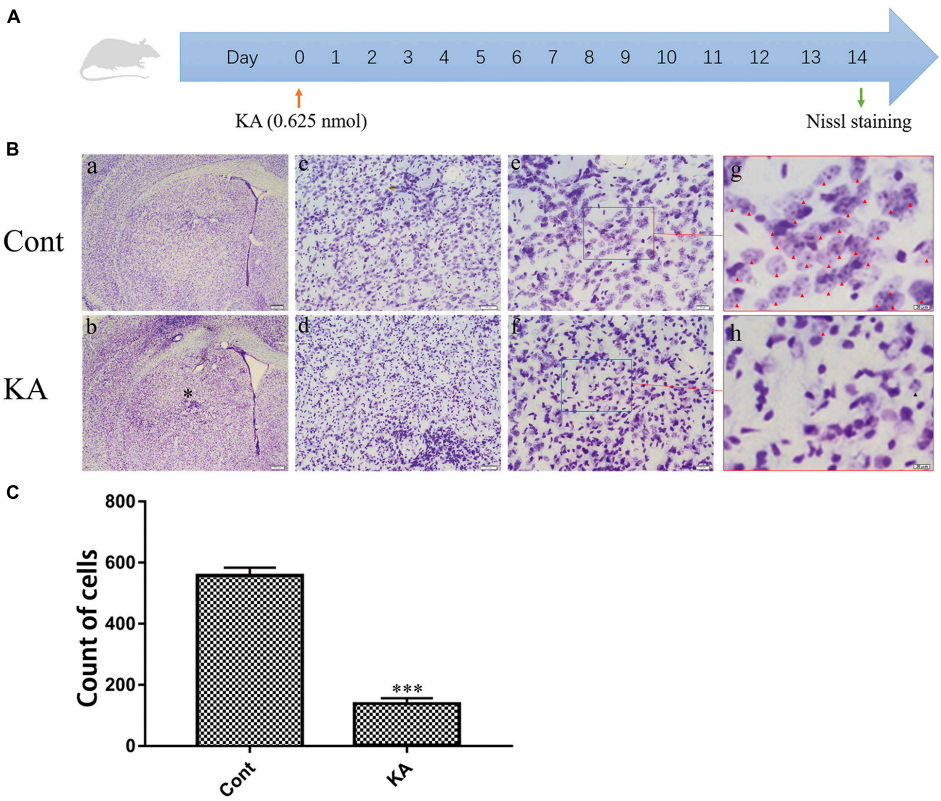

FIGURE 1 | KA causes neuronal injury in the striatum. **(A)** Illustration of experimental design. Mice were sacrificed on day 14 after KA injection. Nissl staining were performed on brain sections. **(B)** Representative microscopic images of Nissl-stained coronal brain section displaying the lesion. **(C)** Quantification of neurons with normal morphological specificity. The histological analyses were single-blinded. Data are expressed as the Mean ± SEM. Scale bar = 500 μm in a, b; 100 μm in c, d; 50 μm in e, f; and 20 μm in g, h ($n = 5$. ***$P < 0.001$ vs control, in Student's *t* test).

was calculated according to the standard curve. The concentration of ATP was converted to nmol/mg based on the protein concentration.

Statistical Analysis

Compare different groups by one-way analysis of variance (ANOVA) with Newman–Keuls *post hoc* test. All data were expressed as means ± SEM. $p < 0.05$ is considered statistically significant.

RESULTS

KA-Induced Excitotoxicity Causes Oxidative Stress and Autophagy Activation, Leading to Neuronal Death in the Striatum

For successfully constructing a neuronal excitotoxicity model *in vivo*, KA (0.625 nmol) was injected into the striatum through stereotactic localization. On the 14th day after the intracranial

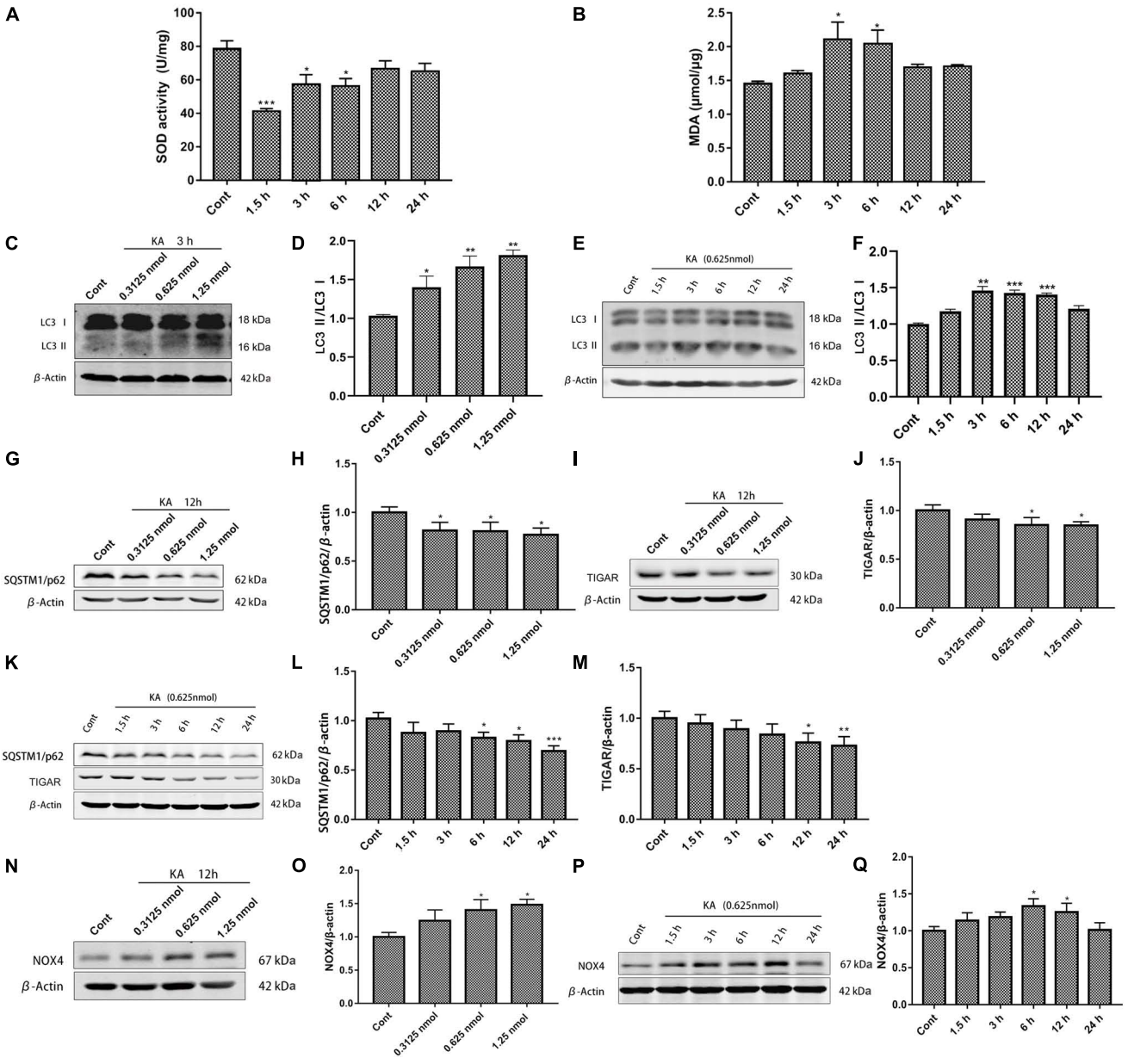

FIGURE 2 | KA-induced excitotoxicity causes oxidative stress and autophagy activation. Mice were given different doses of KA or treated for different periods of time. **(A,B)** The time-course of total SOD activity and MDA content in the striatum. **(C–Q)** Representative bands and semi-quantitation of western blots for detecting LC3-II/LC3-I, SQSTM1/p62, TIGAR, and NOX4 protein levels. Data are expressed as the Mean ± SEM ($n = 5$. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

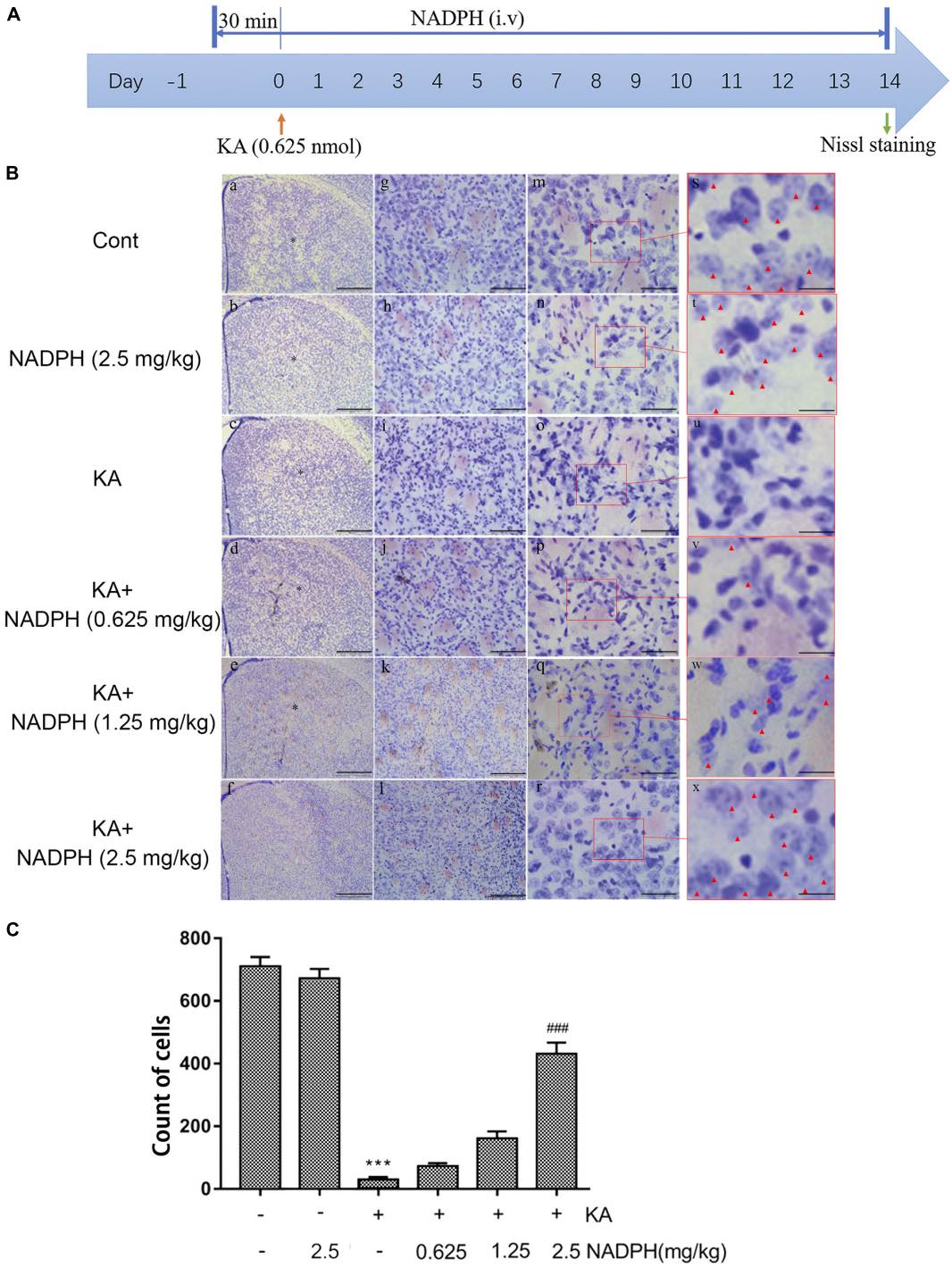

FIGURE 3 | NADPH protects neurons against KA-induced excitotoxicity *in vivo*. **(A)** Illustration of experimental design. Mice were treated with 0.625, 1.25, and 2.5 mg/kg NADPH (*i.v*), 30 min before unilateral intrastriatal injection of 0.625 nmol KA. Administrate NADPH daily until mice were sacrificed 14 days later. Nissl staining were performed on brain sections. **(B)** Representative microscopic images of Nissl-stained coronal brain section displaying the lesion. **(C)** Quantification of neurons with normal morphological specificity. The histological analyses were single-blinded. Data are expressed as the Mean ± SEM. Scale bar = 500 μm in a–f; 100 μm in g–l; 50 μm in m–r; and 20 μm in s–x ($n = 5$. $^{***}P < 0.001$ vs control; $^{###}P < 0.001$ vs KA, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

injection, coronal sections of the brain were prepared for Nissl staining (**Figure 1A**). Representative images displayed that, neurons were hyperchromatic, pyknosis, and karyorrhexis after KA treatment (**Figure 1B**). Quantification of cells with normal morphology in the striatum showed that KA is significantly lethal to neurons (**Figure 1C**). In the subsequent experiments, we determined 0.625 nmol as the dosage in the model group.

The harmful consequences of glutamate receptor overactivation have been shown to be related to a series of events triggered by calcium-dependent enzyme activation, such as oxidative stress and mitochondrial damage (Divakaruni et al., 2017; **Figures 2A,B**). The significant changes of SOD activity and MDA content reflect the occurrence of oxidative stress. Interestingly,

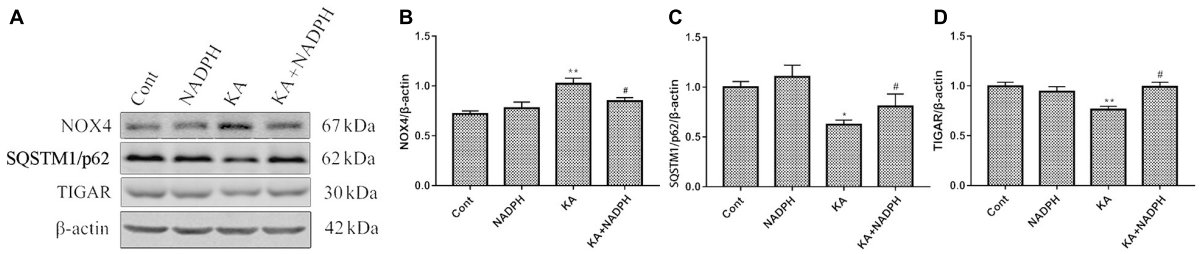

FIGURE 4 | NADPH inhibits KA-induced autophagy activation and upregulation of NOX4. Animals were pre-treated with NADPH (2.5 mg/kg, *i.v*) 30 min prior to KA (0.625 nmol) injection and then they were sacrificed 12 h later for western blotting. **(A–D)** Representative bands and semi-quantitation of western blots for detecting NOX4, SQSTM1/p62 and TIGAR protein levels. Data are expressed as the Mean ± SEM ($n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$ vs KA, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

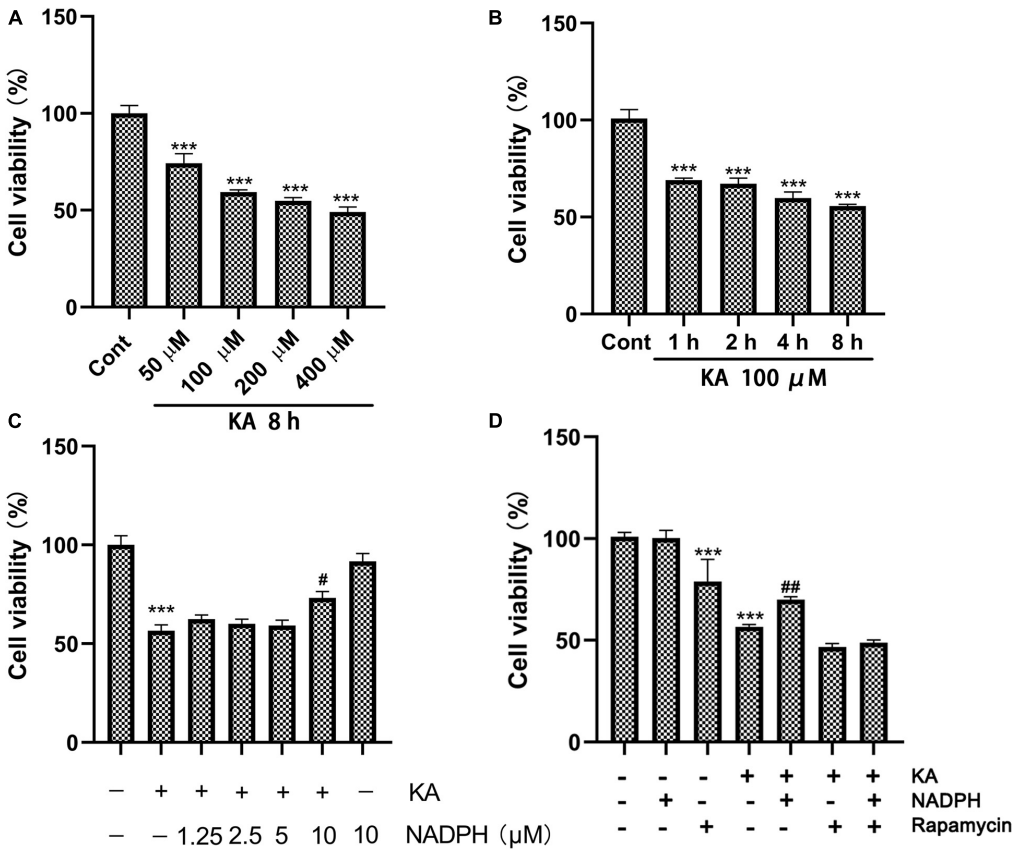

FIGURE 5 | NADPH protects neurons against KA-induced excitotoxicity by inhibiting autophagy *in vitro*. **(A,B)** Cultures were treated with indicated concentrations (100, 200, 400, and 800 μM) of KA for 8 h or KA (100 μM) for 1, 2, 4, and 8 h. **(C)** The effects of NADPH on KA-induced cytotoxicity. Cultures were pre-treated with NADPH (1.25, 2.5, 5, and 10 μM) for 4 h, then treated with KA (100 μM) or 8 h. **(D)** Cultures were pre-treated with NADPH (10 μM) and/or rapamycin (3 μM) for 4 h, then treated with KA (100 μM) for 8 h. Cell viability was measured by CCK8 kit. Data are expressed as the Mean ± SEM. ($n = 5$. $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs KA, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

this change is short-lived. We hypothesized that it might be related to the increased nuclear translocation of Nrf2 that we detected (Tonelli et al., 2018), or due to compensatory stress.

We previously found that autophagy inhibitors partially blocked the toxicity of KA (Wang et al., 2008; Zhang et al., 2009). We examined the effect of KA on autophagy at different doses and treatment times. Increased ratio of LC3-II/LC3-I and decreased SQSTM1/p62 expression level embodied more autophagosomes and enhancive degradation, representing autophagy flows smoothly (**Figures 2C–H,K,L**; Kuma et al., 2017). TIGAR regulates the pathway of glycolysis. TIGAR can inhibit the occurrence of autophagy under nutritional starvation or metabolic stress. In the cellular antioxidant defense system, TIGAR may regulate autophagy as part of its constituent activity, which has a significant influence on the mTOR pathway (Geng et al., 2018; Zhang et al., 2019). It could not maintain the original expression level after KA treatment, which may exert a profound influence on autophagy (**Figures 2I–K,M**). KA injection into the striatum significantly increased NOX4 expression (**Figures 2N–Q**), but did not seem to affect NOX2 (**Supplementary Figure 1**).

There are still few suitable antioxidant drugs clinically available to deal with excitotoxicity in various neurodegenerative diseases. This study provides insights into seeking antioxidants to balance out excess ROS. So we focused on NADPH, which is known as coenzyme II and maintains the reduced GSH.

NADPH Protects Neurons Against KA-Induced Excitotoxicity and Autophagy Activation

We investigated whether NADPH supplementation has an effect on KA-induced neuronal death and autophagy activation *in vivo* first. Mice were pretreated with NADPH (0.625, 1.25, and 2.5 mg/kg, *i.v*) 30 min before intrastriatal injection (**Figure 3A**). NADPH was then administered daily until day 14 through the tail vein. Pretreatment of 2.5 mg/kg NADPH notably reduced the nuclear condensation and abnormal neurons in the unilateral striatum (**Figures 3B,C**). Therefore, we performed the subsequent study with NADPH at 2.5 mg/kg.

Western blot data showed that pre-treatment with NADPH reversed KA-induced upregulation of NOX4 and downregulation of SQSTM1/p62 and TIGAR protein levels (**Figure 4**). In the primary cortical neurons, NADPH treatment also has a certain significant effect (**Figures 5A–C**). We also found that rapamycin, an autophagy activator, was able to negate the protective effect of NADPH in primary cortical neurons (**Figure 5D**). These suggest that NADPH can inhibit KA-induced autophagy activation and that its protective effect is at least partially dependent on this mechanism.

Although NADPH has excellent curative effect, it has the defect of narrow therapeutic window, exposed in the experiment (Qin et al., 2017). This makes practical use and clinical research difficult. Considering its role in both ROS elimination and production, inhibition of NOX appears to be necessary for its future clinical application (Qin et al., 2017). Therefore, we set out to solve this problem.

