MITOCHONDRIA AND ENDOPLASMIC RETICULUM DYSFUNCTION IN PARKINSON'S DISEASE

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MITOCHONDRIA AND ENDOPLASMIC RETICULUM DYSFUNCTION IN PARKINSON'S DISEASE

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Several pathogenic mechanisms are involved in the pathogenesis of Parkinson's Disease (PD), a neurodegenerative disease characterized by the loss of substantial nigra (SN) dopamine (DA) neurons. Alterations in calcium (Ca2+) homeostasis, cellular proteostasis, axonal transport, mitochondrial function, and neuroinflammation are linked to PD. However, research involving inter-organelle communication and their significance as precise mechanisms underlying neuronal death in PD remain to be elucidated.

Evidence showed that perturbations in the mitochondria-endoplasmic reticulum (ER) network play an important role in the pathogenesis of PD. Alterations in the mitochondria-ER interface have been reported in PARK2 knockout mice and patients harboring PARK2 mutations. Enhanced parkin levels maintain mitochondria-ER cross-talk and assure regulated Ca2+ transfer to sustain cell bioenergetics. Several familial PD-related proteins, including Parkin and PINK1, may lead to modifications in the mitochondria-ER signaling. Interestingly, mitochondria-ER tethering suppresses mitophagy and parkin/PINK1-dependent mechanism regulates the destruction of mitochondria-ER contact sites by catalyzing a rapid burst of Mfn2 phospho-ubiquitination to trigger p97-dependent disassembly of Mfn2 complexes from the outer mitochondrial membrane. Mitofusin-mediated ER stress elicited neurodegeneration in Pink1/Parkin models of PD. α-Synuclein, a presynaptic protein, can bind to the ER-mitochondria tethering protein vesicle-associated membrane protein-associated protein B (VAPB) to disrupt Ca2+ homeostasis and mitochondrial ATP production. It has been reported that ER stress and mitochondrial cell death pathways might mediate A53T mutant α-synuclein-induced toxicity.

Mitochondria-ER signaling mechanism is poorly characterized in neurons and its association in neuronal pathophysiology remains uncertain. The presence of mitochondria-ER contacts in neurons, preferentially at synapses, suggests a potential role in regulating synaptic activity. Alterations in mitochondria-ER associations are expected to be potentially detrimental to neurons, especially to SN DA neurons. Compounds from an unbiased chemical screen reverse both ER-to-Golgi trafficking defects and associated mitochondrial dysfunction in different PD models. In addition, a dibenzoylmethane derivative protects DA neurons against ER stress. Thus, mitochondria-ER signaling may represent a possible upstream drug target as potential therapeutic strategy for PD.

In this Research Topic, we bring together knowledge that emphasizes the importance of mitochondria-ER communication and its impact to further dissect the pathogenic mechanisms in PD.

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EDITORIAL
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Editorial: Mitochondria and Endoplasmic Reticulum Dysfunction in Parkinson's Disease

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Editorial on the Research Topic

Mitochondria and Endoplasmic Reticulum Dysfunction in Parkinson's Disease

Endoplasmic reticulum (ER) and mitochondria are distributed in close communication via a dynamic ER-calcium (Ca^{2+}) mitochondria interconnection and regulate a plethora of vital cellular functions, including Ca^{2+} homeostasis, mitochondrial transport and dynamics, bioenergetics, ER stress, apoptotic signaling, and inflammation (Erpapazoglou et al., 2017). Alteration in the ER-mitochondria communication adversely affects overall physiology of the cell (Gómez-Suaga et al., 2018). ER-mitochondria communication is also involved in lipid transport, suggesting that lipidomic approach may be useful to study the potential mechanisms leading to impaired neuropeptidergic signaling (Valadas et al., 2018). Mitochondria-associated membranes (MAMs) are defined as specialized subdomains connecting ER and mitochondria in order to regulate physiological functions, maintain Ca^{2+} signaling and other vital cellular processes (Rodríguez-Arribas et al., 2017). Neurons are highly dependent on MAMs to exchange metabolites and signaling molecules between ER and mitochondria, suggesting that altered function of MAMs due to toxin insults such as rotenone and manganese could play a crucial role in the pathogenesis of neurodegenerative diseases, including Parkinson's disease (PD) (Krols et al., 2016; Harischandra et al.; Ramalingam et al.; Valdinocci et al.). Modifications in the communication between ER and mitochondria cause a reduction in mitochondrial Ca^{2+} homeostasis in several animal models of neurodegeneration, such as PD, an age-dependent neurodegenerative disorder characterized by the progressive loss of dopamine (DA)-producing neurons in the substantia nigra (Paillusson et al., 2016; Lee et al., 2018). Several cellular mechanisms have been identified to be involved in the DAergic neuronal death, including mitochondrial dysfunction, impaired bioenergetics, oxidative stress, autophagy and impaired intracellular Ca^{2+} homeostasis in patient-derived cell models of PD (González-Casacuberta et al.; Segura-Aguilar). However, mechanisms underlying how organelle crosstalk (especially between mitochondria and ER) could affect the progression of pathogenesis in PD still remain unknown. ER stress activates unfolded protein response through the upregulation of the ER chaperone GRP78 and caspases as well as evokes Ca^{2+} flux that induces mitochondrial dysfunction and associated loss of DA neurons (Arduíno et al., 2009; Baek et al.). Interestingly, increased ROS production through PERK/eIF2α/ATF4/CHOP pathway of UPR and concomitant alteration of the mitochondrial network morphology have been reported in PARK20 fibroblasts (Amodio et al.). Emerging evidence supporting significance of altered ER–mitochondria communication suggests that damaged ER–mitochondria signaling could be a potential therapeutic strategy to treat neurodegenerative diseases.

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The present Research Topic is an effort to showcase the significance of MAM in PD pathogenesis. Here, we discuss the recent findings in PD research with main focus on molecular and cellular mechanisms involving mitochondria and ER. Pathophysiological significance of ER-mitochondria interaction has been demonstrated in the case of PD-related genes, such as α-synuclein (α-syn) (Guardia-Laguarta et al., 2014), DJ-1 (Ottolini et al., 2013), PINK1 (Celardo et al., 2016; Gelmetti et al., 2017), and Parkin (Van Laar et al., 2015; Celardo et al., 2016; Gautier et al., 2016; Gelmetti et al., 2017; Zheng et al., 2017). Several clinical cases with diagnosed PD show a well-defined Lewy body pathology (Cookson et al., 2008), which are composed of α-syn. Protein aggregation and imbalanced cellular proteostasis are key factors leading to accumulation of misfolded α-syn (Lehtonen et al.). Within neurons, α-syn is diversely localized to cytosolic and membrane compartments including synaptic vesicles, mitochondria and the ER (Guardia-Laguarta et al., 2015; Colla). Membrane localization of α-syn is well-targeted to lipid rafts (detergent-resistant membranes) that are enriched in cholesterol and acidic phospholipids (Fortin et al., 2004). Interestingly, a subpopulation of α-syn is shown to be enriched in MAM fraction in immortalized cell lines and in the mouse and human brain (Poston et al., 2013; Guardia-Laguarta et al., 2014; Paillusson et al., 2016). Certainly, identification of the A53T mutation in the gene encoding for α-syn (*SNCA*) provides us better understanding of both the genetics and the neuropathology of PD (Polymeropoulos et al., 1997). It has been demonstrated that A53T mutant showed a decreased association with MAM and an elevated mitochondrial fragmentation, as compared to wild-type α-syn (Guardia-Laguarta et al., 2014). Moreover, overexpression of either wild-type or mutant α-syn decreases ER–mitochondria contacts (Paillusson et al., 2016). Thus, substantial accumulation of α-syn aggregates could be linked to the loss of function of this protein at the MAMs. Interestingly, subcellular localization of α-syn to MAM could be related to both normal and pathological states (Guardia-Laguarta et al., 2014, 2015). A recent study demonstrated that α-syn binds to VAPB (an ER-mitochondria tethering protein) to disrupt Ca^{2+} homeostasis and mitochondrial ATP production (Paillusson et al., 2016).

PINK1/Parkin-mediated mitophagy could be an underlying mechanism of nigral DA neuron death in PD (Thomas et al., 2011; Kane et al., 2014; Barodia et al., 2017). ER-mitochondria contact sites were shown to constitute the initiation sites for this process (Yang and Yang, 2013). During mitophagy, PINK1 and BECN1 re-localize at MAM, which induces ER-mitochondria tethering and autophagosome formation (Gelmetti et al., 2017). Parkin expression was significantly increased in the MAM fraction of neurons following glutamate excitotoxicity (Van Laar et al., 2015), which also ubiquitylated several proteins of the ER-mitochondria interface including Mfn2, VDACs and Miro (Sarraf et al., 2013; Pickrell and Youle, 2015). Parkin may regulate ER-mitochondria communication via Mfn2 (Basso et al., 2018). Mitochondrial and ER stress results in an upregulation of Parkin levels via ATF4 (Bouman et al., 2011). ER-mitochondria communication was reported to be increased in fibroblasts from patients with *PARK2* or *PARK6* mutations compared to control group (Celardo et al., 2016; Gautier et al., 2016). This alteration was associated with higher mitochondrial Ca^{2+} absorption, upon IP_3R stimulation. Similar structural changes were observed in MEFs from *PARK2* knock-out mice (Gautier et al., 2016). Parkin has recently been reported to co-regulate ER-mitochondria communication together with the transcription factor peroxisome proliferator activated receptor g coactivator 1a (PGC-1α), a key modulator of mitochondrial biogenesis (Zheng et al., 2017). ER–mitochondria associations have also been linked to the formation of the inflammasome. Cellular stress in neurodegenerative diseases are detected by the innate immune system through pattern recognition receptors (Paillusson et al., 2016). Reactive oxygen species (ROS) from mitochondria are one signal for activation of the NLRP3 inflammasome (Abais et al., 2015). Elevated ROS generation led to NLRP relocation to MAM, which may provide a mechanism whereby NLRP senses damage mitochondria to activate the inflammasome (Zhou et al., 2011). Due to the importance of MAMs in understanding the fundamental mechanisms of PD pathogenesis and their potential use as a therapeutic approach, further research is needed to investigate on the communications between the ER and mitochondria.

AUTHOR CONTRIBUTIONS

SB collected the relevant references and wrote the manuscript. VT, KP, SK, and VM edited the manuscript and provided thorough reviews on the manuscript.

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On the Role of Aminochrome in Mitochondrial Dysfunction and Endoplasmic Reticulum Stress in Parkinson's Disease

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The identity of what triggers the loss of dopaminergic neurons containing neuromelanin in Parkinson's disease (PD) is still unknown. Fifty years since its introduction in PD therapy, L-dopa is still the gold-standard drug despite severe side effects observed after 4 to 6 years of being treated with it. There are no new therapies that can halt or slow down the progression of the disease and much of the research efforts in this context have been destined to treat L-dopa-induced dyskinesia. There is huge concern about the difficulties that have been observed in the translation of successful preclinical results into clinical studies and new therapies in PD. The discovery of genes associated with familiar forms of PD has made an enormous input into basic research, which seeks to understand the degenerative process resulting in the loss of dopaminergic neurons in the nigrostriatal system. Several mechanisms have been suggested to be involved in the degeneration of nigrostriatal neurons in PD, including mitochondrial dysfunction, endoplasmic reticulum stress, lysosomal and proteasomal protein degradation dysfunction, the formation of neurotoxic alpha-synuclein (SNCA) oligomers, neuroinflammation, and oxidative stress.

MITOCHONDRIAL DYSFUNCTION

The brain is completely dependent on chemical energy (ATP) in order to perform the release of neurotransmitters such as dopamine. Therefore, the existence of functional mitochondria is essential to the performed role of a dopaminergic neuron, i.e., to release dopamine. Postmortem brains with PD presented a deficiency in Complex I activity (Shapira et al., 1990; Esteves et al., 2011). Reduced Complex I activity in platelet mitochondria, purified from patients with idiopathic PD, has been observed (Esteves et al., 2011). CHCHD2 mutation in PD patient fibroblasts reduces oxidative phosphorylation in Complexes I and IV and induces fragmentation of the mitochondrial reticular morphology (Lee et al., 2018). A meta-analysis supports the deficit in Complexes I and IV in the case of peripheral blood, the frontal cortex, the cerebellum and the substantia nigra in PD (Holper et al., 2018). Analysis of mitochondria morphology in PD samples compared to controls revealed a significant decrease in the number of healthy mitochondria per cell. Several genes associated with familial forms of PD (PINK-1, DJ-1, Parkin, HTRA2) are linked to mitochondrial impairment (Larsen et al., 2018). Parkinson's disease, associated with vacuolar protein sorting 35 mutation, affects Complex I activity (Zhou et al., 2017). PINK1 and DJ-1 mutation induce energetic inefficiency (Lopez-Fabuel et al., 2017). SNCA induces mitochondrial dysfunction (Devi et al., 2008; Chinta et al., 2010; Nakamura et al., 2011; Martínez et al., 2018).

ENDOPLASMIC RETICULUM STRESS

Endoplasmic reticulum is involved in secretory protein translocation and the quality control of secretory protein folding. Misfolded or unfolded proteins in the lumen accumulate under endoplasmic reticulum stress, which causing an integrated adaptive response identified as the unfolded protein response (UPR), which seeks to restore proteostasis within the secretory pathway (Cabral-Miranda and Hetz, 2018).

The UPR activation markers, phosphorylated eukaryotic initiation factor 2alpha and phosphorylated pancreatic endoplasmic reticulum kinase, were detected in dopaminergic neurons containing neuromelanin in the substantia nigra of PD patients. Interestingly, phosphorylated pancreatic endoplasmic reticulum kinase was colocalized with an increased level of SNCA (Hoozemans et al., 2007). Neuropathological analysis of PD postmortem brain tissue revealed that pIRE1α is expressed within neurons containing elevated levels of α-synuclein or Lewy bodies (Heman-Ackah et al., 2017). SNCA triggers endoplasmic reticulum stress via the protein kinase RNA-like endoplasmic reticulum kinase/eukaryotic translation initiation factor 2α signaling pathway (Liu et al., 2018). N370S mutation and β-glucocerebrosidase-1 retention within the endoplasmic reticulum induce endoplasmic reticulum stress activation, triggering UPR and Golgi apparatus fragmentation (García-Sanz et al., 2017). It has been reported that endoplasmic reticulum stress activates the chaperone-mediated autophagy pathway via an EIF2AK3/PERK-MAP2K4/MKK4-MAPK14/p38-dependent manner (Li et al., 2018).

DOPAMINE OXIDATION AND PARKINSON'S DISEASE

One of the most characteristic features of the pathology of PD, which results in the onset of motor symptoms, is the massive loss of dopaminergic neurons containing neuromelanin in the nigrostriatal system. As mentioned before, several mechanisms, including mitochondrial dysfunction and endoplasmic reticulum stress, have been proposed as being involved in the degeneration of the nigrostriatal neurons in PD, but the question concerns what triggers these mechanisms in dopaminergic neurons containing neuromelanin. Many times, it has been suggested that the involvement of exogenous neurotoxins triggers these mechanisms, but the severe Parkinsonism induced by MPTP in just 3 days in drug addicts who used synthetic drugs contaminated with this compound undermines this idea (Williams, 1986). The rate of the degenerative process in PD takes years (Braak et al., 2004). The extremely slow degeneration of the nigrostriatal neurons and slow progression of the disease challenge the possible role of exogenous neurotoxins in the loss of dopaminergic neurons containing neuromelanin, suggesting that some endogenous neurotoxin must trigger these mechanisms. A neurotoxic event, triggered by an endogenous neurotoxin, will affect a single neuron without propagative effects, which explains the extremely slow rate of this degenerative process in PD. Among possible endogenous neurotoxins are the neurotoxic SNCA oligomers. However, the prion-like hypothesis of SNCA in PD pathogenesis is based on the propagation (neuron-to-neuron transfer) of neurotoxic SNCA oligomers (Brundin and Melki, 2017). According to this prion-like hypothesis, a relatively rapid process is expected, in contrasting with what happens in PD, which takes years. In addition, what triggers the formation of neurotoxic SNCA oligomers inside the dopaminergic neurons containing neuromelanin? Braak stage hypothesis use the intraneuronal inclusion bodies to follow the development of Parkinson's disease where SNCA is one of the aggregated proteins (Braak et al., 2004). What induces SNCA aggregation in other brain region involved in non-motor symptoms remains unclear. A possible explanation is that an endogenous neurotoxin is formed inside dopaminergic neurons containing neuromelanin during dopamine oxidation. The formation of the pigment called neuromelanin in these neurons is the result of dopamine oxidation into ortho(*o*)-quinones, which is a pathway that involves the formation of three *o*-quinones in a sequential manner (dopamine ⟶dopamine *o*-quinone ⟶ aminochrome⟶ 5,6- indolequinone⟶ neuromelanin).

Dopamine *o*-quinone is able to form adducts with proteins, such as ubiquitin carboxy-terminal hydrolase L1 (UCHL-1) and Parkinsonism-associated deglycase (DJ-1, PARK7), as well as ubiquinol-cytochrome c reductase core protein 1, glucose-regulated protein 75/mitochondrial HSP70/mortalin, mitofilin, mitochondrial creatine kinase and glutathione peroxidase-4, and a human dopamine transporter (Whitehead et al., 2001; Van Laar et al., 2009; Hauser et al., 2013). Incubation of purified tyrosine hydroxylase with dopamine and tyrosinase also forms adducts with dopamine (Xu et al., 1998). Dopamine *o*-quinone induces mitochondrial dysfunction (Berman and Hastings, 1999). Exposure of cells to dopamine induced the formation of dopamine adducts with parkin (LaVoie et al., 2005), but the identity of the *o*-quinone involved in this reaction (dopamine *o*-quinone or aminochrome) is not clear. Dopamine *o*-quinone is completely unstable at physiological pH and cyclizes immediately into aminochrome; thus, the question concerns whether dopamine *o*-quinone has the opportunity to form adducts with parkin in the cell cytosol overcrowded with other proteins, molecules and organelles.