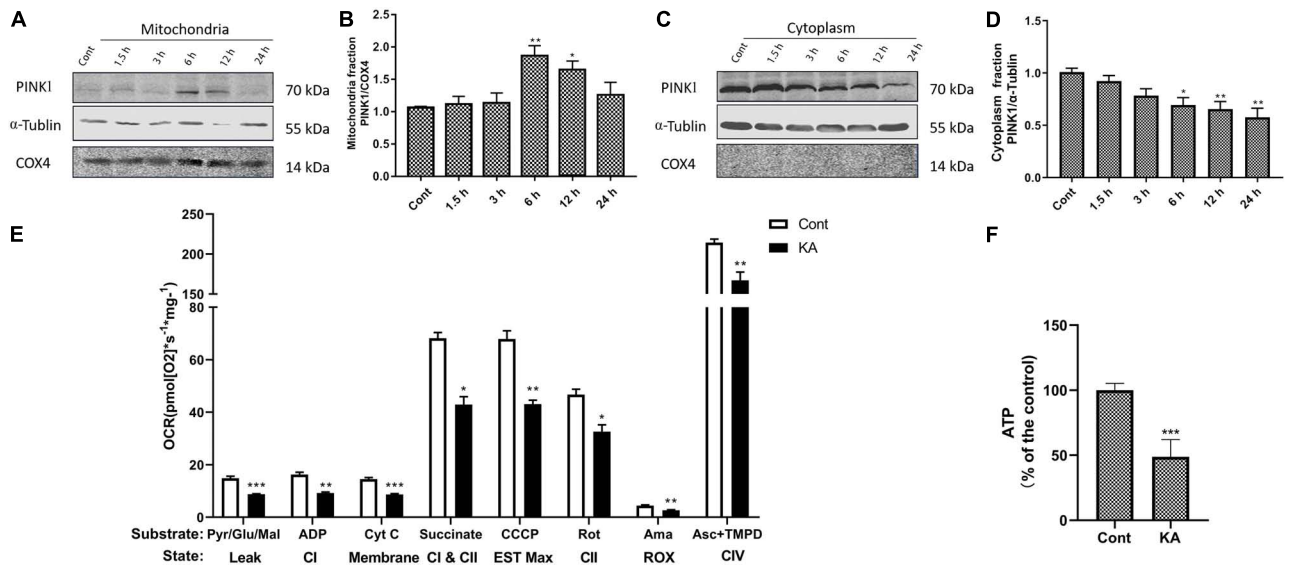

FIGURE 6 | KA induces mitochondria dysfunction *in vivo*. **(A–D)** Mice were given different doses of KA or treated for different periods of time. Representative bands and semi-quantitation of western blots for detecting PINK1 protein levels in the mitochondria fraction and cytoplasm fraction. **(E)** Mitochondrial respiratory parameters determined by O2K. The control group and the KA group were labeled with white and black columns, respectively. **(F)** Quantification of ATP concentration. Data are expressed as the Mean ± SEM. Scale bar = 500 μm (n = 8. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

Mito-Apocynin Inhibits KA-Induced Upregulation of NOX4 on Mitochondria

Then we studied the effect of KA on NOX expression. NOX1, 2, and 4 are the main NOX subtypes expressed in neurons, while the role of NOX2 and NOX4 in neurodegenerative diseases has been relatively well studied (Ma et al., 2017). NADPH has a contradictory role in regulating redox. It can not only generate GSH to remove ROS, but also generate ROS as the substrate of NOX (Ying, 2008). Notably, the reversal of NOX4 expression level indicates that NADPH is less likely to produce ROS as its substrate, suggesting that the combination we are considering is meaningful. We studied the classic NOX inhibitors DPI and apocynin and developed two different combined administration regimens, which have been shown to have better effects when combined with NADPH, both for neuronal protection and motor function recovery, as shown in the (**Supplementary Figures 2–5**).

Mitochondrial dysfunction is one of the important mechanisms of excitotoxicity leading to cell death (Wang et al., 2009). In depolarized mitochondria, PINK1 acted as a molecular sensor for damaged mitochondria, triggering the initiation of mitochondrial autophagy (**Figures 6A–D**). An important physiological function of PINK1 is to increase intracellular resistance to stress. Depletion of PINK1 in the cytoplasm increases the risk of stress-induced cell death. In cells deficient in PINK1, mitochondrial membrane proteins were reduced, affecting the efficiency of mitochondrial transport (Arena and Valente, 2017). The mitochondrial respiratory function and ATP-generating capacity in model group were significantly weaker than those in control group (**Figures 6E,F**). *In vitro*, KA resulted in decreased ATP level, loss of MMP and increased mitochondrial superoxide (**Figure 7**). Considering the regional limitations of NOX4 expression and mitochondrial dysfunction, mito-apocynin-C11, an inhibitor of mitochondrial NOX, was selected for combined treatment with NADPH.

Mito-apocynin-C11 apocynin conjugated to a mitochondria-targeting triphenyl phosphonium cation (TPP^+) selectively target mitochondria NOXs via an alkyl chain consisting of eleven carbon atoms (Ghosh et al., 2016; Langley et al.,

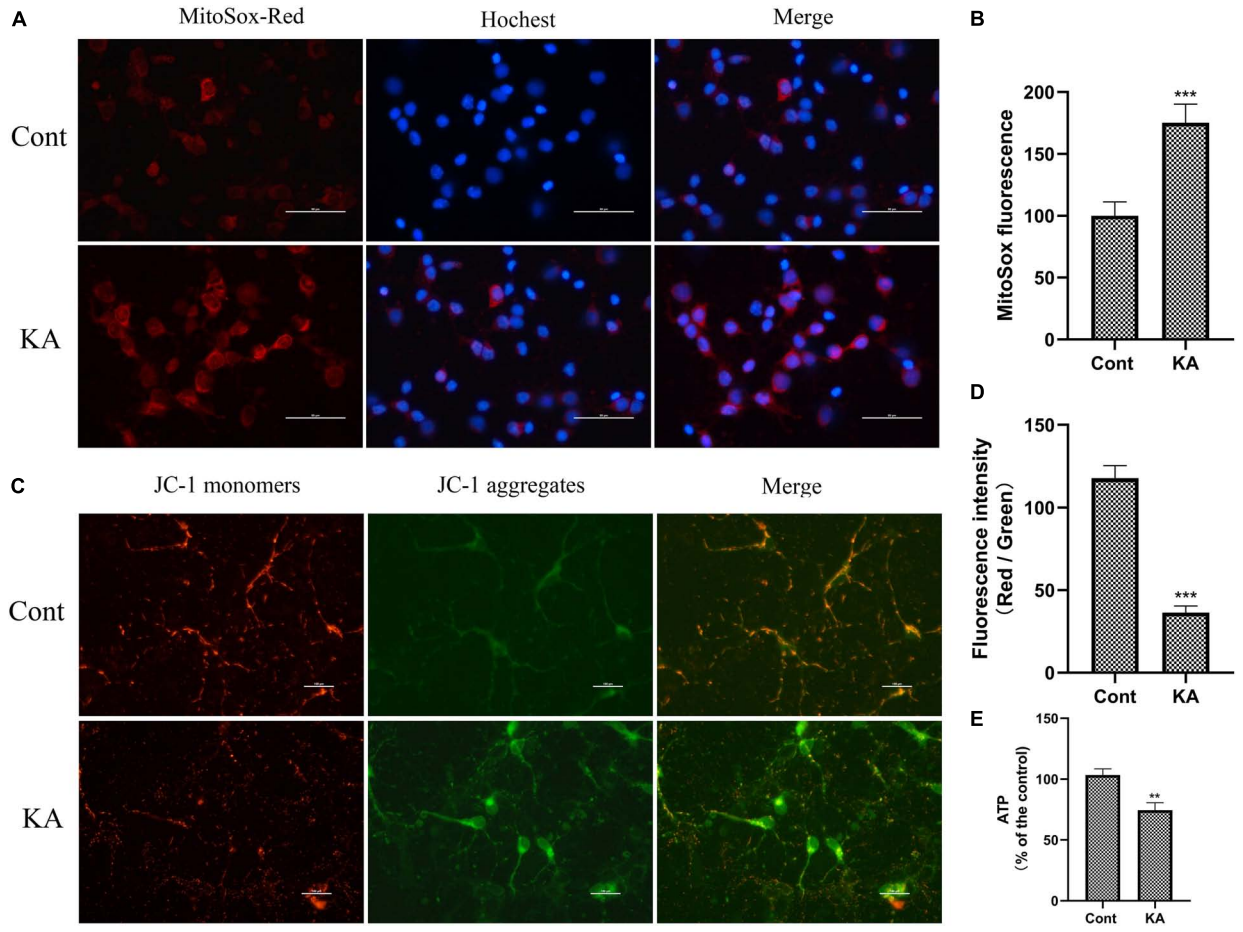

FIGURE 7 | KA induces mitochondria dysfunction *in vitro*. **(A,B)** MtROS was detected and quantified by MitoSOX Red probe. **(C,D)** Mitochondrial membrane potential was examined and quantified by JC-1 staining assays. **(E)** Quantification of ATP concentration. Data are expressed as the Mean ± SEM. Scale bar = 50 μm (n = 8. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

2017). The presence of a highly lipophilic and delocalized cationic moiety in Mito-apocynin-C11 makes it more cell-permeable and selectively target mitochondria (Dranka et al., 2014). Sequestration into mitochondria is facilitated by TPP^+ conjugation to apocynin via long carbon–carbon side chains (Brenza et al., 2017). Mito-apocynin intragastric administration inhibits NOXs activity in the brain (**Supplementary Figure 6**). Further examination revealed that the change in NOX4 expression was mainly attributable to mitochondria, while the level in cytoplasm was relatively stable (**Figure 8**). Mito-apocynin can improve neuronal survival and indirectly inhibit mitochondrial NOX expression (**Figures 8**, **9**).

Combined NADPH and Mito-Apocynin Provides Greater Neuroprotective Effects and Motor Recovery

To determine whether combined Mito-apocynin can enhance the neuroprotective effects of NADPH, we chose their minimum effective dose for further study *in vivo*. The design of the experiment can be seen in **Figure 10A**.

Lesions in the striatum can lead to impaired voluntary movement and muscle tone, especially in the forelimbs (Hidalgo-Balbuena et al., 2019). So we detect impaired motor function as an indicator of neural dysfunction due to excitotoxicity, by performing the adhesive removal test and the cylinder test of forelimb asymmetry (Hernández-Espinosa et al., 2019). Before stereotactic KA injection, the mice could quickly remove the adhesive label and gave priority to touch (Hernández-Espinosa et al., 2019) the container wall with both forelimbs. KA resulted in a significant delay to remove and reduction of bilateral touch in each group. Compared to NADPH, combination therapy group showed similar impaired motor function at 1 h, but better recovery in the later with statistical significance (**Figures 10B,C**). Using NADPH alone, the morphology striatal neurons were not significantly restored as in combination therapy group. Besides, combination administration shrunk the lesion area and increase cell count compared to the monotherapy (**Figures 10D—H**).

The present findings confirm that combination of NADPH and Mito-apocynin can significantly improve the neuronal survival in the neuronal excitotoxicity model, and enhance the rehabilitation of neurobehavioral defects, by speeding up the recovery of muscle tone, fine motor ability and limb coordination. Then we further investigated whether this better effect was associated with further increase in GSH and inhibition of autophagy.

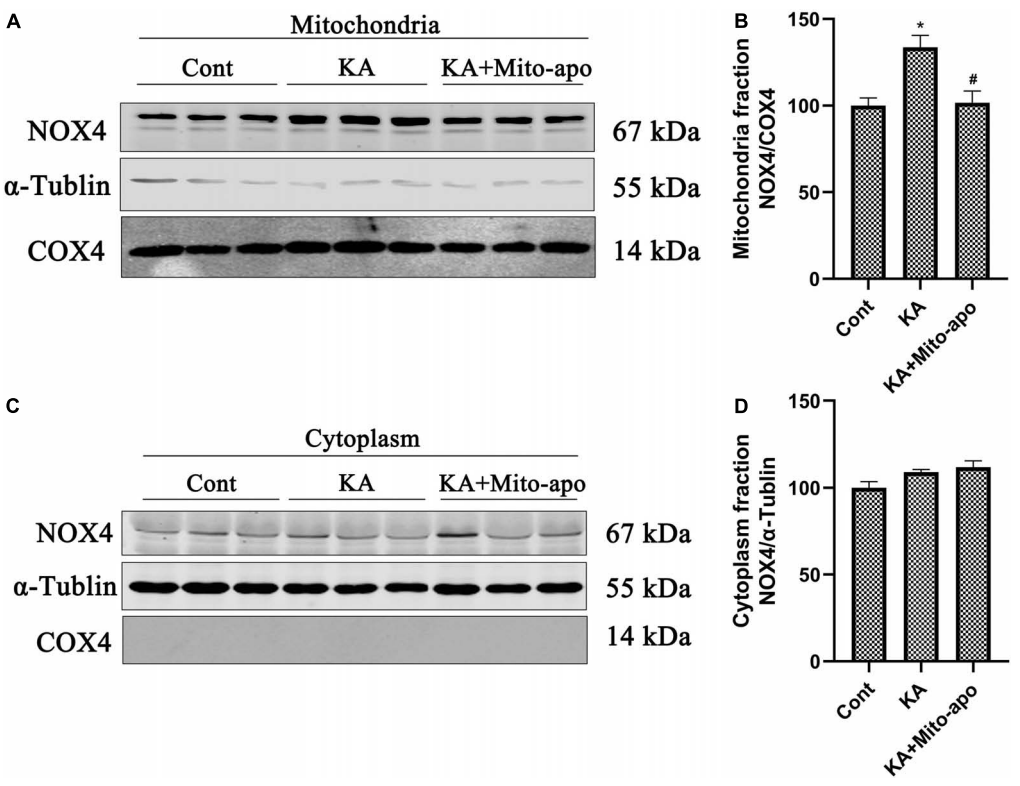

FIGURE 8 | Mito-apocynin reverses KA-induced upregulation of NOX4 in mitochondria fraction. **(A–D)** Animals were pre-treated with mito-apocynin (displayed as Mito-apo in figures; 6 mg/kg, *i.g*) 1 day prior to KA (0.625 nmol) injection and then they were sacrificed 6 h later for western blotting. Representative bands and semi-quantitation of western blots for detecting NOX4 protein levels. Data are expressed as the Mean ± SEM ($n = 5$. $^*P < 0.05$; $^{\#}P < 0.05$ vs KA, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

Combined NADPH and Mito-Apocynin Further Increased GSH Levels and Inhibited Autophagy

.5 NADPH can restore KA-induced GSH level decline *in vivo* and *in vitro*, and play an antioxidant role. In combination, GSH content increases further (**Figures 11A**, **12D**). This indicates that NADPH does, as expected, reduce the proportion of ROS production as a substrate of NOX, and tends to be a reducing agent. In addition, NADPH further reduced LC3-II/LC3-I and increased SQSTM1/p62 expression when combined with Mito-apocynin (**Figures 11B–E**). Mito-apocynin appears to enhance the inhibition of autophagy by NADPH. We concluded that combination of NADPH and mito-apocynin provides better neuroprotection and motor recovery through stronger antioxidant and autophagy balance.

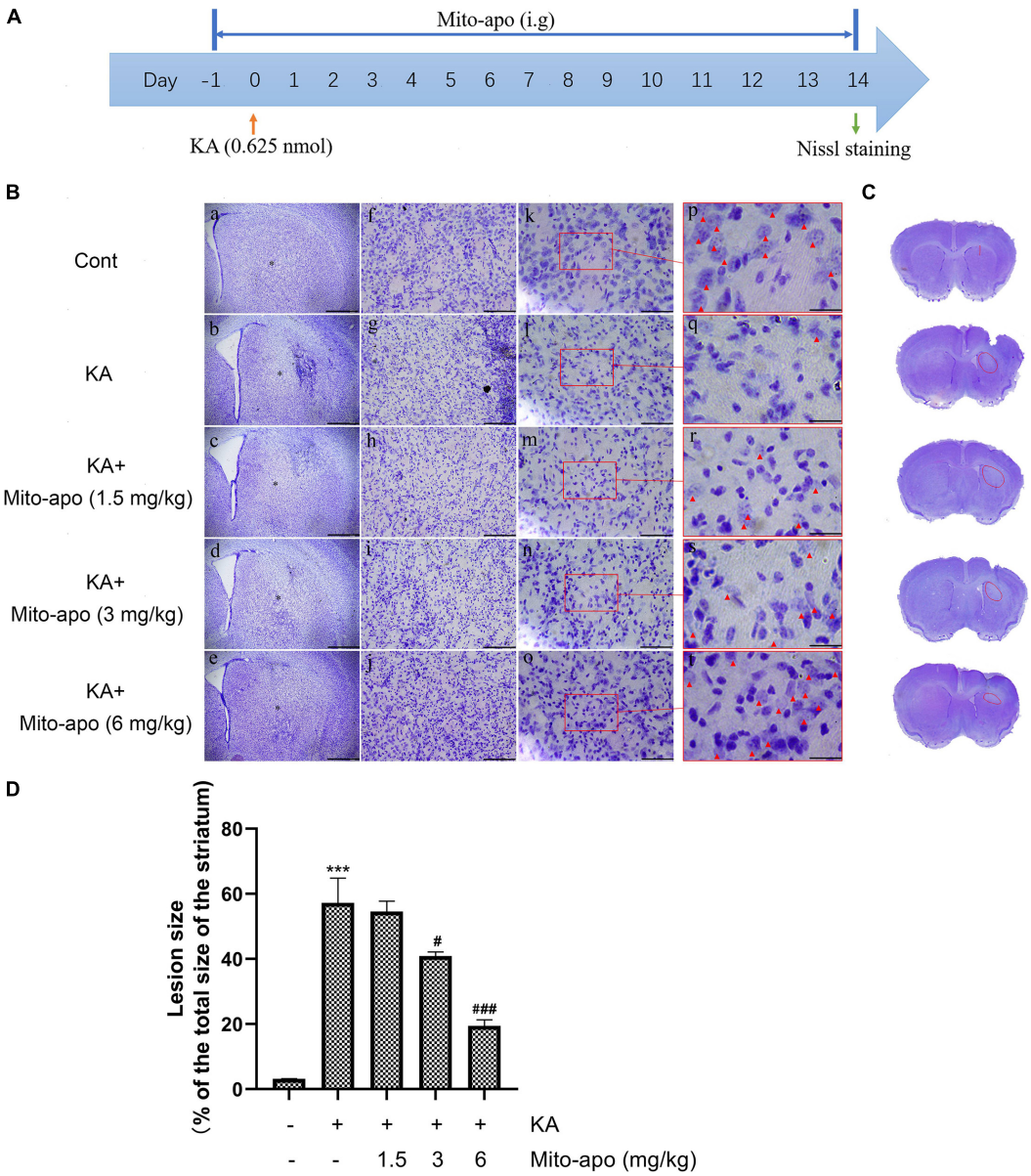

FIGURE 9 | Effects of Mito-apocynin on KA-induced striatal neuronal death. **(A)** Illustration of experimental design. Mice were intragastric administrated with 1.5, 3, and 6 mg/kg Mito-apocynin, 1 day prior to KA (0.625 nmol) injection. Administrate Mito-apocynin daily until mice were sacrificed 14 days later. Nissl staining were performed on brain sections. **(B)** Representative microscopic images of Nissl-stained coronal brain section displaying the lesion. **(C)** Representative photographs of sections. The lession has been redlined. **(D)** Quantification of lesion area. Data are expressed as the Mean ± SEM. Scale bar = 500 μm in a–e; 100 μm in f–j; 50 μm in k-o; and 20 μm in p–t ($n = 3$. $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs KA, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