Aminochrome has been reported to be neurotoxic on account of inducing mitochondrial dysfunction, endoplasmic reticulum stress, autophagy dysfunction, proteasomal dysfunction, oxidative stress, neuroinflammation, the disruption of the cytoskeleton architecture and the formation of neurotoxic SNCA oligomers (Arriagada et al., 2004; Zafar et al., 2006; Fuentes et al., 2007; Zhou and Lim, 2009; Paris et al., 2010, 2011; Aguirre et al., 2012; Muñoz et al., 2012, 2015; Huenchuguala et al., 2014, 2017; Xiong et al., 2014; Briceño et al., 2016; Santos et al., 2017; de Araújo et al., 2018; Segura-Aguilar and Huenchuguala, 2018) (**Figure 1**).

5,6-Indolequinone, the precursor of neuromelanin, is able to form adducts with SNCA (Bisaglia et al., 2010). Dopaminochrome has also been reported to form adducts with SNCA (Norris et al., 2005) and to be neurotoxic in cell cultures (Linsenbardt et al., 2009, 2012). The unilateral injection of dopaminochrome induced degeneration of the

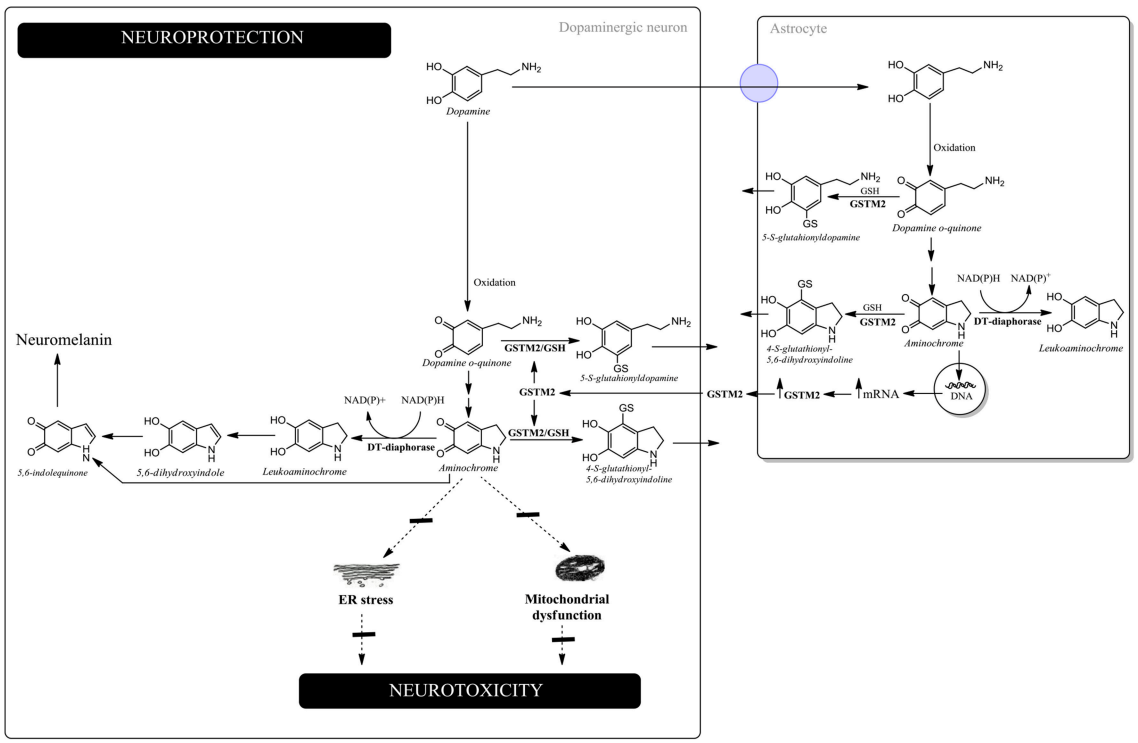

FIGURE 1 | Neuroprotection against aminochrome-induced neurotoxicity. In dopaminergic neurons, DT-diaphorase catalyzes the two-electron reduction of aminochrome into leukoaminochrome, preventing aminochrome-induced endoplasmic reticulum stress and mitochondrial dysfunction. Leukoaminochrome is rearranged into 5,6-dihydroxyindole, which oxidizes into 5,6-indolequinone and polymerizes into neuromelanin. In astrocytes, GSTM2 is able to conjugate both dopamine *o*-quinone and aminochrome with GSH and DT-diaphorase can reduce aminochrome with two-electron to leukoaminochrome. However, astrocytes secrete the enzyme GSTM2, whose dopaminergic neurons internalize in the cytosol. GSTM2 inside the dopaminergic neurons conjugates both dopamine *o*-quinone and aminochrome with GSH, whose stable products are eliminated from dopaminergic neurons.

dopaminergic neurons within the substantia nigra (Touchette et al., 2015). However, the structure of dopaminochrome has not been determined by NMR; nor do we know the nature of this structure. The dopaminochrome structure is different to the aminochrome structure because dopaminochrome has an absorption maximum of 303 and 479 nm (Ochs et al., 2005), while aminochrome has an absorption maximum of 280 and 475 nm and its structure has been confirmed by NMR (Paris et al., 2010).

AMINOCHROME AND PARKINSON'S DISEASE

Dopamine oxidation into neuromelanin is a normal and harmless pathway because neuromelanin accumulates with age, with dopaminergic neurons containing neuromelanin remaining intact in the substantia nigra of healthy seniors (Zecca et al., 2002). Aminochrome is the most stable and studied *o*-quinone formed during dopamine oxidation into neuromelanin. Paradoxically, aminochrome under certain conditions can be neurotoxic as a result of inducing mitochondrial dysfunction (Arriagada et al., 2004; Paris et al., 2011; Aguirre et al., 2012; Huenchuguala et al., 2017; Segura-Aguilar and Huenchuguala, 2018), endoplasmic reticulum stress (Xiong et al., 2014), the formation of neurotoxic SNCA oligomers (Muñoz et al., 2015; Muñoz and Segura-Aguilar, 2017), proteasome dysfunction (Zafar et al., 2006; Zhou and Lim, 2009), autophagy dysfunction (Muñoz et al., 2012; Huenchuguala et al., 2014), lysosome dysfunction (Meléndez et al., 2018), neuroinflammation (Santos et al., 2017; de Araújo et al., 2018), cytoskeleton architecture disruption (Paris et al., 2010; Briceño et al., 2016) and oxidative stress (Arriagada et al., 2004). Aminochrome *in vivo* induces neuronal dysfunction as a consequence of mitochondrial dysfunction, decreased axonal transport resulting in a significant decrease in the number of synaptic monoaminergic vesicles, reduced dopamine release accompanied by an increase in GABA levels, and a dramatic change in the neurons' morphology characterized as cell shrinkage (Herrera et al., 2016). The explanation as to why dopamine oxidation into neuromelanin is not a harmful pathway, despite the formation of potential neurotoxic *o*-quinones, is because the existence of two enzymes [DT-diaphorase and glutathione transferase M2-2 (GSTM2)], which are able to prevent aminochrome neurotoxicity. DT-diaphorase is expressed in dopaminergic neurons and astrocytes and catalyzes the two-electron reduction of aminochrome into leukoaminochrome, preventing aminochrome one-electron reduction into the

leukoaminochrome *o*-semiquinone radical, catalyzed by flavoenzymes that transfer one electron and use NADH or NADPH. DT-diaphorase prevents aminochrome-induced cell death (Lozano et al., 2010), mitochondrial dysfunction (Arriagada et al., 2004; Paris et al., 2011; Muñoz et al., 2012), cytoskeleton architecture disruption (Paris et al., 2010), lysosomal dysfunction (Meléndez et al., 2018), the formation of neurotoxic SNCA oligomers (Muñoz et al., 2015; Muñoz and Segura-Aguilar, 2017), oxidative stress (Arriagada et al., 2004); dopaminergic neurons' degeneration *in vivo* (Herrera-Soto et al., 2017) and astrocytes dell death (Huenchuguala et al., 2016). GSTM2 catalyzes the GSH conjugation of aminochrome into 4-S-glutathionyl-5,6-dihydroxyindoline, which is resistant to biological oxidizing agents such as oxygen, hydrogen peroxide, and superoxide (Segura-Aguilar et al., 1997). GSTM2 also catalyzes the GSH conjugation of dopamine *o*-quinone into 5-glutathionyl-dopamine (Dagnino-Subiabre et al., 2000), which degrades into 5-cysteinyl-dopamine. Interestingly, 5-cysteinyl-dopamine is a stable metabolite that can be eliminated from the cells. 5-Cysteinyl-dopamine has been found in substantia nigra, caudate nucleus, putamen, globus pallidus, neuromelanin, and the cerebrospinal fluid of PD patients (Rosengren et al., 1985; Carstam et al., 1991; Cheng et al., 1996). GSTM2 prevents aminochrome-induced cell death, mitochondrial dysfunction, autophagy, and lysosome dysfunction (Huenchuguala et al., 2014; Segura-Aguilar, 2017a; Segura-Aguilar and Huenchuguala, 2018). The GSH conjugation of aminochrome prevents the formation of neurotoxic SNCA oligomers by generating nontoxic SNCA oligomers (Huenchuguala et al., 2018). GSTM2 is expressed in human astrocytes and it has been reported that astrocytes secrete GSTM2, while dopaminergic neurons are able to internalize this enzyme into the cytosol, protecting these neurons against aminochrome-induced neurotoxicity (Cuevas et al., 2015; Segura-Aguilar, 2015, 2017b).

Mitochondrial dysfunction and endoplasmic reticulum stress are two very important mechanisms involved in the loss of dopaminergic neurons containing neuromelanin in the nigrostriatal neurons in idiopathic PD. However, the question concerns the common denominator in these mechanisms: i.e., what triggers these mechanisms in dopaminergic neurons containing neuromelanin in the nigrostriatal system? We propose that aminochrome is the endogenous neurotoxin that triggers mitochondrial dysfunction and endoplasmic reticulum stress because aminochrome is formed inside dopaminergic neurons of the nigrostriatal system. In addition, aminochrome also triggers other mechanisms involved in the loss of dopaminergic neurons in the nigrostriatal system, such as the formation of neurotoxic SNCA oligomers, oxidative stress, neuroinflammation, and proteasomal and lysosomal protein degradation dysfunction.

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The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Dysfunction of Cellular Proteostasis in Parkinson's Disease

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Despite decades of research, current therapeutic interventions for Parkinson's disease (PD) are insufficient as they fail to modify disease progression by ameliorating the underlying pathology. Cellular proteostasis (protein homeostasis) is an essential factor in maintaining a persistent environment for neuronal activity. Proteostasis is ensured by mechanisms including regulation of protein translation, chaperone-assisted protein folding and protein degradation pathways. It is generally accepted that deficits in proteostasis are linked to various neurodegenerative diseases including PD. While the proteasome fails to degrade large protein aggregates, particularly alpha-synuclein (α-SYN) in PD, drug-induced activation of autophagy can efficiently remove aggregates and prevent degeneration of dopaminergic (DA) neurons. Therefore, maintenance of these mechanisms is essential to preserve all cellular functions relying on a correctly folded proteome. The correlations between endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) that aims to restore proteostasis within the secretory pathway are well-established. However, while mild insults increase the activity of chaperones, prolonged cell stress, or insufficient adaptive response causes cell death. Modulating the activity of molecular chaperones, such as protein disulfide isomerase which assists refolding and contributes to the removal of unfolded proteins, and their associated pathways may offer a new approach for disease-modifying treatment. Here, we summarize some of the key concepts and emerging ideas on the relation of protein aggregation and imbalanced proteostasis with an emphasis on PD as our area of main expertise. Furthermore, we discuss recent insights into the strategies for reducing the toxic effects of protein unfolding in PD by targeting the ER UPR pathway.

Keywords: proteostasis, alpha-synuclein, refolding, ER stress, UPR, protein disulfide isomerase

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INTRODUCTION

In Parkinson's disease (PD), the loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNpc) and subsequent loss of dopamine in the striatum leads to typical motor impairments in PD, such as bradykinesia, rigidity, rest tremor, and postural instability. There are various non-motor symptoms also associated with PD including anosmia, gastrointestinal motility issues, sleep disturbances, sympathetic denervation, anxiety, and depression. These non-motor symptoms generally precede the motor impairments by years (Kalia and Lang, 2015). The presence of Lewy bodies (LBs) with an accumulation of the protein alpha-synuclein (α-SYN) is one of the

pathological hallmarks in PD (Kalia and Lang, 2015; Sveinbjornsdottir, 2016). There is not yet a cure, although, treatments are available to relieve symptoms. Approximately 20 PD-associated genes have been identified to date even though most cases are late onset and sporadic with no evidence for inheritance or genetic cause (Klein and Westenberger, 2012). The phenotypes of both the sporadic and familial forms are essentially indistinguishable, implying that they might share common underlying mechanisms. Moreover, many similarities including protein misfolding and aggregation are also commonly seen in other neurodegenerative diseases. While the exact role of protein aggregation in disease pathology is still under debate, discovering these similarities offers hope for therapeutic advances that could affect many diseases simultaneously. In this review, we summarize recent progress in the studies on the mechanism of endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) in PD, how protein aggregation relates to imbalanced proteostasis and how to remedy the toxic effects of protein unfolding in PD by targeting the ER UPR pathway.

DESCRIPTION OF CELLULAR PROTEOSTASIS DEFICITS IN PD

Physiological Role of α-SYN and Aggregation

α-SYN is a small (14 kDa) protein that is highly expressed in neurons but can also be found in peripheral tissues and blood (Witt, 2013; Malek et al., 2014). A recent report also demonstrated its expression in astrocytes (di Domenico et al., 2019). The physiological function of α-SYN remains mostly undefined (Devine et al., 2011; Liu et al., 2012; Kalia and Kalia, 2015), nevertheless, the involvement in synaptic maintenance, mitochondrial homeostasis, dopamine metabolism, and chaperone activity has been studied. Typically, α-SYN is a monomer with three structural regions (Villar-Piqué et al., 2016). The N-terminal domain (1–60) contains a multi-repeated consensus sequence (KTKEGV) and is responsible for the membrane-binding capacity. The central domain (61–95) is known as the non-amyloid-beta component and contains a highly hydrophobic motif which is involved in α-SYN aggregation. The C-terminal domain's (96–140) proline residues have been found to be acidic. The exact native structure of α-SYN is not completely established, but several studies have described it as a soluble protein with a disordered monomeric structure (Binolfi et al., 2012; Fauvet et al., 2012; Waudby et al., 2013). In addition, soluble tetramers have been identified (Bartels et al., 2011), but the physiologically relevant structure of α-SYN may differ depending on the cellular location and environment. The non-amyloid-beta domain of α-SYN is prone to aggregate, but in its native structure, it appears to be protected by the N- and C-termini (Bertoncini et al., 2005). Changes in environment, mutations and/or post-translational modifications (PTMs) may disrupt the native conformation of α-SYN and induce misfolding and aggregation.

Initially, α-SYN was identified in the nucleus, but this is still in dispute (Huang et al., 2011). It has been proposed that the nuclear protein TRIM28 regulates its translocation into the nucleus and α-SYN may play a role in transcription regulation and histone acetylation (Kontopoulos et al., 2006; Rousseaux et al., 2016). Several studies have shown that PD associated mutations, PTMs and oxidative stress can increase the nuclear localization of α-SYN (Kontopoulos et al., 2006; Xu et al., 2006; Schell et al., 2009; Gonçalves and Outeiro, 2013; Fares et al., 2014). In addition, animal and cellular models and patient studies have shown altered activation of transcription factors upon α-SYN translocation. These include decreased activation of the mitochondrial biogenesis factor PGC-1α, reduced activation of the autophagy-lysosomal pathway (ALP) transcription factor EB (TFEB), and increased activation of calcineurin and subsequent nuclear translocation of nuclear factor of activated T cells (Decressac et al., 2013; Ryan et al., 2013; Luo et al., 2014; Eschbach et al., 2015).

α-SYN is associated with several neurodegenerative disorders, collectively known as synucleinopathies (Wong and Krainc, 2017). α-SYN fibrils are the main component found in LB and Lewy neurites (LNs) in PD and dementia with Lewy bodies (DLBs). LBs are spherical aggregates of α-SYN found in neuronal cell bodies, while LNs are aggregate structures found in neuronal dendrites and axons. Structurally, LBs are made up of insoluble eosinophilic amyloid that is surrounded by fibrils of α-SYN which are typically ubiquitinated (Beyer et al., 2009). In sporadic PD, α-SYN accumulates in neuronal cell bodies and processes resulting in LBs and LNs, respectively. Duplication of *SNCA* results in late-onset autosomal dominant forms of PD and triplication results in early-onset PD (Singleton et al., 2003). This demonstrates that α-SYN levels correlate with the onset of PD. In addition, other mutations causing familial PD, like mutations in leucine-rich repeat kinase 2 (LRRK2), can develop LB pathology (Zimprich et al., 2004). When comparing the pathology of DLB, there are some similarities with PD, but the clinical symptoms are closer to Alzheimer's disease (Spillantini et al., 1998). In PD, the substantia nigra (SN) is affected, while in DLB the pathology is seen in the cortex. In addition to PD and DLB, α-SYN accumulation is present in multiple system atrophy (MSA) and pure autonomic failure (PAF). In MSA the α-SYN inclusions are present in the cytosol of oligodendrocytes. Mutations in α-SYN can cause both PD and MSA symptoms (Fanciulli and Wenning, 2015). LBs and LNs are found in the sympathetic nervous system in PAF (Arai et al., 2000). In addition to synucleinopathies, α-SYN toxicity has been associated with lysosomal storage disorders such as Gaucher's disease, a rare genetic disorder characterized by the deposition of glucocerebroside in cells of the macrophage-monocyte system (Blanz and Saftig, 2016). Mutations in *GBA1*, which encodes glucocerebrosidase (GCase) and causes Gaucher's disease, are the most common risk factors for PD. Some patients carrying these mutations may develop parkinsonism, a clinical syndrome characterized by movement disorders commonly seen in PD, with LB pathology.