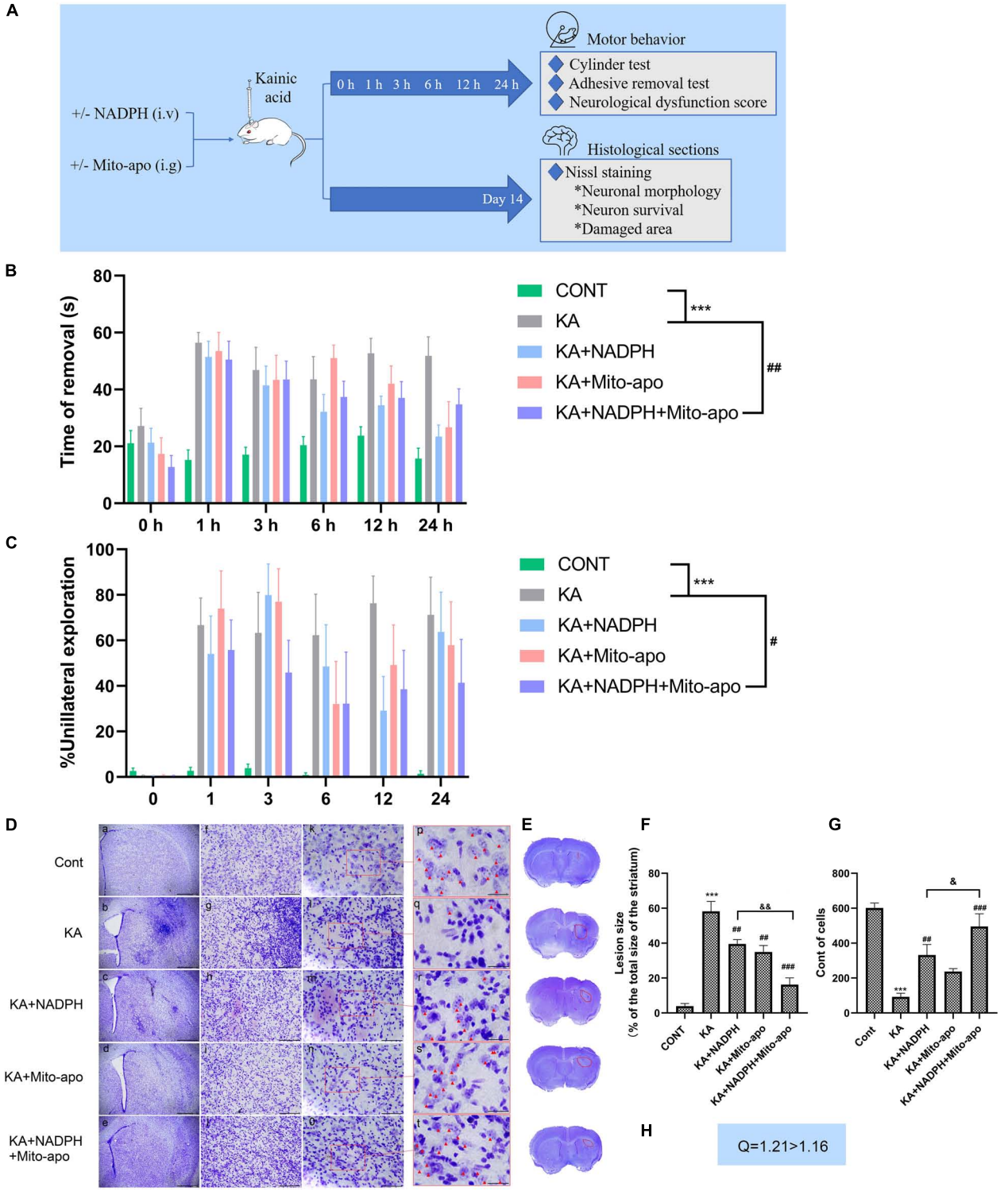

FIGURE 10 | Combined NADPH and Mito-apocynin provides greater motor recovery and neuroprotective effects. **(A)** Illustration of experimental design. Mice were treated with NADPH and/or Mito-apocynin before KA injection. Behavioral tests were performed at 1, 3, 6, 12, and 24 h. The mice were sacrificed on the 14th day to prepare histological sections for Nissl staining. **(B)** Adhesive removal test. **(C)** Cylinder test. **(D)** Representative microscopic images of Nissl-stained coronal brain section displaying the lesion. **(E)** Representative photographs of sections. The lesion has been redlined. **(F)** Quantification of lesion area. **(G)** Quantification of neurons with normal morphological specificity. **(H)** Combination index. Data are expressed as the Mean ± SEM. Scale bar = 500 μm in a–e; 100 μm in f–j; 50 μm in k–o; and 20 μm in p–t ($n = 6$. $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs KA; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ KA + NADPH vs KA + NADPH + Mito-apocynin, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

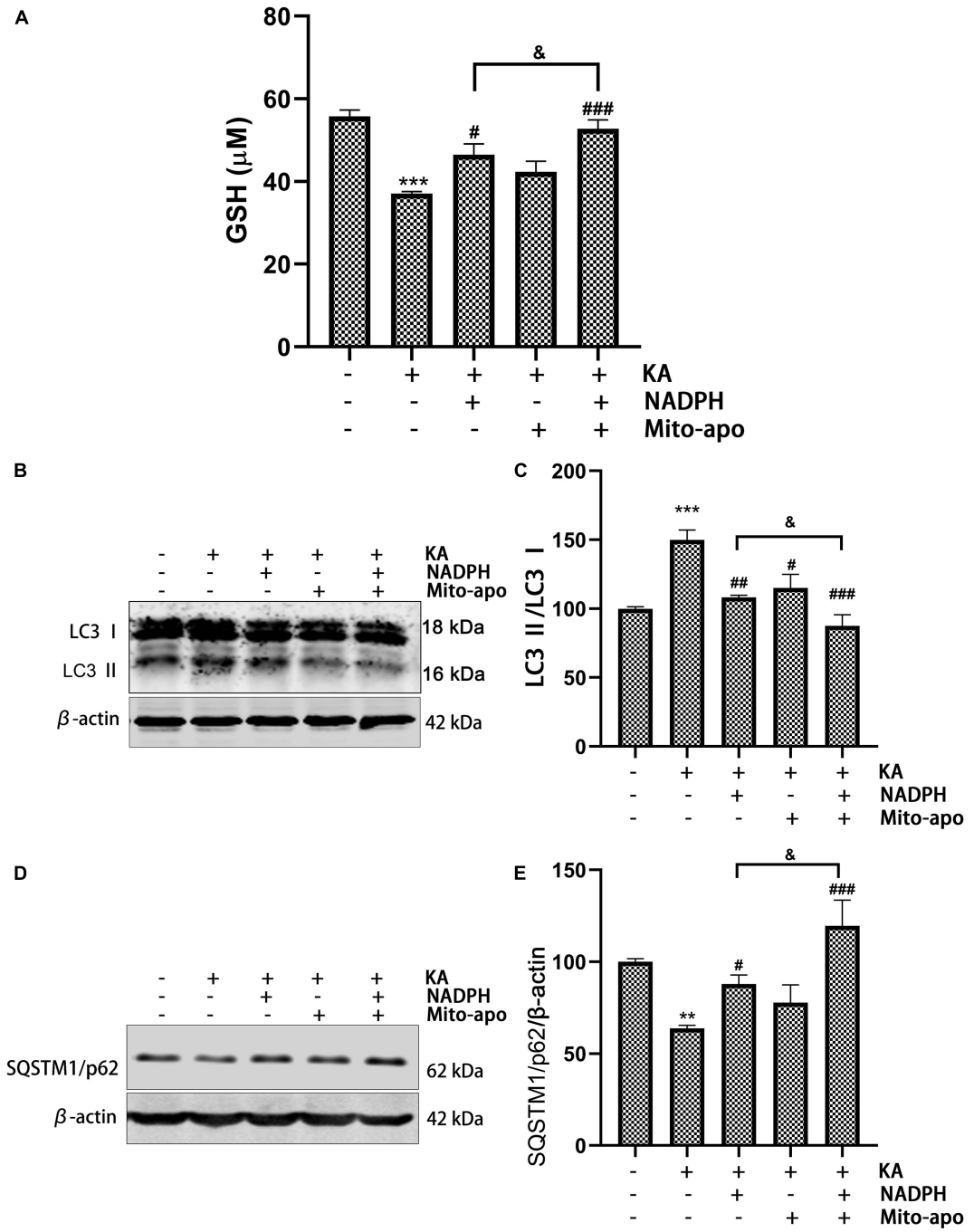

FIGURE 11 | Combined NADPH and Mito-apocynin further increases GSH levels and inhibited autophagy. Animals were pre-treated with Mito-apocynin (3 mg/kg, *i.g*) and/or NADPH (2.5 mg/kg, *i.v*) prior to KA (0.625 nmol) injection. **(A)** They were sacrificed 12 h later for determination of GSH. **(B,C)** They were sacrificed 3 h later. Representative bands and semi-quantitation of western blots for detecting LC3 protein levels. **(D,E)** They were sacrificed 12 h later. Representative bands and semi-quantitation of western blots for detecting SQSTM1/p62 protein levels. Data are expressed as the Mean ± SEM. ($n = 5$. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs KA; $^{\&}P < 0.05$, KA + NADPH vs KA + NADPH + Mito-apocynin, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

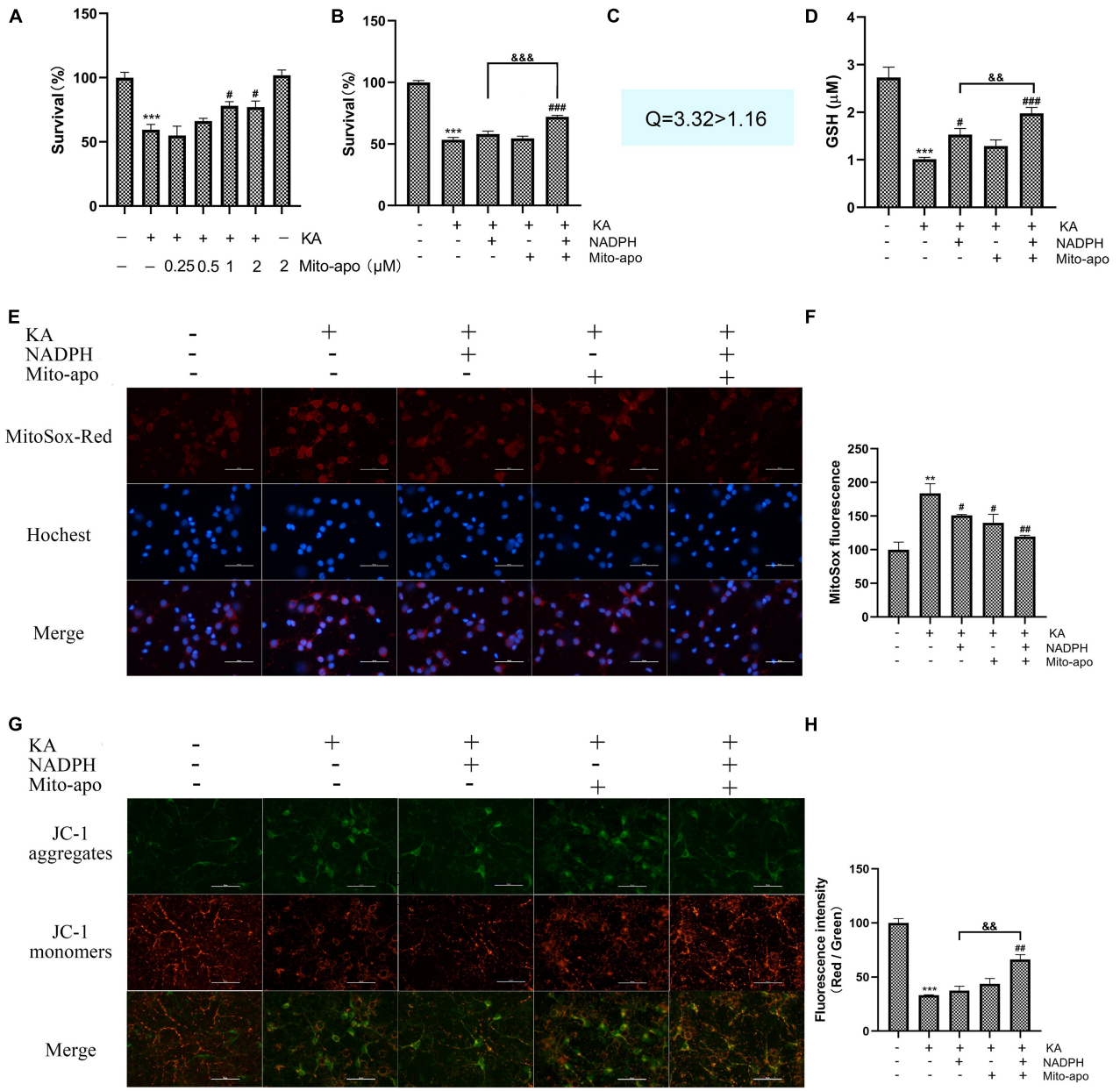

FIGURE 12 | Effects of combined NADPH and Mito-apocynin on KA-induced mitochondria dysfunction *in vitro*. Cultures were pre-treated with NADPH and/or mito-apocynin for 4 h, then treated with KA (100 μM) for 8 h. **(A,B)** Cell survival detected by CCK-8 kit. C_{NADPH} = 5 μM; $C_{mito-apo}$ = 0.5 μM. **(C)** Combination index. C_{NADPH} = 5 μM; $C_{mito-apo}$ = 0.5 μM. **(D)** Determination of GSH. C_{NADPH} = 10 μM; $C_{mito-apo}$ = 1 μM. **(E,F)** MtROS was detected and quantified by MitoSOX Red probe. C_{NADPH} = 5 μM; $C_{mito-apo}$ = 0.5 μM. **(G,H)** Mitochondrial membrane potential was examined and quantified by JC-1 staining assays. C_{NADPH} = 5 μM; $C_{mito-apo}$ = 0.5 μM. Data are expressed as the Mean ± SEM. Scale bar = 50 μm (n = 5. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs KA; $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$, KA + NADPH vs KA + NADPH + Mito-apocynin, one-way ANOVA, followed by a *post hoc* multiple comparison Student-Newman–Keuls test).

Effects of Combined NADPH and Mito-Apocynin on KA-Induced Mitochondria Dysfunction *in vitro*

As mentioned above, we found the phenomena of increased mitochondrial superoxide anions and decreased membrane potential. We preliminarily investigated the effect of combination on mitochondrial dysfunction. Pretreatment with 1 μM Mito-apocynin (**Figure 12A**) or 10 μM NADPH for 4 h significantly improved neuronal survival. When both ineffective dose of NADPH and Mito-apocynin were pretreated, the combination group showed a synergistic effect, as evidenced by combination index (**Figures 12B,C**).

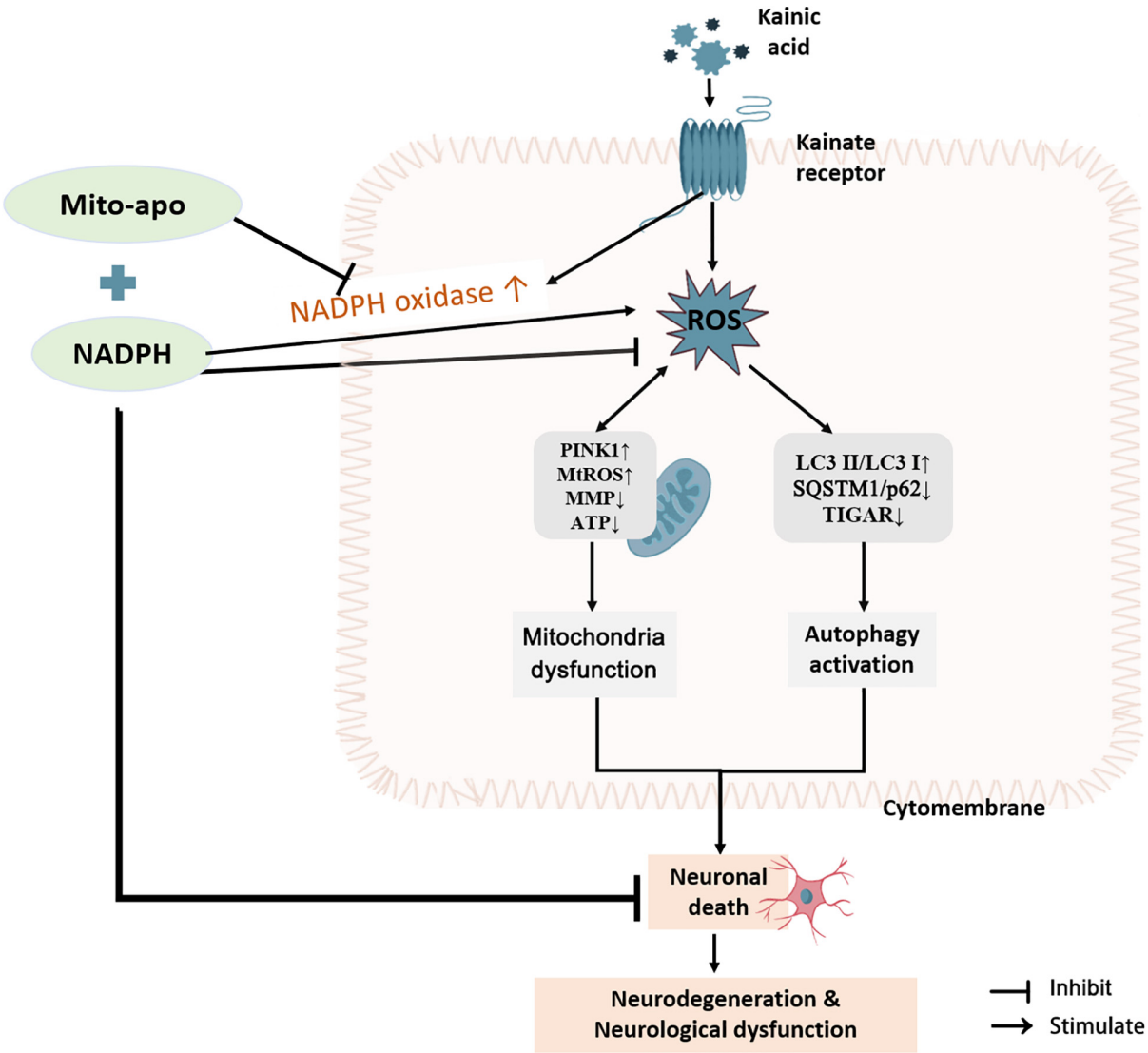

FIGURE 13 | Combination of NADPH and Mito-apocynin offers greater neuroprotection and resistance to mitochondrial impairment on KA-induced excitotoxicity. KA induces the increase of ROS in the striatum, following by autophagy activation and mitochondrial stress, eventually leading to neuronal death, and subsequent neurodegeneration and neurological dysfunction. NADPH can improve neuron survival by reversing the protein expression of SQSTM1/p62 and TIGAR and sheltering mitochondria. When combined with Mito-apocynin, it reduces NADPH as a NOX substrate to generate ROS, and finally performs better neuroprotective effects and resistance to mitochondrial impairment.

In terms of reducing mitochondrial superoxide production, syndication is not so superior (**Figures 12E,F**). In terms of restoring MMP, combined administration is significantly better than monotherapy, (**Figures 12G,H**). The results show that NADPH play a role in reducing mitochondrial dysfunction and, in some ways, combining mito-apocynin is better. Further studies on NADPH and mitochondria are needed.

DISCUSSION

Overstimulation of glutamate receptors increases the production of free radicals, leading to the development of a variety of neurodegenerative diseases (Serwach and Gruszczynska-Biegala, 2019). Some researchers recognize the efficacy of antioxidants on animal models of Huntington's diseases, Parkinson's diseases, Alzheimer's diseases, and stroke (Hughes et al., 2016; Huang et al., 2018; Pinho et al., 2020). In this study, we stimulate glutamate receptors specifically and efficiently to construct representative neurodegenerative disease models, by precisely injecting kainic acid (KA) into the striatum. It mediates ROS accumulation *in vivo*, which can be fatal to neurons. Exogenous NADPH and Mito-apocynin, a mitochondrial targeted NOX inhibitor, both offer efficacy against excitotoxic injury to some extent. The mechanisms by which they operate are associated with maintaining reductive substances and reducing the generation

of free radicals. Our previous studies have shown that systemic administration of NADPH can penetrate the blood-brain barrier (Li et al., 2016). Intracellular NADPH concentration in cultured mouse cortical neurons was significantly increased after the addition of exogenous NADPH. The mechanism of NADPH transmission through the membrane had been a largely under explored domain. We have preliminary research on this issue, but more exploration is needed.

The upregulation of NOX4 expression in striatum, especially in mitochondria, increases the risk of oxidative stress. NADPH could inhibit KA-mediated upregulation of NOX4 expression, indicating that the possibility of ROS generation by NADPH was decreased. Therefore, it is of great significance to study whether the combination of NADPH and NOX inhibitors can play a better neuroprotective role in neuroexcitatory injury. Two NOX inhibitors (DPI and apocynin), two combined schemes (combination of ineffective or effective dose), fully demonstrate the advantages of combined use, both in terms of reducing injury and restoring motor function. Mito-apocynin was selected because of occurrence of NOX4 regional elevations and mitochondrial dysfunction. Its presence greatly increases the potency of NADPH by further increasing the content of GSH that can be produced. Lesions and neuronal death were greatly reduced in the striatum. While any monotherapy did not significantly improve the neurobehavioral of mice, the combination therapy did.

Under some pathological conditions, such as stroke and neurodegenerative diseases, relatively excessive ROS accumulation will destroy cell homeostasis, leading to oxidative stress and mitochondrial dysfunction, and induce autophagy. In this process, oxidative stress promotes autophagy. In turn, autophagy helps to reduce oxidative damage by engulfing and degrading oxidizing substances (Scherz-Shouval and Elazar, 2011). The internal regulation mechanism of ROS autophagy includes various molecular signaling pathways, such as ROS-FOXO3-LC3/BNIP3-autophagy, ROS-NRF2- SQSTM1/p62-autophagy, and ROS-TIGAR-autophagy (Li et al., 2015). NADPH inhibits overactive autophagy caused by excessive ROS in excitotoxicity. And its protective effect can be weakened by autophagy activators. Its dual effect on ROS limits its ability to restore autophagy balance. This drawback seems to be alleviated through a combination of mito-apocynin.

Excitotoxicity resulted in significant mitochondrial impairment *in vitro* and *in vivo*, including enhanced PINK1 signal, increased superoxide, decreased MMP, and reduced ATP production. Antioxidant strategies are abundant, but they do not perform well in neurodegenerative diseases. Mitochondria is recognized as one of the sources of ROS (Dan Dunn et al., 2015). Recent data support that mitochondrial targeted NOX inhibitors have a certain effect on PD, but there are few studies on its mechanism (Dranka et al., 2014; Langley et al., 2017). Mito-apocynin was effective in narrowing the lesion and had a synergistic amplification effect with NADPH. It may reduce NADPH as the substrate of mitochondrial NOX and increase superoxide anions, showing less damage and better movement recovery. The concomitant consequences of excitotoxicity may be in large part related to mitochondrial dysfunction caused by calcium stress. 5 μM NADPH and 0.5 μM mito-apocynin were not significant on the cell activity test. They failed to restore MMP, but had a significant effect when combined. But no significant difference has been found in MtROS reduction.

Collectively, NADPH protects neurons from excitotoxic damage by blocking autophagy activation through antioxidant activity. When used in combination with NOX inhibitors (DPI, apocynin, and Mito-apocynin), the neuronal protection and mobility restoration capabilities are even more powerful. The reason may be the reduction of NADPH as a substrate to promote ROS during the reduction stress. When the NOX inhibitor was targeted at mitochondria, it has an extra miraculous effect on MMP resumption. Maintenance of mitochondrial function may be considered a promising aspect of resisting excitotoxicity (**Figure 13**).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethical Committee of Soochow University.