Oligomers and fibrils are considered to be the toxic species of α-SYN, but there remains some disagreement regarding their

toxicity. Several studies have suggested that soluble oligomers are more toxic than fibrils or aggregates. For example, increased levels of soluble oligomers have been identified in α-SYN transgenic mice and in PD and DLB patient brains. Oligomeric α-SYN caused more severe DA neuron loss than fibrils in rats (Sharon et al., 2003; Winner et al., 2011). By contrast, some studies have shown fibrils to be more toxic compared to the oligomers and caused increased motor impairment, DA cell loss and synaptic impairment (Peelaerts et al., 2015).

In neurons, α-SYN is known to localize in presynaptic terminals and regulate synaptic transmission. The release of neurotransmitters requires cycles of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly and disassembly. α-SYN has been shown to bind to SNARE protein synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP2) and promote SNARE-complex assembly (Burré et al., 2010). The same study also demonstrated that triple knockout mice developed neurological impairments and had decreased SNARE assembly. Subsequently, it was described that α-SYN promotes vesicle-clustering activity, which is dependent on the interaction of α-SYN with synaptobrevin-2/VAMP2 and anionic lipids (Diao et al., 2013). These studies suggest that the major cellular function of α-SYN are interactions of α-SYN with cell membranes, and that the cytosolic state may be transient.

While α-SYN is normally localized in presynaptic terminals, the oligomers and aggregates can be found in cell bodies and neurites, as well as in other cell types, including astrocytes which indicates a widespread toxic action. The pathological effects of α-SYN can affect the function of several different organelles, including synaptic vesicles, mitochondria, lipid bilayers, cell's cytoskeleton, ER, Golgi, proteasomes, lysosomes, and nucleus. α-SYN oligomers can disrupt the SNARE complex formation, dopamine release and synaptic-vesicle motility (Choi et al., 2013; Wang et al., 2014). Increased levels of α-SYN can also decrease the synaptic-vesicle recycling-pool size and mobility leading to a disrupted neurotransmitter release (Nemani et al., 2010; Scott and Roy, 2012). It was discovered that dopamine neurotransmission can be disrupted by high levels of α-SYN. Transgenic mice overexpressing α-SYN showed a DA terminal loss, deficient release and altered synaptic-vesicle distribution (Masliah et al., 2000; Janezic et al., 2013). Moreover, the reduction in dopamine reuptake and defective dopamine transporter function has been linked to increased levels of α-SYN (Lundblad et al., 2012).

The homeostasis of mitochondria can be disrupted by α-SYN toxicity. Mice with an A53T α-SYN mutation have increased mitochondrial DNA damage and upregulated mitophagy (Martin et al., 2006; Choubey et al., 2011; Chen et al., 2015). In contrast, a recent study showed delayed mitophagy in PD patient neurons caused by abnormal accumulation of Miro protein (Shaltouki et al., 2018). α-SYN oligomers also reduced axonal mitochondria transport in induced pluripotent stem cell (iPSC)- derived neurons (Prots et al., 2018). Recent studies have also shown that α-SYN translocated to the mitochondrial matrix and caused impairment of complex I leading to decreased ATP synthesis and increased reactive oxygen species (ROS) production (Martínez et al., 2018). These results suggest that α-SYN can disrupt the mitochondrial homeostasis in several ways.

α-SYN oligomers can interact with and permeabilize lipid membranes causing structural alterations of the intracellular and plasma membranes, increase of intracellular calcium levels, and activation of calpain (van Rooijen et al., 2010; Melachroinou et al., 2013; Ronzitti et al., 2014). Additionally, α-SYN oligomers can inhibit tubulin polymerization and impair neurite network morphology and overexpression in cultured cells and cause microtubule destabilization and neurite degeneration (Lee et al., 2006; Chen et al., 2007; Prots et al., 2013). α-SYN fibrils have also been shown to impair axonal transport of autophagosomes and endosomes but the fibrils didn't affect the transport of synaptophysin or mitochondria (Volpicelli-Daley et al., 2014). However, a recent study found that α-SYN oligomers disrupted anterograde axonal transport of mitochondria and caused subcellular changes in transport-regulating proteins in iPSC-derived neurons (Prots et al., 2018).

Major Pathways of Alpha-Synuclein Clearence in PD

The protein degradation system is part of a protein quality control machinery which clears non-essential misfolded, or damaged proteins. The two major protein degradation systems are the ubiquitin-proteasome pathway (UPP) and ALP. Both are affected in synucleinopathies. These pathways have been shown to be responsible for degrading α-SYN, and failure in one or both can lead to accumulation. The progressive accumulation of α-SYN typical in PD can be linked to the disruption of the UPP by aggregation (Lindersson et al., 2004) as well as different types of autophagy (Winslow et al., 2010; Malkus and Ischiropoulos, 2012). It has been shown that aggregated α-SYN can bind to the membrane proteins of lysosomes and block their function (Malkus and Ischiropoulos, 2012) as well as inhibit certain enzymatic activity domains of proteasomes (Lindersson et al., 2004). α-SYN also inhibits the expression of proteins relevant to autophagosome assembly leading to inefficient removal of aggregated proteins due to impairment in macroautophagy (Winslow et al., 2010).

The Ubiquitin-Proteasome Pathway

In the UPP, short-living proteins that are coupled with ubiquitin molecules are degraded by proteasomes (Pickart, 2001; Glickman and Ciechanover, 2002) (**Figure 1A**). The first evidence of UPP failure in PD came from post-mortem studies that used enzymatic assays to evaluate proteasome activity in brain tissues. These studies showed a significant decrease in the chymotrypsin-like and trypsin-like proteasome activity in the SN of PD patients in comparison to age-matched controls. No evidence of defective proteasome activity was seen in other brain regions but rather increased activity was observed in the unaffected areas (Furukawa et al., 2002). These studies suggested that reduced proteasome activity is specific for certain brain regions, like SN. In contrast, it is probable that the decreased activity of proteasomes could be a consequence of the neurodegeneration in this region.

Reduced levels of proteasome subunits have been observed in PD patients. Several genes that code for proteasome subunits

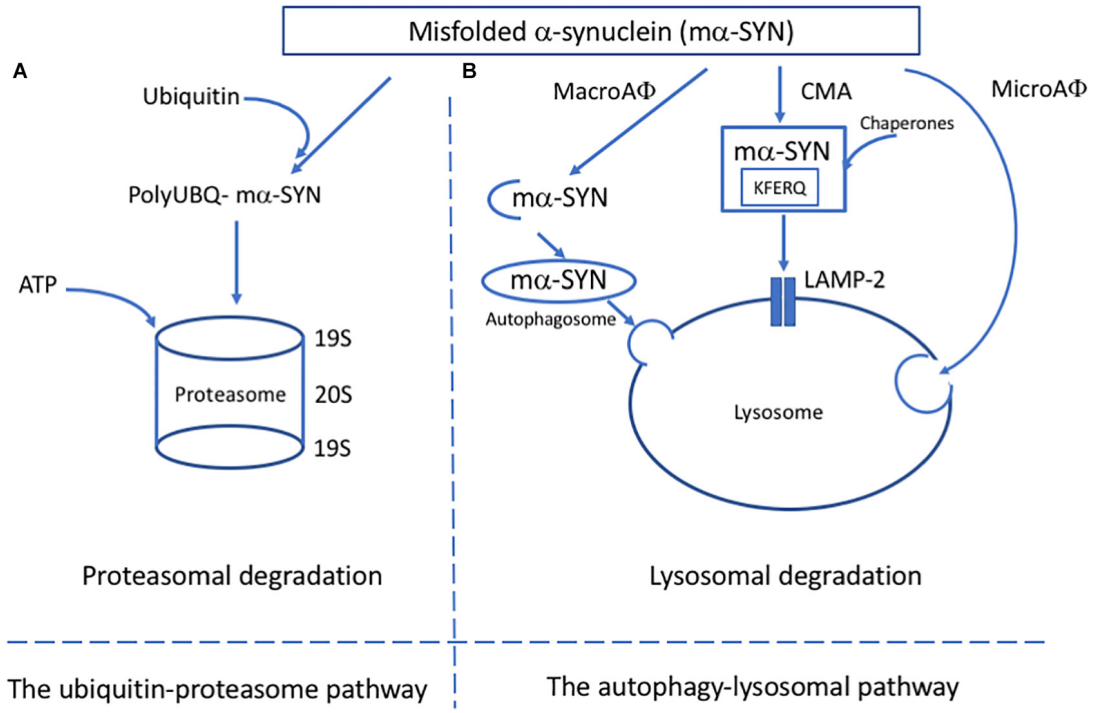

FIGURE 1 | Major pathways of alpha-synuclein clearance in PD. **(A)** In the ubiquitin-proteosome pathway, mα-SYN is tagged with ubiquitin molecules and transferred to the proteosome complex for ATP dependant degradation. **(B)** In the autophagy-lysosomal pathway (ALP), three different pathways have been described: microautophagy (microAφ), macroautophagy (macroAφ) and chaperone-mediated autophagy (CMA). While evidence linking microAφ and mα-SYN is still missing, in macroAφ, mα-SYN is sequestered by a double membrane-organelles called autophagosomes before fusion with lysosome. In CMA, mα-SYN bind to protein chaperones, which help to target them directly to the lysosome for enzymatic degradation.

were downregulated in the SN of PD patients and were linked to reduced levels of 20S proteasome core, α-subunit and 19S regulatory caps (Furukawa et al., 2002; McNaught et al., 2002a, 2003; Grünblatt et al., 2004; Chu et al., 2009; Bukhatwa et al., 2010).

Some studies have demonstrated altered proteasome function in peripheral blood cells of PD patients, but the results were significant only in patients treated with L-DOPA and dopamine agonists (Blandini et al., 2006; Ullrich et al., 2010). This indicates that dopamine levels can alter the proteasome function and is supported by animal and *in vitro* models (Yoshimoto et al., 2005; Berthet et al., 2012).

Studies with disease models have implicated the dysfunction of UPP in PD. Treatment with proteasome inhibitor lactacystin leads to dose-dependent neurodegeneration and formation of ubiquitin and α-SYN positive inclusions in α-SYN-eGFP transfected mouse primary neurons, rat ventral mesencephalic primary neurons, and cultured PC12 cells (McLean et al., 2001; Rideout et al., 2001; McNaught et al., 2002b). McNaught and colleagues used a systemic application of proteasome inhibitor in rats which led to motor deficits and main pathological features typical for PD (McNaught et al., 2004). Also, neurons were found to contain α-SYN and ubiquitin-positive inclusion bodies. Since then, this model has been challenged due to several laboratories inability to replicate the model (more information, see review, Bentea et al., 2017). Besides these studies, toxin-based animal and cellular models have implicated a link between sporadic PD and UPP failure. The toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) appears to target specifically those neurons that are involved in PD. MPTP can easily cross the blood–brain barrier and then is metabolized by astrocytes to become 1-methyl-4-phenylpyridinium (MPP++) ion which is also toxic. MPP+ is released from astrocytes and taken up by DA neurons. MPTP administration causes nigral cell loss, striatal dopamine loss and behavioral deficits (Meredith and Rademacher, 2011). Several *in vitro* experiments have shown decreased proteasome activity after exposure of pesticides and environmental toxins linked to PD (Wang et al., 2006; Caneda-Ferrón et al., 2008; Chou et al., 2010). Consistent with these findings, *in vivo* studies showed reduced proteasome activity after rotenone and MPTP administration (Fornai et al., 2005; Betarbet et al., 2006). The UPP impairment caused by MPTP was alleviated in mice lacking α-SYN suggesting that it increases the detrimental effects of MPTP on the UPP (Fornai et al., 2005).

Numerous *in vitro* studies with purified proteins or cell culture systems, have demonstrated that mutant or wild-type α-SYN can inhibit 20S or 26S proteasome activity, especially in the case of oligomer or fibril formation. In PC12 cells expressing human mutant A53T α-SYN, cells

exhibited accumulation of cytoplasmic ubiquitinated aggregates corresponding with decreased proteasomal chymotrypsin-like activity measured from cell lysates (Stefanis et al., 2001). The finding was confirmed by another study using the same cell line which showed that chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome were all decreased (Tanaka et al., 2001). Also, mutant α-SYN increased cell death in the presence of a proteasome inhibitor. In M17 neuroblastoma cells, mutant α-SYN (A30P or A53T) increased sensitivity to proteasome inhibitors by decreasing proteasome function measured by a GFP reporter system (Petrucelli et al., 2002). These studies indicated reduced proteasome function related to mutant α-SYN, but not to wild-type. However, Snyder et al. (2003) showed inhibition of the proteasome with overexpressing wild-type α-SYN in neuroblastoma M17 cells. A study with yeasts revealed impairment of proteasome-mediated protein degradation in cells expressing wild-type and mutant (A30P) α-SYN (Chen et al., 2005). The cells expressing α-SYN also exhibited a decrease in chymotrypsin-like activity, but no other proteolytic activity of the proteasome was altered.

Contrary to these studies, overexpression of wild-type or mutant (A30P, A53T) α-SYN in PC12 cells or transgenic mice did not result in dysfunction of the UPP (Martìn-Clemente et al., 2004). In a more recent study, Zondler et al. (2017) demonstrated that the impairment of proteasome activity by α-SYN is dependent upon the cellular background. In this study, recombinant α-SYN oligomers and fibrils *in vitro* or transient expression of wild-type or mutant (A30P, A53T) α-SYN in U2OS ps 2042 [Ubi(G76V)-GFP] cells did not affect 20S proteasome function. In contrast, in DA SH-SY5Y and PC12 cells, stable expression of both wild-type and mutant α-SYN resulted in impairment of the chymotrypsin-like 20S/26S proteasomal protein cleavage.

The proposed mechanisms of how α-SYN inhibits the activity of the proteasome may be by direct binding to the S6′ or the Rpt5 subunit of the 19S proteasome, or to the β5 subunit of the 20S proteasome (Ghee et al., 2000; Snyder et al., 2003; Lindersson et al., 2004). In addition, different α-SYN species have been implicated in UPP dysfunction. PC12 cells expressing wild-type or mutant α-SYN produce soluble intermediate sized oligomers that associate with the 26S proteasome and increase in amount after treatment with proteasomal inhibitor, indicating specific degradation of the 26S proteasome (Emmanouilidou et al., 2010). In fact, the expression of α-SYN leads to inhibition of all proteasome activities. This study suggested that only a subset of soluble cell-derived α-SYN oligomers are targeted to the 26S proteasome for degradation. Simultaneously, these species can inhibit the proteasome function.

The Autophagy-Lysosomal Pathway

The ALP is responsible for degrading long-lived proteins, cellular components and organelles through the lysosomal compartment (Parzych and Klionsky, 2014). The ALP has two main purposes: to clear deleterious intracellular components and recycle macromolecules from organelles and proteins to guarantee proteome renewal. Depending on the delivery method, ALP can be divided into three pathways: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy (**Figure 1B**). Because of the lack of evidence linking microautophagy to α-SYN, the focus here is on macroautophagy and CMA.

Macroautophagy is an evolutionary and highly conserved process and the best known of the three autophagic mechanisms (Parzych and Klionsky, 2014; Bento et al., 2016). After the discovery of autophagy-related genes (Atg), the molecular pathway of macroautophagy has been well-characterized. Macroautophagy involves the formation, elongation, and nucleation of double-membrane organelles called autophagosomes that sequester the substrate before fusion with lysosomes. CMA is a particular system based on the recognition of a specific amino acid sequence (KFERQ) (Dice, 1990). The cytosolic chaperone heat shock cognate protein of 70 kDa (hsc70) recognizes the specific motif and translocates the substrate into the lysosome membrane where it interacts with the lysosome-associated membrane protein type 2A (LAMP2A) (Cuervo and Dice, 1996). The final step of the translocation requires the presence of lysosome-associated hsc70 (lys-hsc70) which disassembles LAMP2A into monomers and initiates a new cycle of substrate uptake and degradation (Agarraberes et al., 1997).

Several genetic factors related to PD are involved or interact with ALP (Gan-Or et al., 2015). Mutations in *GBA1* that encode the lysosomal hydrolase GCase can lead to lysosomal dysfunction and disruption of autophagy. For example, SCARB2, which encodes the lysosomal integral membrane protein type 2 and interacts with GCase, has been associated with a reduced risk for PD (Gan-Or et al., 2015). Mutations in the ATP13A2 (*PARK9*) gene, encoding a lysosomal ATPase, causes Kufor-Rakeb syndrome, a rare form of atypical, juvenile-onset autosomal recessive parkinsonism with pyramidal neurodegeneration and dementia.

Along with mutations in genes coding for lysosomal components, other PD-related mutations have been implicated in the process of autophagy. Mutations in the gene encoding vacuolar protein sorting-associated protein 35 (VPS35) cause a rare form of autosomal dominant PD (Gan-Or et al., 2015). VPS35 is involved in endosomal-lysosomal trafficking which is associated with autophagy. Several autosomal recessive PD genes, like parkin (*PARK2*), PINK1 (*PARK6*), DJ-1 (*PARK7*), and FBXO7 (*PARK15*) have been linked to mitophagy, the process of degradation of dysfunctional mitochondria by autophagy (Burchell et al., 2013; Gan-Or et al., 2015; Pickrell and Youle, 2015). Mutations in *LRRK2* are the most common known genetic causes for PD. LRRK2 can be degraded by macroautophagy and CMA, but the most common mutation, G2019S, is poorly degraded by this pathway (Orenstein et al., 2013). In addition, mutated LRRK2 impaired CMA leading to an accumulation of other CMA substrates, including α-SYN. Moreover, mutant LRRK2 caused an increase of autophagic vacuoles in a neuronal cellular model, proposing a more general role of LRRK2 in autophagy (Plowey et al., 2008). In a recent study, Ho et al. (2018) showed that LRRK2 mediates phosphorylation of leucyl-tRNA synthetase leading to impairment of autophagy.