AUTHOR CONTRIBUTIONS

YW conceived and designed the research. NL, M-ML, S-SH, and Z-QL collected data and conducted the research. NL wrote the initial manuscript. YW, J-CW, Z-QL, and Z-HQ revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.612554/full#supplementary-material

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REVIEW
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Understanding the Multiple Role of Mitochondria in Parkinson's Disease and Related Disorders: Lesson From Genetics and Protein–Interaction Network

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As neurons are highly energy-demanding cell, increasing evidence suggests that mitochondria play a large role in several age-related neurodegenerative diseases. Synaptic damage and mitochondrial dysfunction have been associated with early events in the pathogenesis of major neurodegenerative diseases, including Parkinson's disease, atypical parkinsonisms, and Huntington disease. Disruption of mitochondrial structure and dynamic is linked to increased levels of reactive oxygen species production, abnormal intracellular calcium levels, and reduced mitochondrial ATP production. However, recent research has uncovered a much more complex involvement of mitochondria in such disorders than has previously been appreciated, and a remarkable number of genes and proteins that contribute to the neurodegeneration cascade interact with mitochondria or affect mitochondrial function. In this review, we aim to summarize and discuss the deep interconnections between mitochondrial dysfunction and basal ganglia disorders, with an emphasis into the molecular triggers to the disease process. Understanding the regulation of mitochondrial pathways may be beneficial in finding pharmacological or non-pharmacological interventions to delay the onset of neurodegenerative diseases.

Keywords: Parkinson's disease, atypical parkinsonism, Huntington disease, mitochondrial dysfunction, neurodegenerative diseases, Pink1/parkin pathway, alpha-synuclein

INTRODUCTION

Neurodegenerative diseases represent one of the major challenges of our era. Despite their high frequency in populations and impact on our society, pathogenic mechanisms are still widely unknown, and therapeutic approach are only symptomatic nowadays. Movement disorders, together with dementias, are the most frequent neurodegenerative diseases in elderly with Parkinson's disease (PD) affecting up to 2% of the population over 65 years (Ray Dorsey et al., 2018). It is characterized by the progressive loss of dopaminergic neurons in pars compacta of substantia nigra (SN) and the presence of intracellular aggregates of the protein α-synuclein (α-syn). PD is sporadic in most cases (90–95%) but inherited in approximately 5–10% of cases (Bonifati, 2007;

Gasser, 2009; Hernandez et al., 2016). The main clinical features are bradykinesia, rigidity, resting tremor, and gait disturbance, preceded and associated with no-motor symptoms such as rapid eye movement (REM) behavior disorder, hyposmia, and depression.

Although there are many efforts of research in investigating the pathogenesis of PD, primary causes of this disease remain elusive. Mitochondria have often been proposed as potential candidates involved in neurodegeneration. Indeed, the main risk factor for neurodegenerative disease as well as PD is aging in which mitochondria play an important role (Sun et al., 2016; Giannoccaro et al., 2017; Rango and Bresolin, 2018; Theurey and Pizzo, 2018). Experimental models (Schwarze et al., 1995; Khaidakov et al., 2003) and postmortem studies in elderly (Corral-Debrinski et al., 1992; Ojaimi et al., 1999) exhibited an increased load of mitochondrial DNA (mtDNA) mutations and deletions and a reduced respiratory chain activity. The main challenge is establishing whether mitochondria impairment could represent the initial cause of neurodegeneration or an epiphenomenon (Borsche et al., 2020). This review aims to describe the evidence, particularly from genetic field, which highlight the critical role of these organelles in participating and fostering the neurodegenerative processes, especially PD and other neurodegenerative movement disorders. Then, it shows the importance of the relationship between mitochondrial dysfunction and deposits of aggregated α-syn and between mitochondrial dysfunction and impairment of other cellular degradative pathways, particularly lysosomal system.

PD AND ENERGY PRODUCTION FUNCTION OF MITOCHONDRIA

For a long time, the pathogenic role of mitochondria in PD has mainly been considered linked to their function of energy producers for cells and the consequent effect of generators of reactive oxygen species (ROS). Indeed in humans, the brain is the most energy-demanding organ, accounting for approximately 20% of the body's total demand (Clarke and Sokoloff, 1999). Mitochondrial ATP production is very important for multiple neuronal processes such as axonal transport and synaptic neurotransmission, which require high levels of energy. ATP is produced in mitochondria by the respiratory chain where the electrons' flow within the complexes generates superoxide anion. This is a free radical resulting from the reaction between oxygen and a small constitutive leak of high-energy electrons. The production of superoxide anion particularly occurs in complexes I and III. Cells possess antioxidant systems such as superoxide dismutase enzyme (SOD) and glutathione, which hinder the production of free radicals. When an imbalance between these opposite strengths occurs, the result is a state defined "oxidative stress," which indicates an accumulation of excessive ROS and a consequent damage on biological molecules (Morán et al., 2012). Actually, postmortem studies on PD brains showed increased levels of lipid peroxidation, protein carbonyls (Dalfó et al., 2005; Seet et al., 2010), and mtDNA mutations, particularly deletions/rearrangement (Ikebe et al., 1995; Gu et al., 2002; Bender et al., 2006; Dölle et al., 2016) with respect to controls.

In PD, inhibition of respiration, particularly linked to a dysfunction of complex I of respiratory chain, is considered the main source of oxidative stress. Selective deficiency in enzymatic activity of respiratory chain complex I and reduction in glutathione are observed in the SN of PD patients (Schapira et al., 1989, 1990; Mann et al., 1992, 1994; Janetzky et al., 1994), although conflicting results about the evaluation of complex I activity in other PD patients' tissues such as skeletal muscles, platelets, and leukocytes have been provided (Parker et al., 1989; Yoshino et al., 1992; Didonato et al., 1993; Martín et al., 1996; Winkler-Stuck et al., 2005). Several lines of evidences support the specific involvement of complex I. Neuropathological studies confirmed a reduction in complex I within striatum and SN of PD patients (Mizuno et al., 1989; Hattori et al., 1991). Cybrids, mtDNA-depleted human cells repopulated by mitochondria derived from PD patients and controls, respectively, showed deficient activity of complex I and increased oxidative stress in the former with respect to the latter (Swerdlow et al., 1996; Gu et al., 1998). Especially animal models based on chronic administration of toxins specifically inhibiting complex I of respiratory chain such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Rotenone and Paraquat (Betarbet et al., 2000; Fornai et al., 2005; Inden et al., 2011; Bové and Perier, 2012) produced pathological and clinical changes akin to PD (Martinez and Greenamyre, 2012). Why complex I is selectively involved remains to be elucidated.

The importance of mitochondrial energy and ROS-associated productions are furthermore highlighted by some autosomal recessive form of hereditary PD whose genes [*PARKIN*, PTEN-induced putative kinase 1 (*PINK1*), and *DJ1*] have a role in mitochondrial bioenergetics and oxidative stress. *PINK1* is a mitochondrial kinase whose deficit was associated to high levels of ROS and more vulnerability to oxidative stress in *Drosophila melanogaster* (Clark et al., 2006) and mouse models (Gautier et al., 2008). Models of human neurons with deficient expression of *PINK1* showed high levels of ROS in mitochondria and cytosol and reduced ATP production (Gandhi et al., 2009). *Parkin* (*PARK2*) encodes for a E3-ubiquitine ligase, which is localized in cytosol but which translocates in mitochondria under stress conditions. Mitochondria with deficit of *Parkin* showed reduced activity of complexes I and IV of the respiratory chain, higher levels of ROS, and more vulnerability to complex I inhibitors (Whitworth et al., 2005). *Parkin*-knockout *Drosophila* model exhibited a similar phenotype to PINK1-mutated flies (Clark et al., 2006), and overexpression of *Parkin* rescues *PINK1*−/− phenotype but not vice versa, suggesting that *PINK1* acts upstream of *Parkin* in a common, linear pathway (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Fibroblasts from patients affected by *Parkin*-associated PD (Mortiboys et al., 2008) as well as fibroblasts from patients affected by *PINK1*-associated PD (Abramov et al., 2010; Rakovic et al., 2010) exhibited mitochondrial respiratory chain dysfunction and reduced ATP production. *DJ1* is a highly conserved protein encoded by the *PARK7* gene, which likely

works as antioxidant. It owns cysteine residues that can be oxidized, and mitochondria isolated from *DJ1*-knockout mice show high ROS levels (Andres-Mateos et al., 2007). Several other functions have been proposed for *DJ1*, however, all linked to oxidative stress management. Indeed, the protein binds, in an oxidation-dependent manner, multiple RNA targets in cells and brain, including mitochondrial genes, genes involved in glutathione metabolism, and members of the phosphatase and tensin homolog (PTEN)/phosphatidylinositol-3-kinase (PI3K) cascade (Van Der Brug et al., 2008). Further, it stabilizes transcription factor Nrf2, which regulates antioxidant response genes (Clements et al., 2006). *DJ1* could also protect cells from stress linked to heavy metals such as copper and mercury acting as a metal binding protein (Björkblom et al., 2013).

The autosomal recessive forms of PD associated to *PINK1*, *Parkin*, and *DJ1* mutations provided evidence of a primary role of mitochondria at least in the pathogenesis of these genetic diseases. However, the role of mitochondria and oxidative stress in sporadic PD remains to be elucidated because the multiple lines of evidence do not allow to discriminate whether impaired mitochondria bioenergetics and ROS-induced biological molecules damages represent a primary pathogenic process or an epiphenomenon of neurodegeneration. A substantial limit to the resolution of this controversy is the fact that it is possible to observe the phenomenon only many years after the occurrence of the primary events. Besides, the absence of Lewy bodies described in the first neuropathological studies performed on *Parkin*-related PD patients threw some doubts as to whether this hereditary form of PD could have the same disease mechanisms as sporadic PD. Nevertheless, nowadays, several studies described the presence of Lewy bodies in *Parkin* brains (Farrer et al., 2001; Pramstaller et al., 2005; Doherty and Hardy, 2013; Miyakawa et al., 2013; Cornejo-Olivas et al., 2015) as well as in two out of three *PINK1* cases (Samaranch et al., 2010; Steele et al., 2015; Takanashi et al., 2016). It has been suggested that this neuropathological heterogeneity could be related to some specific *PINK1* or *PARKIN* mutations and/or to the age of death (Truban et al., 2017).

MULTIPLE MITOCHONDRIA FUNCTIONS: EVIDENCES FROM PINK1/PARKIN AND OTHER PD-RELATED GENES

It is noteworthy that a more extensive insight into the autosomal recessive models of PD, particularly *PINK1*- and *Parkin*-associated PD, revealed a wider role of mitochondria in PD pathogenesis, beyond the view of a solely ATP- and ROS-producing organelle (**Figure 1**). The autosomal recessive models uncovered the importance of multiple mechanisms that maintain healthy mitochondrial pool and then neurons. These mitochondrial pathways are represented by mitochondrial quality control mechanisms, especially mitophagy, mitochondrial trafficking, and mitochondrial calcium homeostasis maintenance. In the next paragraphs, we will try to explain the relationship between PD and these more recently discovered mitochondrial pathways.

Mitochondrial Quality Control Mechanisms

Studying *PINK1*- and *Parkin*-associated PD, the most frequent autosomal recessive forms of the disease, has been discovered that these two genes are the main responsible of mitochondrial quality control (QC) maintenance, working at various levels. Indeed, they participate both at a molecular level for mild mitochondrial dysfunction and, in a stepwise manner, an organellar level for more severe mitochondrial dysfunction. The former involves molecular chaperones and proteases to correct protein import and folding and to regulate turnover; the latter aims to sequester, sort, and eliminate partially or completely damaged mitochondria. Organellar level involves fusion, fission, and mitophagy processes as well as mitochondrial-derived vesicles (MDVs) pathway.

Mitochondria include over a thousand proteins, the majority of which are encoded by nuclear genome while only approximately 1% of these proteins are encoded by mitochondrial genes. Nuclear-encoded mitochondrial proteins are synthesized in the cytosol and subsequently imported into the organelle by using two complexes localized, respectively, on the outer membrane [translocase of outer membrane (TOM)] and inner membrane [translocase of inner membrane (TIM)] of mitochondria. Inside the organelle, polypeptides fold and assemble into their native functional form these proteins with the involvement particularly of mtHsp60 and mortaline, a member of the Hsp70 family encoded by *HSPA9*. The accuracy of these processes is monitored by chaperones and proteases pertaining to the mitochondrial unfolded-protein response (UPRmt), which is activated by *PINK1*. When proteins are damaged or misfolded, *PINK1* interacts with the serine protease high-temperature-regulated A2 (HtrA2; Plun-Favreau et al., 2007) and the mitochondrial molecular chaperones heat shock protein 75 (Hsp75) also known as tumor necrosis factor (TNF) receptor-associated protein 1 (TRAP1; Pridgeon et al., 2007) promoting their activation by phosphorylation. The relationship between *PINK1* and UPRmt proteases is supported by the finding of a reduction in *HtrA2* phosphorylation in brains of PD patients carrying mutations in *PINK1* (Plun-Favreau et al., 2007). Moreover, brains of *Htra2*-knockout mice exhibit aggregates of misfolded proteins and mitochondrial dysfunction (Moisoi et al., 2009), and these mice develops a phenotype consistent with progressive movement disorder (Jones et al., 2003).

The pathogenic role of this mitochondrial pathway's dysregulation in PD has further been supported by the finding of mutations in genes involved in UPRmt in PD populations. Thus, in HtrA2 gene, by using a candidate gene approach, a new pathogenic mutation, G399S, has been found in a German cohort of PD patients (Strauss et al., 2005). The same mutation has subsequently been found in a large Turkish family where it cosegregated with PD and essential tremor in a dominant manner (Unal Gulsuner et al., 2014; Chao et al., 2015). Further in the German PD cohort, a polymorphism of HtrA2 gene

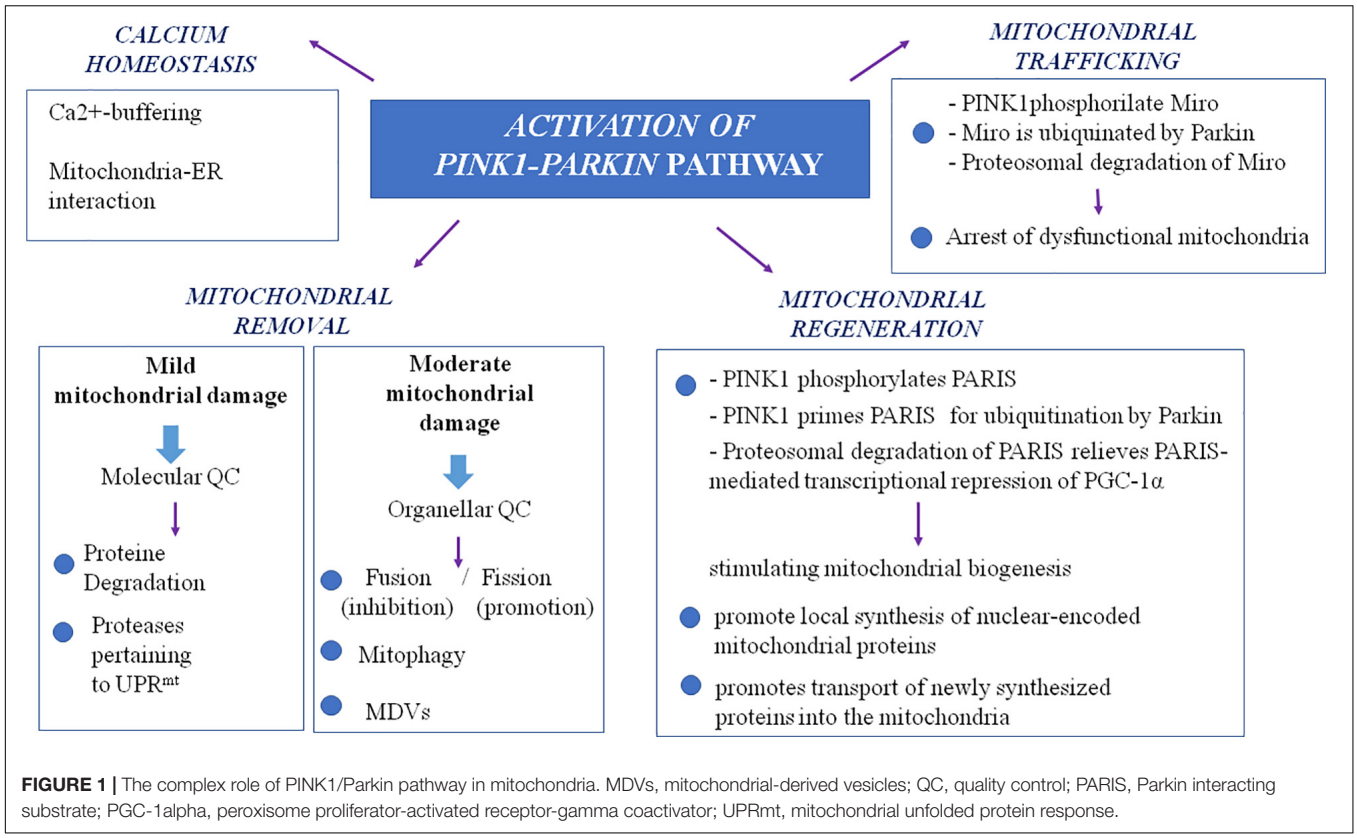

FIGURE 1 | The complex role of PINK1/Parkin pathway in mitochondria. MDVs, mitochondrial-derived vesicles; QC, quality control; PARIS, Parkin interacting substrate; PGC-1alpha, peroxisome proliferator-activated receptor-gamma coactivator; UPRmt, mitochondrial unfolded protein response.

(A141S) increasing the risk for PD was found. Both G399S and A141S mutations resulted in a deficit of HtrA2 protease activity, and immunohistochemistry and functional analysis in transfected cells showed that both mutation (with the risk allele A141S in a lesser extension) induced changes in mitochondrial morphology and mitochondrial dysfunction (Strauss et al., 2005). Recently, the first mutation in the TRAP1 gene was reported in a patient with late onset PD. The mutation caused complete loss of function of the protein, and functional analysis of patient's fibroblasts showed increased ROS production associated with increase in mitochondrial biogenesis, damage of UPRmt, reduction in mitochondrial membrane potential (MMP), and sensitivity to mitochondrial apoptosis (Fitzgerald et al., 2017). Furthermore, a missense mutation (c.3614G > A–p.Arg1205His) in eukaryotic translation initiation factor 4 gamma 1 (EIF4G1; *PARK18*) was reported to be pathogenic in a multi-incident northern French family with autosomal dominant, levodopa-responsive, late-onset parkinsonism (Chartier-Harlin et al., 2011). Although doubts on the relevance of *EIF4G1* in the pathogenesis of PD have been risen by the finding of this mutation also in normal controls (Huttenlocher et al., 2015), *EIF4G1* encodes for a transcription factor of mitochondrial proteins that is involved in the UPRmt.

As already highlighted, in the stepwise manner functioning of mitochondrial QC, when deregulation becomes more severe and overwhelms molecular QC, organellar QC processes are involved, and *PINK1/Parkin* axis has a pivotal role also in their promotion and regulation. In healthy mitochondria, PINK1 is imported within mitochondria by TOM and TIM complexes and is sequentially cleaved first at the mitochondrial targeting sequence (MTS) by mitochondrial proteases in the matrix and then at the transmembrane domain (TM) by presenilin-associated rhomboid-like (*PARL*) protease in the internal mitochondrial membrane (IMM; Jin et al., 2010; Deas et al., 2011; Greene et al., 2012). Cleaved *PINK1* is re-exported and degraded by ubiquitin–proteasome system (UPS). In damaged mitochondria, reduced transmembrane potential does not allow PINK1 to enter the organelle; thus, it accumulates on the outer mitochondrial membrane (OMM) in its not cleaved form (Narendra et al., 2010), and it forms a large multimeric complex with TOM (Plun-Favreau et al., 2007; Pridgeon et al., 2007; Narendra et al., 2010; Becker et al., 2012; Lazarou et al., 2012; Costa et al., 2013; Yamano and Youle, 2013; Zhang et al., 2013; Morais et al., 2014). In this way, stabilized *PINK1* was demonstrated to phosphorylate *Parkin* on its serine-65 (S65) residue of ubiquitin-like domain, and in turn, *Parkin* is activated in its enzymatic function, thus translocating to damaged mitochondria. In its activated form, *Parkin* assembles onto OMM ubiquitin protein's chains, which could be phosphorylated also by *PINK1* itself (Fiesel et al., 2015; Truban et al., 2017). As the results of these events, damaged mitochondria are coated with ubiquitin chains and then embedded in autophagosomes. Here, they are degraded by the autophagy pathway (Okatsu et al., 2010). Therefore, mitophagy is a specialized form of autophagy that manages the turnover of irreversibly damaged and dysfunctional mitochondria and that is mainly regulated by *PINK1/Parkin* pathway. Probably, also F-box

only protein 7 (*FBXO7*), whose gene mutations are responsible for a rare autosomal recessive form of early onset atypical PD (*PARK 15*), is involved in the regulation of mitophagy. Actually, this protein, which is a component of a multimeric E3 ubiquitin ligase complex, has been demonstrated to interact with both *PINK1* and *Parkin*, and cells expressing a deficit of *FBXO7* showed reduced translocation of *Parkin* into mitochondria and deficit in mitophagy (Burchell et al., 2013).