After the initial finding of accumulation of autophagic vacuoles in the SN of PD patients (Anglade et al., 1997), several pathological studies suggested that macroautophagy and CMA are deregulated along with several key proteins related to macroautophagy. Increased levels of beclin-1, which is responsible for the formations and maturation of autophagosomes, and increased levels of autophagosome marker LC3II have been found in the SN of PD patients (Dehay et al., 2010; Miki et al., 2016). Likewise, decreased levels of lysosomal-associated membrane protein type 1 (LAMP1) were evident in nigral neurons in PD patients (Chu et al., 2009; Dehay et al., 2010). The impairment of CMA is associated with the pathogenesis of PD since chaperone Hsc70 and LAMP2 were less expressed in several structures of PD brains (Alvarez-Erviti et al., 2010; Murphy et al., 2015). Decreased levels of several lysosomal markers have been shown in the SN of PD patients. These include the structural protein LAMP1 (Dehay et al., 2010), the lysosomal P-type ATPase ATP13A2 (Dehay et al., 2012), GCase (Gegg et al., 2012; Murphy et al., 2014) and heat shock protein 73 (Chu et al., 2009). In addition, altered activities of lysosomal enzymes, like GCase, Cathepsin A and D have been detected in PD brains (Chu et al., 2009; Gegg et al., 2012; Murphy et al., 2014; Chiasserini et al., 2015).

Transcriptome studies have revealed deregulation of the ALP in PD brains and alterations of several autophagy-related processes, including mTOR, PI3K/AKT, and 14-3-3 protein signalings (Elstner et al., 2011; Mutez et al., 2014; Dijkstra et al., 2015). Increased levels of mTOR protein expression were found in the temporal cortex of patients with DLB, in particular in neurons displaying α-SYN accumulation (Crews et al., 2010). The alteration of other upstream autophagy-related proteins has also been demonstrated, including the immunoreactivity of UNC-51-like kinase 1 (ULK-1), ULK-2, VPS35 and autophagy/Beclin-1 regulator 1 (AMBRA1) within mature LBs, increased levels of Beclin-1, and changed subcellular localization of transcription factor EB (TFEB) (Decressac and Bjorklund, 2013; Miki et al., 2016). Moreover, in a recent study a downregulation of 6 core autophagy genes (*ULK* 3, *Atg*2A, *Atg4B, Atg5, Atg16L1*, and histone deacetylase 6), and increased protein levels of ULK1, Beclin1, and AMBRA1 were detected in peripheral blood mononuclear cells (PBMCs) of PD patients (Miki et al., 2018). These protein levels correlated with increased α-SYN levels in PBMCs. These results suggest a decrease in autophagy properties in PD patients.

The autophagy-lysosomal pathway and α*-SYN*

While α-SYN can be cleared by UPP, the main pathway for its degradation appears to be lysosomal (Webb et al., 2003; Vogiatzi et al., 2008). α-SYN can be degraded by both macroautophagy and CMA, but the structure and mutations may change the final path of degradation. Small soluble forms of α-SYN are more likely to be degraded by CMA but in the pathological condition the burden shifts to macroautophagy even though both pathways can compensate for each other.

Induction of autophagy with rapamycin leads to the clearance of overexpressed wild-type and mutant α-SYN in cell cultures (Webb et al., 2003). This study established that inhibition of macroautophagy with 3-methyladenine causes the accumulation of mutant – but not wild-type – α-SYN. In contrast, a study with PC12 cells showed an increase in both endogenous and overexpressed wild-type α-SYN when macroautophagy was inhibited with 3-methyladenine (Vogiatzi et al., 2008). Other studies with neuronal cells or transgenic mice overexpressing wild-type α-SYN showed accumulation of α-SYN only upon general lysosomal inhibition, and not in suppressed macroautophagy (Lee et al., 2004; Klucken et al., 2012). However, another study has revealed an increase of A53T α-SYN oligomers after pharmacological or molecular inhibition of macroautophagy (Yu et al., 2009). Conditional depletion of *Atg7* in DA neurons caused age-related neuronal loss, the formation of ubiquitinated protein aggregates and increase in monomeric α-SYN (Ahmed et al., 2012). In addition, α-SYN aggregates were detected in striatal axonal swellings of 20-month-old mice after depletion of *Atg7* (Friedman et al., 2012). Both studies suggested a role of macroautophagy in α-SYN turnover *in vivo,* since macroautophagy impairment caused modest alterations in endogenous α-SYN. Overall, these studies indicate that degradation of α-SYN by macroautophagy may depend on the conformation of α-SYN. It is likely that small amounts of wild-type α-SYN are degraded by CMA, but in cases of overexpression or mutations, macroautophagy becomes a more important pathway. The macroautophagic degradation of α-SYN could also depend on PTMs. Phosphorylation and SUMOylation [Small Ubiquitin-like Modifier (SUMO)] have been reported to increase α-SYN degradation by macroautophagy in yeast and PD models (Oueslati et al., 2013; Shahpasandzadeh et al., 2014; Tenreiro et al., 2014). Inside the lysosome, α-SYN is mainly degraded by Cathepsin D, and overexpression of the mutant form of this protease leads to increased levels of α-SYN (Crabtree et al., 2014). In addition, Cathepsin D knockout mice exhibited an accumulation of higher molecular weight α-SYN species (Cullen et al., 2009). Overexpression of mutant A53T α-SYN has been reported to enhance autophagic flux which causes increase in autophagic vacuoles and macroautophagic degradation (Cuervo et al., 2004; Xilouri et al., 2009; Choubey et al., 2011). Similar effects have been reported with wild-type α-SYN, although to a lesser extent. α-SYN can also inhibit macroautophagy via interaction with Rab proteins leading to Atg9 mislocalization (Winslow et al., 2010). Furthermore, α-SYN has been shown to enhance mitophagy. In a transgenic mouse model expressing A53T specifically in DA neurons, the induction of mitophagy was detected (Chen et al., 2015). In the cell culture model, α-SYN overexpression caused increased mitophagy leading to neuronal death (Choubey et al., 2011). However, elevated macroautophagic flux was evident in primary midbrain neurons overexpressing wild-type and A53T α-SYN, without significant alterations in mitophagy (Koch et al., 2015).

Besides neuronal cells, the relation of α-SYN and autophagy has also been demonstrated in other cell types. DJ-1 knockdown microglia exhibited an impaired uptake of α-SYN and had lower autophagy-dependent degradation of p62 and LC3 proteins (Nash et al., 2017). In immortalized astrocyte cell lines overexpressing wild-type, A30P and A53T mutant α-SYN showed decreased LC3-II and increased p62 protein levels, suggesting the

inhibition of autophagy (Erustes et al., 2018). In addition, iPSC-derived astrocytes with *LRRK2* G2019S mutation accumulated α-SYN and had impaired macroautophagy and dysfunctional CMA (di Domenico et al., 2019). PD astrocytes displayed LAMP2A positive vesicles all around the cell body, whereas in control lines the vacuoles were in the perinuclear area. In addition, α-SYN co-localized with LAMP2A receptor in PD astrocytes. LAMP1 -positive vesicles were also found throughout the cell in PD astrocytes, and there was an increase in autophagic vacuoles. Furthermore, higher basal levels of LC3-II, p62 and impaired autophagic flux were detected from PD astrocytes.

The link between α-SYN and CMA was initially established in purified lysosomes demonstrating that α-SYN could be actively degraded by CMA (Cuervo et al., 2004). Interestingly, A30P and A53T α-SYN mutations had higher affinity to LAMP2A and blocked and totally impaired the CMA pathway. Since then, the higher affinity of the mutant α-SYN to LAMP2A was confirmed in neuronal cultures and other cell culture models (Vogiatzi et al., 2008; Alvarez-Erviti et al., 2010). In the neuronal systems, the inhibition of CMA leads to the formation of high molecular weight or detergent-insoluble oligomeric α-SYN conformations (Vogiatzi et al., 2008). Also, in mice where α-SYN expression was enhanced with paraquat or transgenic overexpression, the intralysosomal content of α-SYN was increased as well (Mak et al., 2010). The overexpression of α-SYN in mice also led to upregulation of LAMP2A and hsc70. Another study with mice with VPS35 deficiency or expression of PD-linked mutation D620N showed accumulation of α-SYN in DA neurons and DA degeneration (Tang et al., 2015). This was accomplished by an impaired endosome-to-Golgi retrieval of LAMP2A leading to decreased levels of LAMP2A and a reduced α-SYN clearance. In Drosophila, which lacks CMA, neuronal expression of human LAMP2A protected against starvation and oxidative stress and delayed the locomotor decline in aging flies (Issa et al., 2018). LAMP2A also alleviated the progressive locomotor and oxidative defects induced by neuronal expression of PD-associated human A30P α-SYN. LAMP2A stimulated autophagy in adult Drosophila, and neuronal expression of LAMP2A upregulated levels of Atg5.