Nevertheless, to avoid global dysfunction and maintain mitochondrial integrity, mitochondria can resort to fusion and fission (mitochondrial dynamics), which are regulated again by the *PINK1/Parkin* pathway. These two mechanisms, which are hypothesized to be paired, ensure a healthy population of mitochondria to cells replacing damaged component (mtDNA, proteins and respiratory chain components) of the organelle during the turnover. Mitochondrial fusion, by promoting exchanges with neighboring mitochondria, supplies dysfunctional mitochondria with restored proteins and not-damaged mtDNA, avoiding mitophagy (Twig and Shirihai, 2011). Main proteins participating fusion include three GTPases: mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) involved in OMM fusion and optic dominant atrophy (OPA1) involved in IMM fusion (Chan, 2006). Mitochondrial fission allows to segregate dysfunctional parts of the mitochondria by generating two mitochondria daughters, and the impaired mitochondria daughter then undergoes mitophagy (Celardo et al., 2014). Particularly, during fission, mitochondria endure a drop of membrane potential, which, beyond a certain level, leads the impaired mitochondria daughter to mitophagy (Twig and Shirihai, 2011). Fission mechanism mainly involves the dynamin-related GTPase protein 1 (Drp1), the mammalian Drp1 homolog dynamin-like protein 1 (DLP1), and the mitochondrial fission 1 protein (Fis1) (Eisner et al., 2018). Taking into account that fusion prevents mitophagy whereas fission promotes it, a correct balance between these two mechanisms is required for an efficient mitophagy and, subsequently, the maintenance of a healthy mitochondria pool. *PINK1/Parkin* pathway is involved in the regulation of mitochondrial dynamics, inhibiting mitochondrial fusion. Indeed, both Mfn1 and Mfn2 are substrate of *PINK1* and *Parkin*; thus, when they are in active state on the OMM, they are ubiquitinated, phosphorylated, and degraded. This event prevents refusion of damaged mitochondria with the healthy network (Chen and Dorn, 2013).

More recently, the *PINK1/Parkin* pathway has been found to be involved also in an alternative organellar QC mechanism for degradation, which prevents global mitophagy: the formation of the so-called MDVs (Truban et al., 2017), a mechanism in which the fission protein Drp1 is not involved (Neuspiel et al., 2008). It has been suggested that a local increase in oxidized proteins and lipids or higher ROS levels that induce a localized reduction of MMP could lead to local *PINK1/Parkin* pathway activation by preventing inactivation of *PINK-1*, which is not imported within mitochondria (Sugiura et al., 2014). This local activation of the *PINK1/Parkin* pathway could lead to the formation of vesicles from mitochondria containing oxidized proteins. These vesicles are directly degraded within the lysosome without the involvement of autophagic machine (Soubannier et al., 2012). Aside from MDVs directed to lysosomes, MDVs directed to peroxisomes have also been identified. Nowadays, only one protein is known to travel by using this type of MDVs, a mitochondrial-anchored protein ligase (MAPL or Mul1) (Braschi et al., 2010). It may likely be important in the regulation of balance between mitochondrial fusion and fission events taking into account that MAPL is responsible for the stabilization of Drp1 and for the degradation of Mfn2, conjointly thus increasing mitochondrial fission (Braschi et al., 2009). Braschi et al. (2010) also showed that binding of MAPL and recruitment of peroxisome MDVs need the involvement of vacuolar protein sorting 35 (VPS35) and vacuolar protein sorting 36 (VPS36), two components of retromer complex that mediates retrograde transport from endosomes to the *trans*-Golgi network. Interestingly, several mutations of the *VPS35* gene were described in PD families with an autosomal dominant inheritance of the disease (*PARK17*; Vilariño-Güell et al., 2011; Zimprich et al., 2011). It is supposed that they cause an imbalance in mitochondrial dynamics by an impairment in fusion with a consequent fragmentation of mitochondria (Tang et al., 2015; Wang et al., 2016). Indeed, functional analysis of *VPS35*-depleted-dopaminergic neurons and neurons expressing D620N mutation, the most frequent in *VPS35*, showed that in these cells, MAPL is not bound anymore, and thus, it has not been degraded. Consequently, increased stabilization of Drp1 and degradation of Mfn2 happen, and fission increases causing fragmentation of mitochondria. Cells expressing D620N mutation in *VPS35* exhibited increased levels of MAPL and decreased Mfn2 levels (Tang et al., 2015). These results suggest the possible role of the dysregulation of fission in pathogenesis of familial and maybe sporadic PD. However, mutations in *VPS35* are rare and explain only 0.2% of sporadic PD cases (Hernandez et al., 2016). The important role of mitochondrial QC in PD pathogenesis is supported also by the finding of mutations in the vacuolar protein sorting 13C (*VPS13C*) gene, which causes an autosomal recessive early onset parkinsonism with rapid progression and early cognitive decline (*PARK 23*; Lesage et al., 2016). Indeed, the *VPS13C* gene encodes for a protein that is partly localized to the outer membrane of mitochondria and whose deficit was associated in cell models with lower mitochondrial membrane potential, mitochondrial fragmentation, exacerbated *PINK1/Parkin*-dependent mitophagy, and transcriptional upregulation of PARK2 in response to mitochondrial damage (Lesage et al., 2016).

The failure of both molecular and organellar levels of mitochondria QC mechanisms leads to irreversible damage of mitochondria and consequent release of its components and proapoptotic proteins (including cytochrome c) into the cytosol. These events, by the apoptotic pathway, lead to cell death (Youle and Strasser, 2008), which could also be strengthened by the activation of neuroinflammatory mechanisms (Hirsch and Hunot, 2009). Indeed, the release into the cytosol of mtDNA together with other compartmentalized mitochondrial molecules could activate a Toll-like receptor (TLR) 9-mediated inflammatory response (Oka et al., 2012), which induces the production of interleukin (IL)-1β and other proinflammatory cytokines (Miura et al., 2010). These events could represent the

way by which *PINK1* and *Parkin* mutations provoke loss of dopaminergic neurons and PD.

Mitochondrial Trafficking

Another important mitochondrial function is their trafficking within the cells, according to local energy demand. It is particularly important in neurons where synapses, which are highly-demanding energy structures, are often located at long distances from cell bodies. Thus, to provide ATP along the entire neuron, mitochondria travel from soma to axons (anterograde axonal transport) through the association with the kinesin family motor proteins KIF1Bα and KIF5, while the transport back to the cell soma (retrograde transport) is mediated by cytosolic dynein (Hollenbeck and Saxton, 2005; Hirokawa et al., 2010). Following studies revealed the presence on the mitochondrial surface of a motor/adaptor complex involved in mitochondrial transport. This complex is formed by the proteins Miro, the heavy chain of kinesin-1 (KHC), Milton, and dynein. Miro is a mitochondrial Rho-like GTPase, which is attached to the OMM and directly interacts with Milton, which in turn recruits the KHC to mitochondria. Thus, the Miro–Milton complex links KHC to mitochondria for anterograde transport of the organelle. Dynein is a cytoplasmic protein mainly involved in retrograde transport by the interaction with kinesin-1 (Stowers et al., 2002; Fransson et al., 2003, 2006; Guo et al., 2005; Hollenbeck and Saxton, 2005; Glater et al., 2006).

It is supposed that impairment in mitochondria trafficking could have a role in the pathogenesis of PD. Indeed, *PINK1* and *Parkin* have been found to be associated with the Miro/Milton complex, suggesting their involvement in mitochondrial trafficking (Weihofen et al., 2009; Wang et al., 2011). *PINK1* is able to phosphorylate Miro on multiple sites, and this event promotes ubiquitination by *Parkin*. This results in proteosomal degradation of Miro and lacking formation on mitochondria surface of the complex, which is essential for the movement of the organelle along the axons. This interaction between *PINK1*/*Parkin* and Miro is important because it arrests dysfunctional mitochondria, preventing corruption of healthy mitochondria via fusion and the spreading of ROS within neurons. Thus, it is conceivable that *PINK1* and *Parkin* mutations, producing a loss of function of these proteins, could promote dopaminergic cells' death and PD also by an impairment of mitochondrial trafficking. In line with this observation, *Drosophila* model of PD expressing a deficit of *PINK1* exhibited mitochondrial transport defects (Liu et al., 2012). To strengthen the possible role of altered mitochondria trafficking in PD pathogenesis, the protein leucine-rich repeat kinase 2 (*LRRK2*), which is involved in the most frequent autosomal dominant form of PD, has been demonstrated to interact with Miro1 in human-induced pluripotent stem cell (iPSC)-derived neurons. It forms a complex with Miro 1 and removes it from damaged mitochondria, stopping them and preventing their motion along the axons. In this model, the most common pathogenic mutation of *LRRK2* gene, G2019S, suppresses this function of arresting mitochondria, thus slowing the initiation of mitophagy (Hsieh et al., 2016).

Taking into account these observations, *RhoT1*, the gene that encodes for Miro1, has been proposed as a candidate gene for PD. However, to date, it has not found any significant association between polymorphisms of this gene and PD (Anvret, 2012).

Mitochondrial Calcium Homeostasis

Mitochondria are also involved in calcium homeostasis, which is critical for appropriate neuronal functioning considering that calcium signal regulates several processes including not only synaptic release of neurotransmitters and vesicle recycling but also neuronal plasticity and axonal transport. Mitochondria represent an important buffer for Ca^{2+} as demonstrated by the rapid increase in cytosolic Ca^{2+} concentration after stimulation of these organelles with specific agonists. Thus, mitochondria allow the accumulation of high levels of Ca^{2+} in specific subcellular domain as observed in neurons where, for example, they localize in the synaptic terminal near Ca^{2+} channels to accumulate this ion and prevent its spreading (Rizzuto et al., 2012). Mitochondria also present tight contacts with endoplasmic reticulum (ER), which is the main calcium stock in cells. This Ca^{2+}-buffering function of mitochondria is particularly important in dopaminergic neurons of the substantia nigra, which have an activity of autonomous pacemaker in order to allow the sustained release of dopamine. As L-type calcium channels are essential for this mechanism, these neurons are exposed to high fluxes of Ca^{2+} (Surmeier et al., 2011). Dopaminergic neurons need higher levels of ATP to maintain adequate cytosolic calcium concentration. Deregulation of calcium homeostasis with mitochondrial calcium overload could lead to dramatic consequences for cells, such as apoptosis and death. A link between altered calcium homeostasis and PD has been suggested by the finding of mitochondrial calcium overload in Rotenone models of PD (Yadava and Nicholls, 2007) as well as in *PINK1*-deficient neurons where excess of mitochondrial calcium leads to apoptotic cell death (Gandhi et al., 2009). Furthermore, some authors have demonstrated that α-*syn*, *Parkin*, and *DJ1* are important for the ER–mitochondria interaction aimed to the transfer of calcium (Calì et al., 2012a,b). Therefore, mutations in the genes encoding for these proteins, involved in inherited forms of PD, could promote pathogenesis of the disease also through abnormal mitochondrial calcium accumulation.

Mitochondrial Biogenesis

Another aspect of mitochondrial functions, which could be involved in PD pathogenesis, is mitochondrial biogenesis, as demonstrated by *PINK1*/*Parkin* pathway role also in this field. In the dopaminergic neurons of substantia nigra, a Krüppel-associated box domain zinc finger protein (KRAB-ZFP) has been identified and denominated Parkin-interacting substrate (PARIS). Its name is explained by the fact that the expression of PARIS is regulated by Parkin via the UPS. Indeed, *in vitro Parkin* is able to ubiquitylate the transcriptional repressor PARIS, inducing its proteasomal degradation (Shin et al., 2011). A known function of PARIS is to repress the expression of the peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1α (PGC-1α) (Shin et al., 2011), which is an

important inducible coactivator of nuclear receptor. PGC-1α stimulates mitochondrial biogenesis through the induction of expression of nuclear respiratory factors and mitochondrial transcription factor A, a regulator of mtDNA replication and/or transcription (Wu et al., 1999). Therefore, loss of function mutations in *Parkin* gene could promote cell death by impairing mitochondrial biogenesis (Stevens et al., 2015). Indeed loss of Parkin could induce overexpression of PARIS with consequent excessive repression of PGC-1α.

The many evidence explained thus far describe an involvement of mitochondria in PD pathogenesis at multiple levels (for summary, see **Table 1** and **Figure 2**). Several mitochondria insults, such as toxins, aging, and gene mutations, could promote mitochondrial dysfunction by increasing oxidative stress and impairing energy production, dysregulating calcium homeostasis and mitochondrial trafficking, and inducing apoptosis as the result of abnormal QC mechanisms and mitochondrial biogenesis. Nonetheless, recently, within the neurodegenerative process, high consequence has been ascribed to the interaction between mitochondria and amyloidogenic proteins, i.e., α-syn in PD pathogenesis.

RELATIONSHIP BETWEEN MITOCHONDRIAL DYSFUNCTION, α-SYN AGGREGATES, AND LYSOSOMAL SYSTEM IN PD

Considering the role of mitochondria in the pathogenesis of PD, it is necessary to evaluate the relationship between this organelle and α-syn, which represents the pathological hallmark of PD. Indeed, this protein represents the principal constituent of Lewy bodies, and the point mutation A53T in its gene (*SNCA*) was the first to be identified in 1997 in hereditary PD (Polymeropoulos et al., 1997). Alpha-syn is a 140-amino-acid long protein that is mainly located in the presynaptic terminal of neurons where it is involved in synaptic vesicle turnover and endocytosis. Particularly, it is involved into the synthesis, storage, and release of dopamine within neurons of substantia nigra (Yavich et al., 2004).

Mitochondrial dysfunction and α-syn aggregates are linked by a bidirectional relationship, where the former can promote the production of pathological α-syn oligomers, and the latter can cause mitochondrial impairment. However, it is hard to distinguish the primary event in PD (**Figure 3**). On the one side, several studies demonstrated that excess of ROS production, for instance because of mitochondrial insults, promotes intracellular α-syn misfolding and aggregation (Perfeito et al., 2014; Ganguly et al., 2017). On the other side, wild-type α-syn in high concentration or mutated α-syn tends to acquire a cross-beta amyloid conformation and self-aggregate thus forming oligomers. Larger oligomers are highly toxic because of their greater stability (Parnetti et al., 2014; Pieri et al., 2016) and can impair multiple cellular functions including mitochondrial energy production. Experimental evidence suggest an interaction of α-syn with complex I of respiratory chain. Indeed, in cell models overexpressing α-syn, oxidative stress and mitochondria changes were associated to depolarization of mitochondrial membrane and decreased activity of complex I of mitochondrial respiratory chain (Hsu et al., 2000). Thus, an inhibitory effect on complex I has be suggested for α-syn that could act in a dose-dependent manner as reported in a study on rat brains (Liu G. et al., 2009). Besides α-*syn*, knockout mice are resistant to the effects of MPTP, suggesting that α-syn is required downstream of complex I (Dauer et al., 2002).

More recently, a new understanding of the role of mitochondrial dysfunction in the pathogenesis of neurodegenerative disease has been proposed. Indeed, these organelles could directly be involved in importing and degrading amyloidogenic proteins such as α-syn (Lautenschäger and Schierle, 2019). Many evidence showed that α-syn can tie OMM. Particularly, an interaction with Tom 20 and Tom 40, proteins pertaining to TOM complex, has been demonstrated (Di Maio et al., 2016; Martínez et al., 2018; Ryan et al., 2018). Alpha-syn has also been demonstrated to interact with ATP synthase, thus suggesting a localization of this protein at the IMM (Ludtmann et al., 2016). In line with these evidence, α-syn includes in its N-terminus domain an amino acidic sequence similar to those with mitochondrial targeting properties (Miraglia et al., 2018). Changes into the N-terminus of α-syn influence the affinity for lipids, thus modifying the ability of the protein to bind mitochondrial membranes and conditioning the subcellular localization of α-syn (Lin et al., 2019). Particularly, α-syn derived from point mutation A53T of *SNCA* gene exhibit high affinity for mitochondrial membrane, and it is supposed that α-syn could influence mitochondrial size and shape. Actually, the analysis of human-derived neurons expressing A53T-α-syn showed a higher fraction of this protein localized into mitochondria as well as mitochondria fragmentation and defect in mitochondrial transport (Pozo Devoto et al., 2017). A role of mitochondria in degrading α-syn aggregates is supported also by the demonstration of an interaction between the mitochondrial protease HtrA2 and α-syn (Liu M.L. et al., 2009). Besides, in yeast, protein aggregates formed under heat shock conditions are imported into mitochondria for degradation (Ruan et al., 2017). Some authors proposed that α-syn enters mitochondria to perform a specific and physiological activity. Actually, a study performed on mice brains showed that monomeric α-syn modulates ATP synthase function by interacting with its α subunit (Ludtmann et al., 2016). Alpha-syn aggregates could also obstruct the mitochondrial import protein system preventing the import of other proteins important for proper functioning of mitochondria. This fact could interfere with mitophagy preventing proper activation of PINK1/Parkin pathway. Thus, proteins involved in genetic forms of PD could also participate the pathogenesis of the sporadic form of disease (Di Maio et al., 2016). However, further investigations are required. Taking into account these observations, Lautenschäger and Schierle (2019) speculated that mitochondrial insults could impair the ability of mitochondria to degrade amyloidogenic proteins. The deficit in α-syn degradation could lead to an increase in the cytosolic levels of protein that tend to aggregate. An impairment of import and degradation of physiologically relevant mitochondrial proteins

TABLE 1 | Multiple mechanisms underlying mitochondrial dysfunction in the pathogenesis of PD.

Mitochondrial dysfunction	Molecular mechanisms involved	Effects	PD genes potentially involved
Impairment in bioenergetics	Mitochondrial respiratory chain (especially inhibition of *complex I*)	• Reduced ATP production; • Oxidative stress	PARKIN, PINK1, DJ1
Impairment in quality control mechanisms	• UPRmt (e.g., *mtHsp60, mortaline, HtrA2, TRAP1*); • Mitochondrial dynamics: fusion (*proteins Mfn1 andMfn2, OPA1*) and fission (proteins *Drp1, DLP1, Fis1*); • Mitophagy (*mitochondrial proteases in matrix, PARL, Ubiquitin*); • MDVs (*directed tolysosomes and peroxisomes, MAPL or Mul1 protein*)	• Altered proteostasis with accumulation of damaged proteins; • Fragmentation of mitochondria; • Altered turnover, accumulation of dysfunctional mitochondria	PARKIN, PINK1, FBXO7, VPS35, VPS13C, *EIF4G1*
Impairment in Mitochondrial trafficking	Altered axonal transport (involvement of complex *Miro/KHC/Milton/dynei n*)	Unmeet local demands for energy, calcium, redox balance, and other mitochondrial functions	PINK1, PARKIN, LRRK2
Impairment in calcium homeostasis	Mitochondrial calcium overload	• Mitochondrial permeability transition pore opening (e.g., with cytochrome c release); • Oxidative stress	PINK1, α-syn, Parkin, DJ1
Impairment in mitochondrial biogenesis	Reduced synthesis of new mtDNA, protein, and membrane (involvement of *PARIS, PGC1α*)	Imbalance of mitochondrial health, apoptosis	PINK1, Parkin

α-syn, alpha-synuclein; ATP, adenosine triphosphate; Drpl, dynamin-related GTPase protein 1; DLP1, dynamin like protein 1; Fisl, mitochondrial fission 1 protein; HtrA2, serine protease high-temperature-regulated A2; KHC, kinesin heavy chain; MAPL or Mull, mitochondrial-anchored protein ligase; MDVs, mitochondria-derived vesicles; Mfnl, mitofusinl; Mfn2, mitofusin2; mtDNA, mitochondrial DNA; OPA, optic atrophy protein; PARIS, PARkin interacting substrate; PD, Parkinson's disease; PGCla, peroxisome proliferator-activated receptor gamma coactivator-la; TRAP1, TNF receptor-associated protein 1.

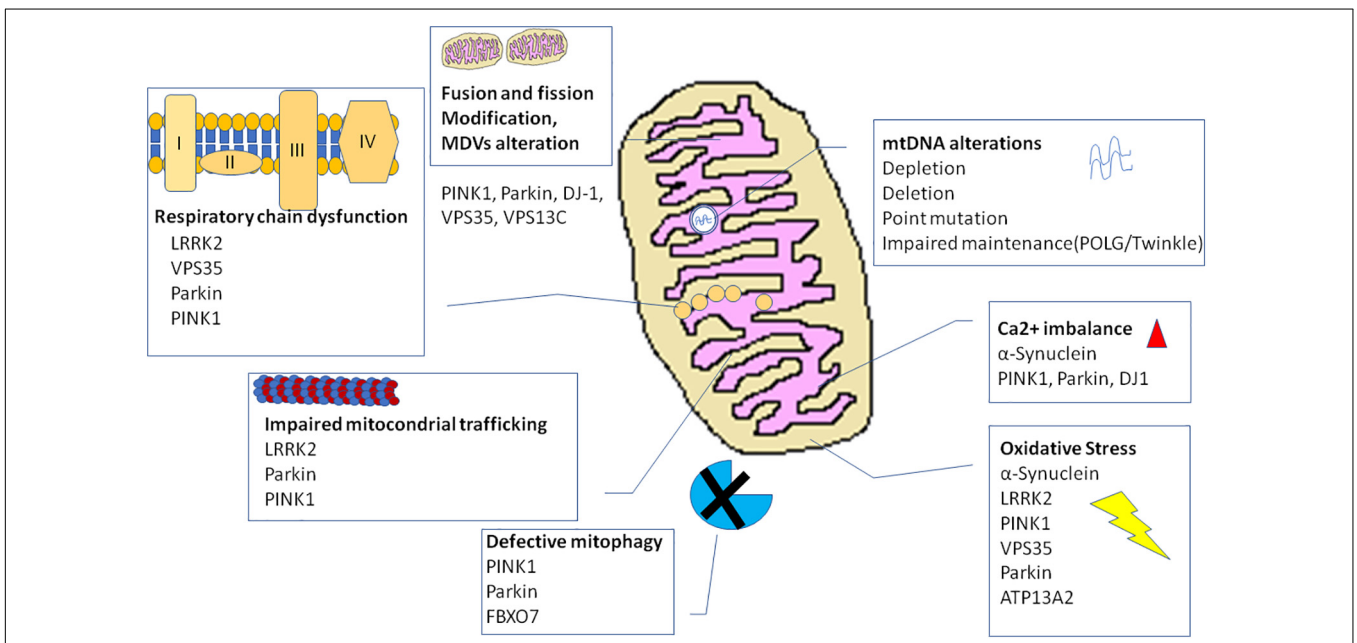

FIGURE 2 | Mechanisms and genes involved in mitochondrial dysfunction in Parkinson's disease. ATP13A2, ATPase cation transporting 13A2; DJ-1, protein deglycase; LRRK2, leucine-rich repeat kinase 2; *PINK1*, phosphatase and tensin homolog (PTEN)-induced putative kinase 1; VPS35, vacuolar protein sorting 35.

could also occur, thus intensifying mitochondrial dysfunction (Lautenschäger and Schierle, 2019).

Alpha-syn has also been demonstrated to interact within the interconnection between ER and mitochondria, which are essential to neuron survival (Lin et al., 2019). In stress conditions, ER transfers high levels of Ca^{2+} to the mitochondria, increasing the production of ROS (Melo et al., 2018).

It has been proposed that dopaminergic neurons are particularly vulnerable to the formation of highly toxic α-syn oligomers considering their high density of synapses and above

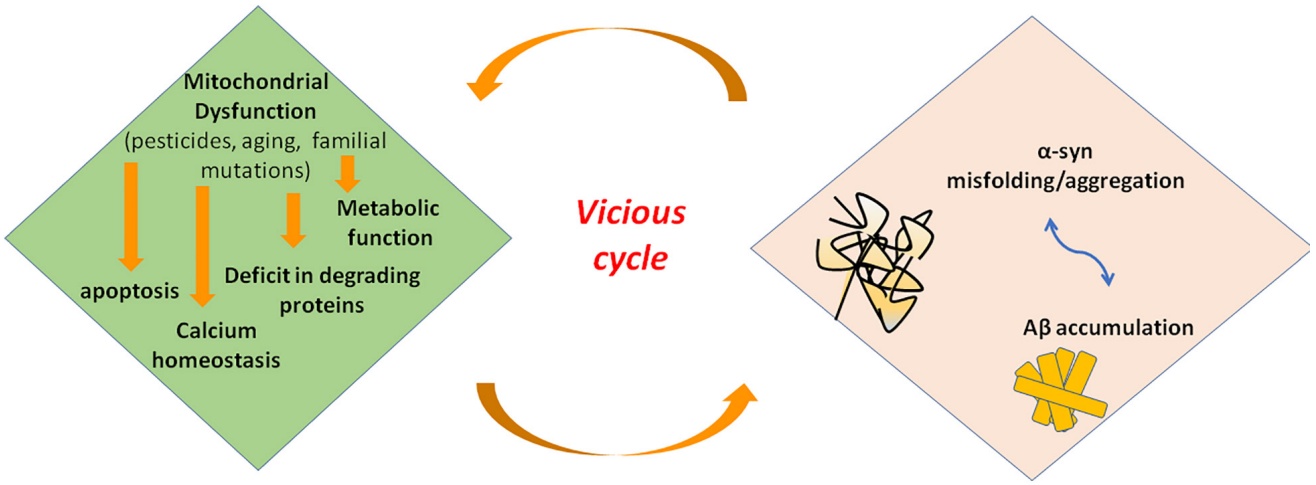

FIGURE 3 | Interaction of misfolded proteins and mitochondria in neurodegenerative disorders. Mitochondrial dysfunction leads to deficit in metabolic function and calcium homeostasis, apoptosis, and possibly impairment of the ability of mitochondria to degrade and import proteins. Amyloidogenic proteins such α-syn and Aβ accumulate in cytosol and aggregate, preventing also mitochondrial import of other proteins. These events increase mitochondrial dysfunction in a vicious cycle. α-syn, alpha-synuclein; Aβ, amyloid beta; ROS, reactive oxygen species.

all the increased oxidative stress related to dopamine production. The high ROS production, which is also increased by the oxidized dopamine, further promotes the aggregation of α-syn and its consequent accumulation in Lewy bodies in a vicious cycle manner (Mor et al., 2017).

Furthermore, the relationship between α-syn and monoamine oxidase-B (MAO-B), which is an outer mitochondrial membrane protein that oxidizes arylalkylamine neurotransmitters, is thought to be significant in the degeneration of dopaminergic neurons (Kang et al., 2018). The expression of MAO-B increases with aging and in neurodegenerative processes, and it is associated with higher levels of ROS and free radical damage (Mahy et al., 2000). Recently, the metabolite produced by the enzymatic activity of MAO-B on dopamine, 3,4-dihydroxyphenyl acetaldehyde (DOPAL), has been demonstrated to eventually further stimulate enzymatic activity of MAO-B itself by triggering the activity of asparagine endopeptidase (AEP or leguminase). This enzyme cleaves α-syn at its residue N103 producing a fragment (α-syn 1–103) that binds MAO-B and stimulates its enzymatic activity, more efficiently than α-syn wild type. The consequence is an increased activation of MAO-B and an enhanced production of the neurotoxic methabolite DOPAL in a vicious cycle leading to dopaminergic neurodegeneration. Virally mediated expression of α-syn 1–103 induces PD pathological changes but only in mice expressing MAO-B, underlining the importance of this mitochondrial enzyme in PD pathogenesis (Kang et al., 2018).

It is also important to consider that an excess of α-syn as well as an impairment in mitochondrial function could also be promoted by a dysfunction of one or both the main proteolytic pathways in cells, the UPS and the lysosome system, the latter including macroautophagy (or simply autophagy), chaperon-mediated autophagy (CMA), and endocytosis (Rott et al., 2017; Grassi et al., 2018; Limanaqi et al., 2019). All of these autophagy routes converge on the only lysosome that is involved in proteostasis and degradation of several classes of macromolecules. This function is particularly important in long-lived, postmitotic cells such as neurons. An impairment at each level of this network can lead to insufficient clearance of pathogenic proteins, defective membrane trafficking and signaling, and cell damage (Nixon, 2020). Misfolded proteins are targeted for protease degradation, including toxic forms of α-syn. These, as well as non-toxic monomers, are degraded through macroautophagy. However, a dysfunction in these pathways could cause a reduced removal of damaged wild-type proteins such as α-syn (or mutated proteins), which accumulates in degrading organelles. Thus, toxic species damage proteolytic pathways in a vicious cycle manner. According to this hypothesis, mutations in genes involved in lysosomal system have been identified in several forms of PD, such as *ATP13A2* and *GBA*, the gene encoding for glucocerebrosidase 1, a hydrolase that catalyzes the metabolism of glucosylceramide and whose loss of function represents one of the main genetic risk factor for PD (Aharon-Peretz et al., 2004). Further, patients affected by lysosomal storage diseases with mutations in lysosome system genes can exhibit parkinsonism as a clinical feature (Stamelou et al., 2013). Recently, also the gene leucine-rich repeat kinase 2 (LRRK2) has been linked to lysosomal function. Mutations in this gene represent one of the most frequent genetic cause of PD, and they are found in 1–2% of sporadic PD cases (Healy et al., 2008). Despite that, the function of the protein encoded by this gene remains unclear, with evidence for a role in synaptic transmission, endolysosomal trafficking, cellular proliferation, and cytoskeleton dynamics. A recent study (Ysselstein et al., 2019) showed that mutations in LRRK2, which gain the kinase activity of the encoded protein, act as negative regulator of the lysosomal glucocerebrosidase-1 activity. The augmentation of LRRK2 kinase activity could reduce the glucocereborsidase-1

activity by increasing the level of phosphorylated Rab10, a GTPase potentially involved in endolysosomal function and lysosomal homeostasis, which is demonstrated to be an LRRK2 substrate (Eguchi et al., 2018). Interestingly, increased LRRK2 kinase activity was observed in idiopathic PD, suggesting a possible role of this gene in sporadic PD pathogenesis (Di Maio et al., 2018).

Nonetheless, mutated species of α-syn could primarily impair UPS and/or lysosome system. Several authors suggest a trilateral relationship of mitochondrial dysfunction, α-syn aggregation, and degradation of the endolysosomal and proteasome systems in the neurodegenerative process of PD (Pozo Devoto and Falzone, 2017; Grassi et al., 2018; Vicario et al., 2018). Both mitochondria dysfunction and endolysosomal impairment are involved in the development of α-syn pathology and vice versa. An intricate interplay is supposed, in which dysregulation of one system influences the proper activity of the other two. However, which is the starting factor is not known. They could interact with each other simultaneously in promoting PD process, or most likely, in each patient, the interaction between the genetic equipment and environmental factors defines the most involved system and so the initiating factor. This is supported also by the high variability in clinical presentations of PD. Then, a vicious cycle could develop once one system becomes dysfunctional (Lin et al., 2019).

MITOCHONDRIAL MONOGENIC PARKINSONISMS

Since the first discovery of primary mtDNA mutations in 1988, only a few case reports have linked mtDNA mutations with parkinsonism. These include m.11778G > A in MT-ND6, a heteroplasmic 4 bp deletion in MT-CYB21, and m.8344A > G in MT-TK (Giannoccaro et al., 2017). Furthermore, parkinsonism has not been observed in several national cohort studies of common mtDNA mutations (Mancuso et al., 2013, 2014; Nesbitt et al., 2013), suggesting that the association with primary mtDNA mutations is more likely to be an incidental co-occurrence. We have also failed to demonstrate the connection of m.8344A > G mutation in 159 patients with PD (Mancuso et al., 2008). Moreover, neuropathological studies have not identified significant mtDNA mutations in the substantia nigra of patients with idiopathic PD (IPD) compared to controls. Based on these findings, we believe that primary mtDNA point mutations do not play a role in the pathogenesis of PD.

However, while parkinsonism is an extremely rare clinical feature of a mitochondrial disorder caused by a primary mitochondrial DNA mutation, in those mitochondrial diseases determined by mutations in nuclear genes involved in mtDNA replication and maintenance, parkinsonism is quite a common clinical feature, usually associated with mitochondrial myopathy and chronic progressive external ophthalmoplegia (CPEO) (Giannoccaro et al., 2017). In a large, national cohort United Kingdom study, parkinsonism accounted for 43% of all extrapyramidal movement disorders in patients with mitochondrial disease, making the most common extrapyramidal movement disorders identified in this population. The most common genetic defect associated with parkinsonism is POLG mutations (Martikainen et al., 2016).

MITOCHONDRIAL DYSFUNCTION IN ATYPICAL PARKINSONISMS

The role of mitochondrial dysfunction in the pathogenesis of atypical parkinsonism has been recently investigated. The involvement of mitochondria in pathogenetic pathways is demonstrated in both synucleinopathies and tauopathies. Multiple system atrophy (MSA), a synucleinopathy together with PD and dementia with Lewy bodies, is a neurodegenerative disorder in which a variable degree of parkinsonism, cerebellar ataxia, dysautonomia, and pyramidal features coexist. Considering the predominant symptomatology, parkinsonian (MSA-P) or cerebellar (MSA-C) subtype has been described (Fanciulli and Wenning, 2015; Krismer and Wenning, 2017). In neuropathological studies, MSA shows an accumulation of α-syn in neurons and oligodendrocytes (Jellinger, 2018). Although the aberrant protein localization is known for many years, pathogenic mechanisms are almost unclear, and several processes such as α-syn overexpression, cell-to-cell transfer, inflammation, and mitochondrial functioning have been proposed (Jellinger, 2018). A reduced complex I activity in MSA patients' skeletal muscle (Blin et al., 1994), but not in platelets or SN (Gu et al., 1997), has been observed. Additionally, an impairment of enzymatic activities related to respiratory chain complex II in fibroblast primary cultures of MSA patients has also been demonstrated (Monzio Compagnoni et al., 2018a).

Recently, an increasing interest has been drawn to coenzyme Q2, polyprenyltransferase (COQ2). COQ2 is involved in mitochondrial respiratory chain, playing an important role in transferring electrons from complexes I and II to complex III (Monzio Compagnoni et al., 2020). Mutations of COQ2 gene are thought to have a causative role in familial and sporadic cases of MSA (Mitsui et al., 2013). Recessive COQ2 mutations cause primary CoQ10 deficiency, leading to an infantile encephalomyopathy/nephropathy with cerebellar atrophy. Similarly, other primary CoQ10 deficiency syndromes are characterized by cerebellar ataxia as the main clinical feature (Quinzii et al., 2006; Schottlaender and Houlden, 2014). However, it is still controversial whether variants in the gene encoding COQ2 increase the risk of MSA (Jeon et al., 2014; Schottlaender and Houlden, 2014; Ronchi et al., 2016). Although conflicting results are available about the role of COQ2 mutations in MSA, a reduced CoQ10 amount has been observed in the MSA cerebellum (Barca et al., 2016; Schottlaender et al., 2016), cerebrospinal fluid (Compta et al., 2018), plasma (Mitsui and Tsuji, 2016), serum (Kasai et al., 2016), and fibroblasts (Monzio Compagnoni et al., 2018a), independently from COQ2 mutational status. Nevertheless, in MSA brains, a reduction in two enzymes involved in CoQ10 synthesis (decaprenyl diphosphate synthase subunit 1—PDSS1 and coenzyme Q5, methyltransferase—COQ5) has been found (Barca et al., 2016; Monzio Compagnoni et al., 2018b). A recent interesting study

demonstrated an impairment in autophagy and mitochondrial functioning in MSA neurons (Monzio Compagnoni et al., 2018b). In particular, the authors not only identified an impaired activity of respiratory chain complexes (specifically complex II and complexes II + III), but they also observed an increase in mitochondrial mass and the upregulation of several enzymes involved in multiple mitochondrial pathways, including the CoQ10 synthesis, suggesting that it might be related to a mitochondrial attempt to compensate the functional deficit (Monzio Compagnoni et al., 2018b). Although data about the role of mitochondrial dysfunction in the pathogenesis of MSA are encouraging, it is still unclear whether it is a cause or a consequence of misfolded proteins accumulation. Up to now, how mitochondrial dysfunction and the α-syn accumulation in MSA are related is not completely clarified.

Evidence from laboratory and *in vivo* studies suggest a mitochondrial dysfunction also in progressive supranuclear palsy (PSP). A reduction in complex I activity in PSP cybrid lines, which are transmitochondrial cytoplasmic hybrid (cybrid) cell lines expressing mitochondrial genes from patients with PSP, was demonstrated several years ago (Swerdlow et al., 2000). Using the same techniques, increased activity of antioxidant enzymes and oxidative damage to lipids was revealed (Swerdlow et al., 2000; Albers et al., 2001; Chirichigno et al., 2002). Moreover, the presence of oxidative stress in PSP brains is found in postmortem immunochemical studies (Dexter et al., 1992; Sian et al., 1994; Jenner and Olanow, 1996; Odetti et al., 2000; Cantuti-Castelvetri et al., 2002). A reduction in high-energy metabolites in the brains of PSP patients was also shown using combined phosphorus and proton magnetic resonance spectroscopy (Stamelou et al., 2009). In this study, there was a decreased concentration of high-energy phosphates (=ATP + phosphorylated creatine) and inorganic phosphate in the basal ganglia of PSP patients, whereas no significant differences in low-energy phosphates (=ADP + unphosphorylated creatine) were found in comparison to controls. The frontal lobe, but not the occipital lobe, showed similar alterations. A peak of lactate, which is related to increased anaerobic glycolysis, was found in 35% of PSP patients (Stamelou et al., 2009).

An interesting association was found in the French West Indies between atypical parkinsonism and the habitual intake of *Annona muricata*, which is a plant belonging to the Annonaceae family. About one-third of the affected patients developed a PSP-like parkinsonism (Lannuzel et al., 2007). Annonaceous plants produce Annonaceous acetogenins, a family of lipophilic complex I inhibitors (Ries et al., 2011). The major acetogenin contained in *A. muricata* is annonacin. It has been demonstrated to be ∼1,000 times more toxic to cultured mesencephalic neurons than 1-methyl-4- phenylpyridinium (MPP+) (A. Lannuzel et al., 2003). In rats treated with chronic systemic infusion of annonacin, reduced brain ATP levels, along with neuronal cell loss and gliosis in the brainstem and basal ganglia reflecting a PSP-like pattern, were found (Champy et al., 2004). Moreover, a redistribution of tau from the axons to the cell body is induced by annonacin, leading to cell death. Further compounds to which humans are potentially exposed are able to inhibit the complex I of the mitochondrial chain. They were also demonstrated to decrease ATP levels, induce neuronal cell death, and cause the redistribution of tau from axons to the cell body; therefore, their potency in inhibiting the complex I correlated with their potency in inducing tau redistribution (Escobar-Khondiker et al., 2007; Höllerhage et al., 2009). A loss of neurons in the SN and in the striatum, associated with abnormally high levels of tau immunoreactivity in the cytoplasm of neurons, oligodendrocytes, and astrocytes, was demonstrated in rats treated with rotenone (complex I inhibitor) (Höglinger et al., 2005). The toxicity of rotenone is attenuated by CoQ10, which preserves the mitochondrial membrane potential in cultured neurons (Menke et al., 2003; Moon et al., 2005). These preliminary results led to investigating the effect of CoQ10 on PSP. However, although a short-term effect of CoQ10 in 21 clinically probable PSP patients was found in terms of a mild clinical improvement along with significant increase in cerebral energy metabolism (Stamelou et al., 2008), a following 12-month study on 61 PSP patients using high doses of CoQ10 did not significantly improve PSP symptoms or affect disease progression (Apetauerova et al., 2016).

MITOCHONDRIAL DYSFUNCTION IN HUNTINGTON'S DISEASE

Mitochondrial dysfunction has been reported also in Huntington's disease (HD), and mitochondrial defects could be involved in the region-specific pattern of HD degeneration (Damiano et al., 2010). HD is an autosomal-dominant inherited progressive and eventually fatal neurodegenerative disease, the typical manifestations of which are involuntary movements, psychiatric symptoms, and cognitive decline (Walker, 2007). The etiological basis is the deleterious expansion of polyglutamine (PoliQ) encoding CAG repeats in the exon 1 of the huntingtin (*HTT*) gene, leading to the expression of neurotoxic mutant huntingtin (mHTT) (MacDonald et al., 1993). The disease usually starts in midlife, with age of onset inversely correlating to CAG repeat number. The greater the number of CAG repeats, the earlier the age of onset, and the greater the severity of the disorder (Tabrizi et al., 2013). Extensive degeneration of neurons primarily occurs in the striatum and cortex. Striatal medium spiny GABAergic neurons (MSNs) are the most vulnerable in front of a relative sparing of the large striatal neurons, including the striatal interneurons. Moreover, different degrees of degeneration could be also observed within the striatal neuronal population with a more severe involvement of the indirect pathway expressing predominantly D2 receptors (Vonsattel and DiFiglia, 1998). Although the HD mutation has been identified, the molecular processes that determine HD pathogenesis are not yet fully understood. Several lines of evidence indicate that the CAG expansion predominantly leads HTT to gain a toxic function (Cisbani and Cicchetti, 2012). However, HTT is a cytoplasmic protein expressed widely throughout the body but with the highest expression in the brain and testes, which has been shown to interact with a wide variety of transcription factors and to serve as a scaffold to coordinate complexes of other proteins. As a result, its depletion seems to disrupt several processes that are fundamental for the

survival and functioning of the neuron, including endocytosis, vesicle trafficking, RNA biogenesis, endocytosis, mitosis, transcriptional regulation, postsynaptic signaling, apoptotic signaling pathway, and defects in energy metabolism (Ross and Tabrizi, 2011). Additionally, the resultant longer polyQ tracts of HTT are prone to aggregate with ubiquitin-positive proteins, which are a pathological hallmark of HD. Overall, decades of intense research using cell models, animal models, and postmortem HD brains have implicated a critical role of mitochondrial dysfunction in HD progression and pathogenesis (Yan et al., 2020). The point at which mitochondrial involvement begins is unclear, but there is evidence that mitochondrial impairment occurs even in asymptomatic HD carriers (Saft et al., 2005). In HD, dysfunctional mitochondria have been shown to trigger both neuronal apoptosis and necrosis, disrupt glia, and initiate the inflammatory cascade (Lin and Beal, 2006). Other crucial cellular changes involved in HD pathogenesis, including *N*-methyl-D-aspartate receptor (NMDAR) activation, caspase activation, calcium dyshomeostasis, and abnormal axonal trafficking, require a normal mitochondrial function. It has been suggested that mHTT could have direct or indirect effects on mitochondria, compromising energy metabolism and increasing oxidative damage. There is extensive indirect evidence for bioenergetic deficits in HD, such as a body weight loss despite sustained caloric intake, nuclear magnetic resonance spectroscopy showing increased lactate in the cerebral cortex and basal ganglia, and defective cerebral glucose metabolism in PET studies of the brains of HD patients (Reddy and Shirendeb, 2012). Postmortem striatum samples of HD patients showed first a reduced activity of mitochondrial complexes II–IV of the electron-transport chain and aconitase (Gu et al., 1996; Browne et al., 1997), correlating with reduced levels of ATP in the mutant neurons and reduced uptake of substrates by mitochondria. Later, studies in the brain tissue of HD transgenic and knock-in mice confirmed a decreased activity of complexes I–IV (Pandey et al., 2008). In keeping with this, mitochondrial toxins that selectively inhibit succinate dehydrogenase and complex II, such as rotenone and 3-nitropropionic acid, induce a clinical and pathological phenotype that closely resembles HD (Beal et al., 1993). Notably, cellular energy metabolism seems to be impacted extremely early in the cascade of HD pathogenic events (Browne, 2008). Several authors argued that striatal neurons are particularly sensitive to defects of the mitochondrial oxidative phosphorylation due to their high-energy demand (Pickrell et al., 2011). However, mitochondrial alterations in HD result from a combination of disease-promoting pathways. mHTT associates with the outer mitochondrial membrane in different HD models, resulting in mitochondrial membrane potential loss, cytochrome c release, protein import deficit, and increased sensitivity to calcium-induced mitochondria permeabilization (Bossy-Wetzel et al., 2008; Lautenschäger and Schierle, 2019). Their major function is energy metabolism, but they also play an important role in buffering and shaping cytosolic calcium rises and in mediating cell death by apoptosis. Furthermore, mitochondrial dysfunction could result in overproduction of ROS and nitrogen-reactive species (RNS) and/or failure of the antioxidant defense leading to oxidative/nitrative stress, which is associated with HD. Oxidative damage is fluid (Stack et al., 2008). mtDNA is a major target of the oxidative stress associated with mHTT. Accordingly, higher frequencies of mtDNA deletions were found in HD patients than healthy controls (Banoei et al., 2007). Mitochondrial loss and altered mitochondrial morphology and dynamics have been observed in HD brain and worsen with increasing disease severity (Kim et al., 2010). The balance between fission and fusion is important for maintaining normal mitochondrial function. High levels of fission genes (e.g., *Drp1*), low levels of fusion genes (e.g., Mfn1), and high levels of cyclophilin D have been selectively found in striatum and cortex specimens from HD patients (Shirendeb et al., 2011). Specifically, induction of mitogen-activated protein kinase 1 (MAPK1) can upregulate Drp1 activity causing mitochondrial fragmentation (Roe and Qi, 2018). Additionally, mHTT also translocates to the nucleus, where it binds and increases the level and transcriptional activity of p53, which interacts with Drp1 leading to mitochondria fragmentation (Guo et al., 2014). Recently, Zhao et al. (2019) have found in HD that mitochondrial protein ATPase family AAA-domain containing protein 3A (ATAD3A), an interactor of Drp1, exhibits a gain of function that causes mitochondrial fragmentation and impairs mitochondrial biogenesis. A new peptide inhibitor has also been developed to decrease Drp1 interaction with ATAD3A suppressing mitochondrial fragmentation and mtDNA damage, as well as reducing HD neuropathology (Zhao et al., 2019). Finally, in HD neurons, the increase in mitochondrial free radicals activates another fission protein called Fis1, which promotes an increase in mitochondrial fragmentation. Both *PINK* and *parkin* are involved in mitophagy, and previous studies in *Drosophila* models of HD displayed that *PINK1* overexpression can affect the efficiency of the mitophagy process by inhibiting mHTT activity (Khalil et al., 2015). mHTT has been also found to activate autophagy by inhibiting the mechanistic target of rapamycin (mTOR) (Lee et al., 2015). Expression of PGC-1α, which provides neuroprotective effects by activating autophagy and is a coregulator of mitochondrial biogenesis and antioxidant enzymes (as seen in PD), is reduced in HD, contributing to mitochondrial impairment (Johri et al., 2013). Indeed, mice knockout for *PGC-1α* shows a clinical phenotype similar to HD (Lin et al., 2004). As mentioned, mHTT demonstrated to directly interact with various cellular proteins, and the stress-responsive transcription factor (HSF1) has been reported as the major transcriptional regulator factor impaired in HD (Gomez-Pastor et al., 2017). Recently, Intihar et al. (2019) proposed the existence of alterations in a common p53-HSF1-PGC-1α axis in mediating transcriptional dysregulation and mitochondrial dysfunction in HD. Additionally, the association of the valosin-containing protein (VCP), a multifunctional protein implicated in protein degradation, with mHTT at mitochondria, caused perturbation in mitophagy and increased cell death (Guo et al., 2016). Furthermore, disorders of the mitochondrial dynamics lead to failure of mitophagy. A toxic effect of mHTT could be to compromise ubiquitin–proteasome activity (Wanker et al., 2019), but mHTT aggregates impair also transport of mitochondria in axons (Chang et al., 2006). To this end, recent studies identify HTT and adaptor protein huntingtin-associated

protein-1 (HAP-1) as regulators of autophagosome transport in neurons, hypothesizing that an abnormal stabilization of the mHTT–HAP-1 interaction through the expanded polyQ tract may disrupt the movement of autophagosomes to cell bodies and lead to inefficient clearance of mitochondrial fragments in neurons (Wong and Holzbaur, 2014). Thus, the modulation of molecular pathways that include mitochondrial dysfunction, oxidative stress, and process of autophagy might represent very valuable therapeutic targets. Additionally, there is a need for reliable biomarkers to assess disease progression and to evaluate therapeutic interventions, especially in view of the upcoming HTT-lowering strategies, and mitochondrial signatures in HD could be used as potential biomarkers.

CONCLUSION

In vitro and *in vivo* researches as well as studies in genetic models of PD revealed that mitochondrial dysfunction is not restricted to an imbalance in respiratory chain for ATP production with ROS generation. Failure of several mechanisms involved in mitochondrial health such as QC pathways, calcium homeostasis, and mitochondrial trafficking could cause cell death and neurodegeneration in PD and related disorders. Taking into account this idea, an important issue is whether a failure in mitochondrial homeostatic mechanisms is necessary and sufficient or only necessary but not sufficient to cause PD. Many PD genes are linked to mitochondrial dysfunction with a large number of them directly or indirectly involved in PINK1/Parkin pathway (Ryan et al., 2015). Many other PD genes are related to lysosomal system impairment. It is conceivable that the same genes involved in hereditary PD could exist, in the sporadic form of the disease, variants with weaker effects that could increase susceptibility to other external or internal factors thus leading to sporadic PD. Many of these genes likely act through PINK1/Parkin signaling (Truban et al., 2017).

Another question is about the primary event in mitochondrial dysfunction. Experimental models with cell cultures allow to study exclusively each single mitochondrial pathway involved in the homeostasis of the organelle. Nevertheless, it is conceivable that these multiple processes described are strictly interconnected and interact almost simultaneously, making difficult to discriminate the initiating event. Considering the high phenotypic variability of PD, different forms of disease (or the disease in different patients) could present different primary events in mitochondrial dysfunction, each converging anyway on a final way of action, which causes neuronal death. Nonetheless, in our opinion, the most important issue is whether mitochondrial dysfunction could represent the initiating factor in neurodegeneration. The finding of common mitochondrial dysfunctions, such as altered quality control mechanisms, imbalance in calcium homeostasis, impairment in trafficking in PD as well as in atypical parkinsonism and HD, the involvement of PINK1/Parkin both in PD and HD, and the possibility of producing animal models of all these neurodegenerative diseases by using toxins acting on mitochondrial respiratory chain, suggests that these events are probably important but not the trigger of neurodegeneration. We think that these events could feed neurodegeneration generating vicious cycles but could not represent the primary event. Instead, the observation that mitochondria could participate in degrading the proteins whose accumulation in cytosol generates pathological aggregates deserve great attention. The failure of this role and the impairment in import physiological proteins necessary for normal mitochondria functioning represent the strongest link of mitochondria with neurodegeneration. Nonetheless, mitochondrial impairment has a tight relationship with UPS and lysosome system. These factors are clearly demonstrated to be able to induce and/or worsen mitochondrial dysfunction in a vicious cycle.

Finally, the recognition in mitochondria of multiple pathways that could be affected in PD should lead to a different approach in therapeutic options for these diseases. Reflecting on the failure of drugs aiming to improve respiratory chain's efficiency and scavenge ROS such as Co-Q10 and vitamin E (Weber and Ernst, 2006), a complex therapeutic approach considering the multiple processes that could cause mitochondrial impairment could be required.

AUTHOR CONTRIBUTIONS

VN substantially contributed to the conception and design of the manuscript and interpreting the relevant literature and drafted and revised the manuscript. GP and EP substantially contributed to the design of the manuscript and interpreting the relevant literature and drafted and revised the manuscript. MM revised the manuscript critically for important intellectual content. RC contributed to the design of the manuscript and revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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PARK Genes Link Mitochondrial Dysfunction and Alpha-Synuclein Pathology in Sporadic Parkinson's Disease

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Parkinson's disease (PD) is an age-related neurodegenerative disorder affecting millions of people worldwide. The disease is characterized by the progressive loss of dopaminergic neurons and spread of Lewy pathology (α-synuclein aggregates) in the brain but the pathogenesis remains elusive. PD presents substantial clinical and genetic variability. Although its complex etiology and pathogenesis has hampered the breakthrough in targeting disease modification, recent genetic tools advanced our approaches. As such, mitochondrial dysfunction has been identified as a major pathogenic hub for both familial and sporadic PD. In this review, we summarize the effect of mutations in 11 *PARK* genes (*SNCA, PRKN, PINK1, DJ-1, LRRK2, ATP13A2, PLA2G6, FBXO7, VPS35, CHCHD2,* and *VPS13C*) on mitochondrial function as well as their relevance in the formation of Lewy pathology. Overall, these genes play key roles in mitochondrial homeostatic control (biogenesis and mitophagy) and functions (e.g., energy production and oxidative stress), which may crosstalk with the autophagy pathway, induce proinflammatory immune responses, and increase oxidative stress that facilitate the aggregation of α-synuclein. Thus, rectifying mitochondrial dysregulation represents a promising therapeutic approach for neuroprotection in PD.

Keywords: Parkinson's disease, mitochondria, mitophagy, α-synuclein pathology, *PARK* genes

INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disorder with an insidious onset and a substantial preclinical phase (estimated as >20 years). The progressive aggregation of α-synuclein (Lewy pathology) through the brain and the loss of midbrain dopamine neurons are the pathological landmarks of PD (Surmeier et al., 2017; Fares et al., 2021). Although the etiology of PD is multifactorial, mitochondrial dysfunction has been recognized as a main neuropathogenic mechanism that can affect energy provision and biological pathways (e.g., autophagy, proinflammatory, and antioxidant) to potentially facilitate the Lewy pathology and neuronal loss (Poewe et al., 2017; Park et al., 2018; Grunewald et al., 2019; Pang et al., 2019; Fenton et al., 2021; Onishi et al., 2021).

High-throughput next-generation sequencing and genome-wide association studies have revealed PD risk-associated genes, including 23 *PARK* genes (Schulte and Gasser, 2011; Marras et al., 2017; Poewe et al., 2017) and others (e.g., *HLA-DRA*, *EIF4GI, GBA, MAPT, BSTI, TMEM230, APOE,* and *POLG*). Most *PARK* genes have been revealed as low prevalence (Tran et al., 2020), perhaps a reason of limited research on their involvement in sporadic PD. We reviewed 11 *PARK* genes (**Table 1**) relevant to mitochondrial function, aiming to highlight their potential roles in the etiology of sporadic PD.

PARK GENES IN MITOCHONDRIAL FUNCTION AND HOMEOSTATIC CONTROL

PARK1/4 (*SNCA*): α-Synuclein

SNCA was the first *PARK* gene discovered to cause PD (Polymeropoulos et al., 1996). The prevalence of *SNCA* mutations is estimated as ∼0.05% in a cohort size of more than 2,000 sporadic PD patients (Tan et al., 2019). *SNCA* mutations can cause early-onset PD of variable clinical phenotypes and diverse Lewy pathologies (Campelo and Silva, 2017). Duplications or triplications of *SNCA* cause gene dosage effect on PD severity (Ibanez et al., 2004; Miller et al., 2004).

The physiological function of α-synuclein is not fully understood. Consistent with its many functions, this native disordered protein locates in multiple cellular organelles and sites: mitochondria, nucleus, synapse, endoplasmic reticulum (ER), Golgi, and lysosomes (Bernal-Conde et al., 2019; Shahmoradian et al., 2019).

α-Synuclein directly and indirectly interacts with mitochondria (Ganguly et al., 2018; Grunewald et al., 2019). It maintains mitochondrial membrane composition and structure and its deposition in neurons alters mitochondrial morphology and fragmentation, membrane potential, respiratory chain complex I function, and increases oxidative stress (Di Maio et al., 2016; Zambon et al., 2019; Hannestad et al., 2020). Overexpression of mitochondria-targeted α-synuclein results in mitochondrial reactive oxygen species (ROS) formation, reduced ATP levels, and neuronal death (Ganjam et al., 2019). *Vice versa*, mitochondrial dysfunction causes α-synuclein pathology as shown in traditional PD models induced by paraquat and rotenone. In addition, α-synuclein interacts with a number of critical mitochondrial proteins, including voltage-dependent anion-selective channel 1, PINK1, Parkin, and DJ-1 proteins (Bernal-Conde et al., 2019). It is also associated with mitochondrial Sirtuin 3, a nicotinamide adenine dinucleotide (NAD+)-dependent enzyme critical in mitochondrial quality control and the prevention of oxidative stress (Park et al., 2020).

PARK2 (*PRKN*): Parkin RBR E3 Ubiquitin Protein Ligase (*parkin*)

PRKN, the second identified PD gene (Matsumine et al., 1997) is the most common autosomal recessive gene causing early onset PD (Klein and Lohmann-Hedrich, 2007; Jiang et al., 2020). There are over 100 known mutations in *PRKN* that lead to either a dysfunctional small Parkin protein being rapidly degraded or defective parkin without activity (Abbas et al., 1999). Some, but not all *PRKN* mutations cases have Lewy Pathology (Farrer et al., 2001; Shimura et al., 2001; Pramstaller et al., 2005; Miyakawa et al., 2013; Johansen et al., 2018).

Parkin is a HECT/RING hybrid ligase that receives ubiquitin on its catalytic center and passes ubiquitin onto its substrates (Trempe et al., 2013). It regulates mitochondrial quality control through mitophagy and mitochondrial biogenesis. A loss of Parkin function contributes to the pathogenesis of PD through affecting mitochondria, innate immunity, and interactions with α-synuclein. In human cell models, the lack of *parkin* altered mitochondrial respiratory chain function, oxidative stress levels, mitochondrial morphology and motility, mitophagy (Koentjoro et al., 2017; Bonello et al., 2019), and mitochondrial biogenesis [by upregulating PARIS and subsequently downregulating PGC-1α (Kumar et al., 2020)]. Notably, loss of *Parkin* alone is not sufficient to induce dopaminergic (DA) neuron loss or motor deficits in mouse models (AguiarJr., Tristao et al., 2013). However, in combination with a *POLG* mutation (a proofreading-defective mtDNA polymerase), *Parkin*-deficient mice have both DA neuron loss and motor defects (Sliter et al., 2018).

PARK6 (*PINK1*): PTEN Induced Kinase 1 (*PINK1*)

PINK1 is the second most common autosomal recessive gene for PD identified in 2004 (Valente et al., 2004). Heterozygous pathogenic mutations were found in both sporadic and familial PD (Klein et al., 2007). More than 70 mutations have been identified in *PINK1* (Siuda et al., 2014; Puschmann et al., 2017). Heterozygous G411S mutation cells have normal PINK1 levels but reduced kinase activity, altered ubiquitin phosphorylation, parkin recruitment, and mitophagy, whereas heterozygous Q456X mutation cells have reduced levels of PINK1 with decreased kinase activity, but their mitochondrial response to damage remains intact (Puschmann et al., 2017). Most but not all *PINK1* mutation cases have Lewy pathology, gliosis, and DA neuronal loss in the substantia nigra (Samaranch et al., 2010; Takanashi et al., 2016; Nybo et al., 2020).

PINK1 is a mitochondrial serine/threonine-protein kinase that recruits parkin to depolarized mitochondria for mitophagy (Matsuda et al., 2013). *Pink1*-deficient mice show significantly impaired mitochondrial respiration in the brain with aging, but no altered mitochondrial morphology, DA neuron loss, or Lewy pathology (Kitada et al., 2007; Gautier et al., 2008). Interestingly, *Pink1*-deficient rats exhibit DA neuron loss, altered neurotransmitters, and Lewy pathology at 12 months (Creed and Goldberg, 2018, 2020; Creed et al., 2019). Although PINK1/parkin are essential in the mitophagy pathway, they are not required in basal mitophagy (McWilliams et al., 2018). PINK1/parkin are significantly involved in regulating the basal inflammatory response (Sliter et al., 2018; Wang et al., 2019). Human cell models that lack PINK1 have altered mitochondrial

TABLE 1 | *PARK* genes that are linked with mitochondrial function.

Locus	Genes	Mutation	No. of cases reported	Mean age at onset	Disease progression/ disease duration	Initial signs and symptoms (Top 5 whenever available)	L-Dopa response (out of PD patient tested)	α-synuclein pathology	Brain region M vs Y Up-regulated (≥2 folds)	Brain region M vs Y Down-regulated (≥2 folds)
Autosomal dominant inheritance										
PARK1/PARK4	*SNCA*	Missense (A30P^, E46K^, H50Q^, G51D^, A53E^, A53T^, A32V^) or multiplication^#	146	40s	Rapid (<10 years)	Bradykinesia; Rigidity; Depression	Good (50/103)	Yes	ATZ, BLA, Pu	BMA, 10, RaM
PARK8	*LRRK2*	Missense (R1441G^, Y1699C^ G2019S, I2020T, G2385) or gain of function	724	50s	Rapid (15–20 years)	Tremor; Bradykinesia; Rigidity	Good (276/476)	Yes	CgGf,10, RaM, LC, Rpn, FuG	ATZ, BLA, BMA, Dt, CgGr, Rpn, Acb, VT
PARK17	*VPS35*	Missense (D620N#)	67	50s	Slow	Bradykinesia; Postural instability; Rigidity	Good (8/45)	Yes	BLA, Pu	BMA, PrG, RPN, FuG
Autosomal recessive inheritance										
PARK2	*PRKN*	Missense (K161N#, R256C#, R275W^, T415N#, 202–203 delAG#, 255delA# and 321–322insGT#, W453STOP#), multiplication, deletion ^#, or loss of function	1,000	30s	Slow (27–50)	Tremor; Bradykinesia; Dystonia; Tremor at rest; Rigidity;	Good (192/427)	Yes	Dt	BMA, VTA
PARK6	*PINK1*	Missense (G411S#, Q456X#), deletion ^# or loss of function	151	30s	Median (6–28)	Tremor; Bradykinesia; Rigidity; Dystonia; Tremor at rest	Good (84/113)	Yes	Dt,CA4,	ATZ, BMA, CgGr, SN,10, RaM, RPn, Pu, VT
PARK7	*DJ1*	Missense (A104T, M26I, L10P#, L166P#, L172Q^, and P159DEL#) or loss of function	33	20s	Slow	Bradykinesia; Dystonia; Tremor;	Good (5/25)	Yes	Crus II, CgGf, SN	BMA, CgGr, 10, RaM, RPn, Acb
Atypical Parkinsonism										
PARK9	*ATP13A2*	Missense (F182L, G504R, G877R, T12M#, G533R#, A746T#) or loss of function	36	10s	Slow	Bradykinesia; Intellectual development disorder; Cognitive decline; Gait difficulties; Rigidity	Good (9/30)	Yes (*in vitro*)	ATZ, BLA, BMA, CA4, VT	CgGf, CgGr, SPL, RPn, FuG, STG
PARK14	*PLA2G6**	Missense (G31A, D331Y/M3581IfsX) or loss of function	7	20–30s	Rapid (1–15)	Bradykinesia; Rigidity; Spasticity; Hyperreflexia	Moderate	Yes	CgGf, CA2, SPL, Acb, Pu, FuG, STG	BMA, Dt, VTA, RaM, RPN,
PARK15	*FBXO7*	Missense (R378G#, R498X#, and T22M#)	26	10–20s	Rapid	Bradykinesia; Tremor; Gait difficulties; Rigidity; Behavioral abnormalities	Good (6/18)	Yes	CgGf	10, SPL, Pu, STG
PARK22	*CHCHD2**	Missense (T61I#)	19	50s	Long	Bradykinesia; Resting tremor; Posture instability;	Good	Yes	Dt, VTA, LC	BMA, PrG,10, SPL, RPn, Pu, FuG, STG
PARK23	*VPS13C*	Missense (A269S, W395C^, A444P#^, G1389R#^, Q1593L, and E3109STOP and deletion (V452-K3035)	4	20–30s	Rapid	Bradykinesia; Rigidity; Dystonia; Depression	Moderate	Yes	CgGf, VTA, LC	CgGr, SPL, RPn, Acb

*Information for SNCA, LRRK2, VPS35, PRKN, PINK1, DJ1, ATP13A2, PLA2G6, FBXO7, and VPS13C in this table were extracted from MDS gene, International Parkinson and Movement Disorder Society (http://msdgene.org). MDSGene currently collects data on 1651 different mutations in 6628 movement disorder patients extracted from 1250 publications (Klein et al., 2018). *Information for PLA2G6 and CHCHD2 gene extracted from Online mendelian Inheritance in Man® (http://omim.org). #Mutations were shown to affect mitochondria function. ^Mutations were shown to involve in the formation of α-synuclein pathology. Abbreviations: Y-Young age (age of 24, 31, and 39), M-Middle age (age of 49, 55, and 57). M vs Y up-regulated: up-regulated gene expression with a fold change of greater or equal to 2. M vs Y down-regulated: down-regulated genes expression with a fold change of greater or equal to 2 (M vs Y).*

respiratory chain function, morphology, motility, and mitophagy [reviewed in Grunewald et al. (2019)].

PARK7 (*DJ-1*): Parkinsonism Associated Deglycase (*DJ-1*)

Mutations in *DJ-1* were identified as a rare cause of early onset recessive PD in 2003 (Bonifati et al., 2003). Around 20 pathogenic *DJ-1* mutations have been identified with reduced protein due to rapid degradation (Ramsey and Giasson, 2010), and less dimerization into its functional form (Kumar et al., 2019). The autopsy of a patient with L172Q mutation showed severe DA neuronal loss in the substantia nigra with Lewy pathology (Taipa et al., 2016). About 57% of *DJ-1* mutation carriers exhibit non-motor symptoms, a higher proportion than *PRKN* or *PINK1* mutation carriers (Kasten et al., 2018).

DJ-1 is involved in cellular transformation, oxidative stress response, and mitochondrial function (Di Nottia et al., 2017; Raninga et al., 2017). DJ-1 responds to oxidative stress by accumulating on the outer mitochondrial membrane (OMM) in a PINK1/parkin dependent manner (Thomas et al., 2011; Joselin et al., 2012) which may be neuroprotective (Piston et al., 2017). Depletion of DJ-1 leads to increased ROS, decreased mitochondrial membrane potential, and accumulation of dysfunctional mitochondria, which can be rescued by increasing parkin (Andres-Mateos et al., 2007; Trempe and Fon, 2013; Ozawa et al., 2020). DJ-1 directly interacts with α-synuclein monomers and oligomers in mouse brains (Zondler et al., 2014) and DJ-1 deficiency increases α-synuclein aggregation in human and mouse models (Shendelman et al., 2004; Xu et al., 2017). Notably, the loss of DJ-1 does not induce nigral DA neuron demise in mice (Goldberg et al., 2005). *Dj-1*-deficient rats show DA neuron loss and evident motor abnormalities (Dave et al., 2014). Similar to *SNCA*, *LRRK2*, and *UCHL1*, mutations in *DJ-1* block or reduce the activity of chaperone-mediated autophagy (Sala et al., 2016).

PARK8 (*LRRK2*): Leucine-Rich Repeat Kinase 2 (*LRRK2*)

LRRK2, discovered in 2004, is the most frequent autosomal dominant gene causing PD with more than 100 mutations (Paisan-Ruiz et al., 2004; Rui et al., 2018). Genome Aggregation Database predicts LOF in *LRRK2* variants cause an 82.5% reduction in protein level, with no change in lifespan or clinical phenotype (Whiffin et al., 2020). *LRRK2* mutations generally develop later in life and are clinically similar to sporadic PD, although up to 50% do not have Lewy pathology. Mouse models studying G2019S, R1441G, and *Lrrk2*-deficient failed to show correlation between loss of function (LOF) of LRRK2 and α-synuclein pathology (Daher et al., 2012; Xiong et al., 2017) but impaired parkin-mediated mitophagy is found in fibroblasts from patients with the G2019S mutation (Bonello et al., 2019).

LRRK2 has multiple domains including a kinase and GTPase enzyme. It is involved in a wide range of cellular processes (Berwick et al., 2019; Marchand et al., 2020) and interacts with Miro on OMM to promote its removal, stopping mitochondrial motility and initiating mitophagy (Hsieh et al., 2016). RAB10, a substrate of LRRK2 kinase activity, accumulates on depolarized mitochondria and interacts with the autophagy receptor OPTN (optineurin) to mediate mitophagy in a PINK1/parkin-dependent manner (Wauters et al., 2020). The toxic gain in function of LRRK2 kinase activity inhibits the accumulation of RAB10 on mitochondria (Wauters et al., 2020). The lack of LRRK2 in macrophages induces oxidative stress and dynamin-related protein 1 (DRP1)-dependent mitochondrial fragmentation (Weindel et al., 2020).

PARK9 (*ATP13A2*): ATPase Cation Transporting 13A2 (*ATP13A2*)

Loss of function of *ATP13A2* was initially reported in Kufor-Rakeb syndrome (KRS; Ramirez et al., 2006) and in three other distinct neurodegenerative conditions: juvenile-onset neuronal ceroid lipofuscinosis (Bras et al., 2012), juvenile-onset hereditary spastic paraplegia (Estrada-Cuzcano et al., 2017), and amyotrophic lateral sclerosis-like phenotype (Spataro et al., 2019). More than 30 mutations have been identified in *ATP13A2* and rare variants may contribute to PD risk (Cristina et al., 2020). Mutations in *ATP13A2* cause decreased protein stability, increased proteasomal degradation, impaired polyamine transport and accumulation in lysosomes, and cell death (Podhajska et al., 2012; van Veen et al., 2020).

ATP13A2 is a lysosomal protein, located in the ER, endosomal and lysosomal membranes of neurons (de Tezanos Pinto and Adamo, 2018; Spataro et al., 2019). Loss of *ATP13A2* in mouse and human cell models increase mitochondrial fragmentation and increase ROS and cell death (Gusdon et al., 2012; Park et al., 2014). Cell models from KRS patients and cells with silenced *ATP13A2* show α-synuclein oxidation and accumulation (Tsunemi and Krainc, 2014). Over-expression of ATP13A2 reduces intracellular α-synuclein via the release of exosomes (Kong et al., 2014).

PARK14 (*PLA2G6*): Phospholipase A2 Group VI (*PLA2G6*)

PLA2G6 mutations was discovered in a large family with neurodegeneration in 2006 (Morgan et al., 2006). Mutations in *PLA2G6* can cause autosomal recessive PD with high clinical variability, but all show cerebral and cerebellar atrophy, iron accumulation in the basal ganglia, cognitive decline (Khateeb et al., 2006; Ferese et al., 2018) and marked Lewy pathology (Paisan-Ruiz et al., 2012). A total of 16 mutations have been reported. Although most mutations of this gene are homozygous, a heterozygous missense mutation (G31A) has been reported to increase the risk of PD (Ferese et al., 2018). In contrast, compound heterozygous mutations (D331Y/M358IfsX) cause dystonia-parkinsonism with a poor response to levodopa (Chu et al., 2020).

PLA2G6, a calcium-independent phospholipase A2, is involved in maintaining mitochondrial function (Chiu et al., 2017). Overexpression of PLA2G6 exerted neuroprotection in human cells by increasing the level of mitophagy proteins in response to rotenone (Chiu et al., 2017). The loss of PLA2G6 results in shortened acyl-chains in phospholipids, which affects

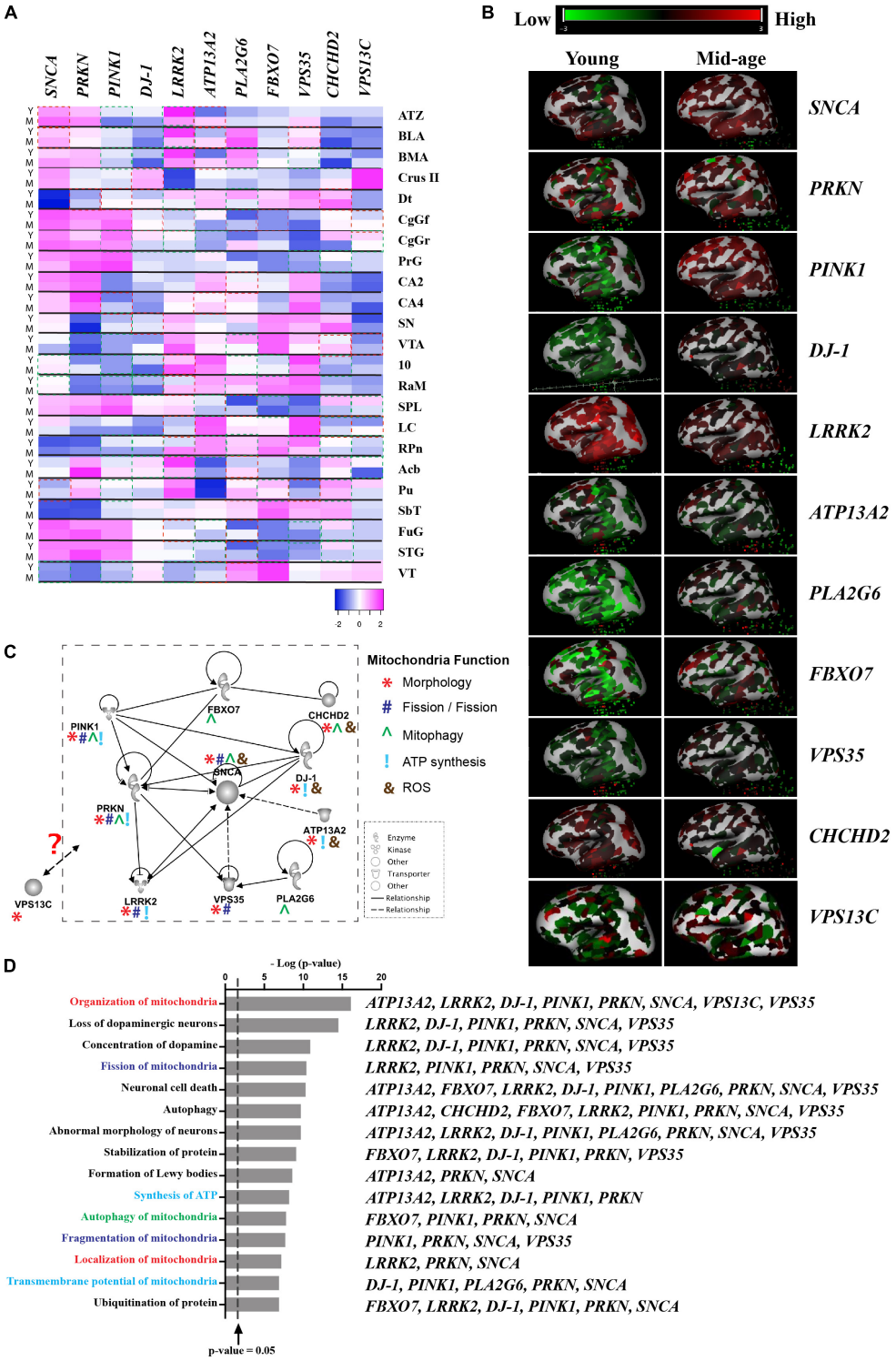

FIGURE 1 | Interaction of proteins encoded by mitochondrial-related *PARK* genes. **(A)** Gene expression data was obtained from Allen Brain Atlas, Allen Institute. *n* = 2–3/group. Y-Young (age of 24, 31, and 39), M-Middle age (age of 49, 55, and 57). Heatmap showing the z-score was generated using online Heatmap program (Babicki et al., 2016). Red boxes indicates up-regulated gene expression with a fold change of greater or equal to 2 (M vs Y) and green boxes indicates down-regulated genes expression with a fold change of greater or equal to 2 (M vs Y). **(B)** 3D heatmap of the 11 genes from Allen Brain Atlas, Allen Institute. Young (age of 24) and mid-age (age of 57); **(C)** The interactive pathways of proteins encoded by the 11 *PARK* genes generated from IPA (Ingenuity Systems Inc., Redwood city, CA, United States). Symbols are used to show mitochondrial function: *Morphology; #Fission/Fusion; ^Mitophagy; !ATP synthesis; and &ROS. **(D)** Top 35

(Continued)

FIGURE 1 | Continued
biological functions identified to be related to mitochondrial function and DA neuron survival and relevant *PARK* genes listed next to functions. The biological functions were plotted against the negative log10 *p*-value [–log(*p*-value)] as measured by Fischer's exact test determined by IPA. Line represented *p*-value = 0.05. Abbreviations: ATZ, amygdalohippocampal transition zone; BLA, basolateral nucleus; BMA, basomedial nucleus; Cb-Crus II, crus II; Dt, dentate nucleus; CgGf, cingulate gyrus; frontal part; CgGr, cingulate gyrus, retrosplenial part; PrG, precentral gyrus; CA2, CA2 field; CA4, CA4 field; SN, substantia nigra; VTA, ventral tegmental area; 10, dorsal motor nucleus of the vagus; RaM, raphe nuclei of medulla; SPL, superior parietal lobule; LC, locus ceruleus; RPn, pontine raphe nucleus; Acb, nucleus accumbens; Pu, putamen; SbT, subthalamus; FuG, fusiform gyrus; STG, superior temporal gyrus; and VT, ventral thalamus.

ER homeostasis, neurotransmission, and promotes α-synuclein aggregation (Mori et al., 2019). Elevated expression of α-synuclein in neuronal mitochondria is observed in PLA2G6 deficiency (Sumi-Akamaru et al., 2016).

PARK15 (*FBXO7*): F-Box Protein 7 (*FBXO7*)

A homozygous mutation in *FBXO7* was reported to cause autosomal recessive PD (Shojaee et al., 2008). Mutations in *FBXO7* have not been detected in sporadic PD (Conedera et al., 2016). Mutations in *FBXO7* promote the aggregation of the toxic form of this protein in mitochondria, resulting in impairment of mitophagy and the ubiquitin-proteasome system (Zhou et al., 2015). *FBXO7* mutations and *SNCA* G51D mutation have been implicated in Parkinsonian-pyramidal syndrome with early onset and rapid progression (Joseph et al., 2018).

FBXO7 is an adaptor protein in Skp-Cullin-F-box (SCF) SCF^{FBXO7} ubiquitin E3 ligase complex, which recognizes substrates and mediates their ubiquitination and translocation to mitochondria following cellular stress (Winston et al., 1999; Joseph et al., 2018). FBXO7 recruits parkin into damaged mitochondria and facilitates its aggregation, but overexpression of FBXO7 can still rescue DA neuron degeneration in parkin null Drosophila (Burchell et al., 2013; Zhou et al., 2016) and restore PD phenotype in the absence of parkin, indicating FBXO7 mediates neuroprotective effects via a parkin-independent pathway (Burchell et al., 2013). Both soluble and insoluble FBXO7 are increased in PD (Zhou et al., 2015). FBXO7 immunoreactivity is detected in most α-synuclein aggregates in PD and in glial cytoplasmic inclusions of multiple system atrophy (Zhao et al., 2013). In contrast, only occasional tau-positive inclusions in Alzheimer's disease and progressive supranuclear palsy contain FBXO7.

PARK17 (*VPS35*): Vacuolar Protein Sorting 35 Ortholog (*VPS35*)

Mutations in *VPS35* were identified in 2008 (Wider et al., 2008) and are reported in patients with autosomal dominant PD. A heterozygous missense mutation D620N has been confirmed as pathogenic (Williams et al., 2017; Chen et al., 2019) and has been found in 0.056∼0.91% of the sporadic PD patients (Ando et al., 2012; Kumar et al., 2012). The D620N mutation did not affect the stability, assembly, or subcellular location of the retromer (Tian et al., 2015), instead it enhanced LRRK2 kinase activity (Mir et al., 2018). D620N mutant mice show no motor disorders but have increased mitochondrial fission and fragmentation (Wang et al., 2017; Cataldi et al., 2018).

VPS35 forms part of a retromer cargo-recognition complex involved in intracellular retrograde transport from endosomes to the *trans*-Golgi network (Hierro et al., 2007; Tabuchi et al., 2010). Loss of iPLA2-VIA (the Drosophila homolog of human PLAG2A) destabilizes VPS35 and impairs retromer function, resulting in ceramide accumulation and cell stress (Lin et al., 2018). VPS35 is implicated in the formation of mitochondria-derived vesicles directed to the peroxisome or lysosome for degradation of mitochondria proteins (Braschi et al., 2010; Wang et al., 2016). Lack of VPS35 in human cells with *VPS35* mutations exhibit defective mitochondrial fusion and increased mitochondrial fragmentation (Tang et al., 2015). Mitochondrial dysfunction induced by *VPS35* mutation can be restored by inhibition of mitochondrial fission (Wang et al., 2016). α-Synuclein is transported by the retromer complex (Miura et al., 2014). Heterozygous *Vps35* KO mice show α-synuclein aggregation, DA neuron degeneration, impaired locomotor behavior, and altered lysosomal morphology (Tang et al., 2015). Overexpression of VPS35 reduces α-synuclein accumulation in mice overexpressing α-synuclein (Dhungel et al., 2015). Moreover, knockdown *Vps35* in Drosophila results in α-synuclein accumulation (Miura et al., 2014).

PARK22 (*CHCHD2*): Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 2 (*CHCHD2*)

Mutations in *CHCHD2* are a rare cause of autosomal dominant PD, originally found in 3/340 PD patients in 2015 (Funayama et al., 2015). To date, there is only one brain autopsy of a PD patient carrying *CHCHD2* T61I mutation that revealed widespread Lewy pathology with additional amyloid plaques and neurofibrillary tangles in the brainstem, limbic regions, and neocortex (Ikeda et al., 2019). α-Synuclein aggregation was accelerated by *CHCHD2* T61I in human cells and in Drosophila. Human cells from T61I patients show accumulated CHCHD2 in the mitochondrial intermembrane space (IMS), resulting in increased ROS and apoptosis (Cornelissen et al., 2020).

CHCHD2 (also called mitochondria nuclear retrograde regulator 1) contains at least one CHCH domain (Modjtahedi et al., 2016). The protein locates in the IMS (Aras et al., 2015). Loss of CHCHD2 function causes an abnormal mitochondrial matrix structure and impaired oxygen respiration in mitochondria resulting in oxidative stress, DA neuron loss, and motor dysfunction with aging (Meng et al., 2017). Importantly, overexpression of CHCHD2 rescues the phenotype of PD. In addition, CHCHD2 binds to cytochrome *c* and Bax inhibitor-1, suggesting the role CHCHD2 in regulating apoptosis and cell death (Liu et al., 2015). In Drosophila, CHCHD2 interacts

with the mitochondrial protein P32 and indirectly regulates the level of mitochondrial fusion protein, Opa1, highlighting the role of CHCHD2 in regulating mitochondrial fusion and cristae morphology (Liu et al., 2015). Moreover, human cells lacking CHCHD2 have altered mitochondrial respiration (Harjuhaahto et al., 2020).

PARK23 (*VPS13C*): Vacuolar Protein Sorting-Associate Protein 13C

Mutations in *VPS13C*, identified in 2016 cause an autosomal recessive early onset PD, characterized by early cognitive decline and rapid disease progression (Lesage et al., 2016; Schormair et al., 2018). The post-mortem examination of the brain of the affected patient displayed reduced protein levels of VSP13C and the presence of α-synuclein pathology (Lesage et al., 2016; Smolders et al., 2021).

VPS13C acts at membrane contact sites on multiple organelles such ER, mitochondria, and late endosome and lysosome for lipid delivery, which is important for mitochondrial biogenesis and function (Kumar et al., 2018). VPS13C was localized to the OMM as shown in HEK293 cells (Lesage et al., 2016) and was found between lipid droplets and mitochondria (Ramseyer et al., 2018). LOF in VPS13C in COS-7 monkey cells resulted in abnormal mitochondrial morphology, increased vulnerability to stress and the activation of PINK1/parkin-dependent mitophagy (Lesage et al., 2016). Overexpression of W395C or A444P VPS13C in Hela or SH-SY5Y cells showed the ER-endosomal/lysosomal localization of VPS13C was lost, suggesting these mutants might affect the stability of the protein thereby influencing its localization (Smolders et al., 2021).

DISCUSSION

Over the past 20 years, great progress has been in our understanding of PD with the identification of 23 *PARK* genes. No doubt there will be more that await discovery. The 11 *PARK* genes highlighted here collectively emphasize the mechanistic importance of mitochondrial function underlying that pathobiology of PD. These genes are involved in multiple pathways affecting mitochondrial morphology, quality control, respiratory chain function, release of ROS, and biogenesis (fission/fragmentation). More importantly, proteins encoded by five genes (*PRKN, PINK1, DJ-1*, *LRRK2,* and *FBXO7*) closely interact with α-synuclein. Mutations in *LRRK2, ATP13A2, PLA2G6, VPS35, CHCHD2,* and *VPS13C* lead to increased α-synuclein accumulation, and mutations in *SNCA*, *PRKN, PINK1, DJ-1*, *LRRK2,* and *VPS35* are responsible for the loss of DA neurons.

Although there is a lack of topographical mapping of these 11 gene coding proteins, the heatmap of their RNA expression is available for the human brain (**Figures 1A,B**; © 2010 Allen Institute for Brain Science. Allen Human Brain Atlas. Available from: human.brain-map.org), suggesting *PARK* gene expression is both age and brain region related, which further highlight regional vulnerability in the profiling of these proteins. Assessment of pathways affected by these 11 *PARK* genes using Ingenuity® Pathway Analysis software (Ingenuity Systems Inc., Redwood city, CA, United States) reveals links to DA neuron survival, mitochondrial function, formation of Lewy body pathology, and their mutual protein interactions (**Figures 1C,D**). Hitherto, cell type specific expression of these gene coding proteins remains unknown. This review suggests the knowledge gap in the field and highlights the importance of studying these genes in sporadic PD, which is essential before targeting these mitochondrial pathways for disease modification.

AUTHOR CONTRIBUTIONS

WL and YF conceived of the presented data. WL and YF wrote the manuscript in consultation with GH and CS. GH and CS were in charge of overall direction and planning. All authors contributed to the article and approved the submitted version.

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