PTMs can affect the degradation of α-SYN through CMA. Oxidation and nitration of α-SYN slightly inhibited the CMA, whereas phosphorylation and exposure to dopamine almost completely block the CMA degradation system. However, only dopamine-modified α-SYN blocks the degradation of other substrates (Martinez-Vicente et al., 2008). The same study reported that CMA could degrade only monomeric or dimeric α-SYN, but not oligomers. Blocking the CMA by aberrant forms of α-SYN can also have a toxic effect and impact other degradation pathways. PD-linked mutations like A30P and A53T or dopamine-modified wild-type α-SYN can inhibit the function of CMA leading to activation of macroautophagy and increased toxicity in cells (Martinez-Vicente et al., 2008; Xilouri et al., 2009).

Recently, micro-RNAs (miRNAs) have been implicated in CMA function and α-SYN clearance. Several miRNAs have been described to target LAMP2A and Hsc70 and decrease α-SYN degradation (Alvarez-Erviti et al., 2013; Li et al., 2014). The initial study found four miRNAs that reduce LAMP2A levels, and three that decreased Hsc70 levels. This was accompanied by increased accumulation of α-SYN in SH-SY5Y neuroblastoma cells. These miRNAs were also found up-regulated in brains of PD patients and correlated with decreased protein levels of CMA (Alvarez-Erviti et al., 2013).

Autophagy enhancing agents as a potential therapeutic strategy for PD

Because ALP is an important pathway in α-SYN degradation, the opportunity to use autophagy enhancement as a strategy against α-SYN aggregation in PD has raised considerable interest. Pioneering studies with rapamycin and other macroautophagy enhancing agents have demonstrated an increased α-SYN clearance in several PD models. However, the selectivity of these early autophagy enhancers is limited. Selective targeting of ALP components, like TFEB, lysosomes, and CMA, may provide more potential for development of new therapies for PD. The main findings are listed in **Table 1**, and for more details see the literature (Moors et al., 2017).

The most studied and used macroautophagy-enhancer is rapamycin which inhibits mTORC1 signaling (Bové et al., 2011). Rapamycin has been shown to reduce α-SYN accumulation in wild-type, A30P, or A53T α-SYN expressing PC12 cells and in mice (Crews et al., 2010) and rats (Decressac and Bjorklund, 2013) with overexpressed α-SYN. Rapamycin also improved the motor function in mice with overexpressed A53T α-SYN (Bai et al., 2015). The drawback of mTORC1 inhibition is the interference with numerous other pathways. Prolonged treatment with rapamycin can inhibit mTORC2 and stimulate other cellular pathways, including cell survival mechanisms (Bové et al., 2011). The activation of macroautophagy can be achieved by activating AMPK, leading to downstream inhibition of mTORC1. Several agents which act through this pathway have been described, such as metformin, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and resveratrol (Curry et al., 2018). Metformin, commonly used to treat *Diabetes Mellitus*, showed neuroprotective effects in *in vitro* and *in vivo* models of PD (Ng et al., 2013; Dulovic et al., 2014; Patil et al., 2014). Metformin also decreased phosphorylated levels of α-SYN in SH-SY5Y cells and MPTP-treated mice (Pérez-Revuelta et al., 2014; Katila et al., 2017). Another agent that affects the AMPK signaling is trehalose, which inhibits members of the SLC2A (*GLUT*) family of glucose transporters leading to AMPK-dependent increase of macroautophagy (DeBosch et al., 2016). Trehalose-induced autophagy has shown to increase cell survival and α-SYN clearance in cell lines and in multiple *in vivo* models (Sarkar et al., 2007, 2014; Rodríguez-Navarro et al., 2010; Lan et al., 2012; Tanji et al., 2015; He et al., 2016). However, a recent study did not find improvement in neuronal survival after exposure to α-SYN pre-formed fibrils (Redmann et al., 2017). Although increasing autophagy by AMPK pathway has shown beneficial effects, AMPK is involved in several other cellular functions, and its modulation is likely to induce unwanted effects.

Recently, several other agents acting through an mTOR-dependent pathway have been studied in cell cultures and PD animal models. Sheng et al. (2017) showed that uric acid treatment increased autophagy in PC12 cell in dose- and

TABLE 1 | Commonly used autophagy enhancing agents.

	Target	Agent	PD model	Effect	References
Autophagy	mTORC1	Rapamycin and analogs	α-SYN overexpressed SH-SY5Y	↓Phospho-Ser129 α-SYN levels	Pérez-Revuelta et al., 2014
			Rotenone-exposed SH-SY5Y cells	↓Cell death, ↓Mitochondrial dysfunction	Pan et al., 2009; Xiong et al., 2011; Hou et al., 2015
			6-OHDA and MPTP treated PC12	↓Cell death	Malagelada et al., 2010
			MPTP mice	↓Cell death	Dehay et al., 2010; Malagelada et al., 2010
			αα -SYN transgenic mice	↓Cell death	Crews et al., 2010
			α -SYN transgenic rats	↓Cell death	Decressac et al., 2013
			A53T α-SYN transgenic mice	↓Cell death	Bai et al., 2015
			6-OHDA mice	↓Levodopa-induced dyskinesia	Santini et al., 2009
			6-OHDA rats	↓Levodopa-induced dyskinesia	Decressac and Bjorklund, 2013
	AMPK	Metformin	MPTP mice	↓Cell death, α-SYN levels ↑Neurotrophic factors	Katila et al., 2017
			α-SYN overexpressed SH-SY5Y	↓Phospho-Ser129 α-SYN levels	Pérez-Revuelta et al., 2014
	Beclin-1	PREP inhibitor Isorhynchophylline	A30P α-SYN transgenic mice	↓Oligomeric α-SYN, ↑Striatal DA levels	Savolainen et al., 2014
			N2a cells transfected with WT, A53T and A30P α-SYN	↑α-SYN clearance	Lu et al., 2012
			Embryonic DA neurons	↑α-SYN clearance	Lu et al., 2012
			WT, A30P, and A53T α-SYN expressing PC12 cells	↑α-SYN clearance, ↓α-SYN accumulation	Webb et al., 2003
			α-SYN transgenic mice	↑α-SYN clearance, ↓α-SYN accumulation	Miki et al., 2016
	TFEB	2-HPβCD	Human neuroglioma cells transfected with α-SYN	↑α-SYN clearance	Kilpatrick et al., 2015
	SLC2A	Trehalose	Rotenone-treated rats	↓Cell loss	
			Rotenone-treated PC12 cells	↓Cell loss, ↑α-SYN clearance	Wu et al., 2015
			M MPTP mice	↓Cell loss, ↓Neuroinflammation, ↓Motor deficits	Sarkar et al., 2014
			A53T α-SYN overexpression in rats	↓Cell loss, ↓Motor deficits, ↑α-SYN clearance	He et al., 2016
			WT and A53T α-SYN expressed PC12 cells	↑α-SYN clearance	Sarkar et al., 2007; Lan et al., 2012
			NB69 human neuroblastoma cells	↑α-SYN clearance	Casarejos et al., 2011
			A53T α-SYN overexpressing mice	↑Detergent-insoluble α-SYN clearance	Tanji et al., 2015
Lysosomes	GCase	Ambroxol	GBA1 mutant fibroblasts	↑Lysosomal function, ↑GCase activity ↓Oxidative stress	McNeill et al., 2014; Ambrosi et al., 2015
			Primary cortical neurons	↑GCase activity, ↑TFEB	Magalhaes et al., 2018
			α α-SYN transgenic mice	↑GCase activity, ↓α-SYN levels	Migdalska-Richards et al., 2016

mTORC1, mammalian target of rapamycin complex 1 or mechanistic target of rapamycin complex 1; PREP, prolyl oligopeptidase; 2-HPβCD, 2-hydroxypropyl-β-cyclodextrin; α-SYN, alpha-synuclein; DA, dopaminergic; ROS, reactive oxidative stress; WT, wild-type; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-fenyl-1,2,3,6-tetrahydropyridine; Gcase, glucocerebrosidase; TFEB, transcription factor EB; SLC2A, (GLUT) family of glucose.

time-dependent manners. Moreover, uric acid reduced α-SYN accumulation in PC12 cells overexpressing wild-type or A53T mutant α-SYN. *In vivo*, uric acid modulated autophagy markers increased the autophagosome/autolysosome formation and reduced α-SYN accumulation in the midbrain of SNCA A53T transgenic mice. Suresh et al. (2017) showed that a novel autophagy modulator 6-Bio alleviated α-SYN toxicity. In yeast and mammalian cell lines, 6-Bio induced autophagy and enhanced autolysosome formation which resulted in α-SYN degradation and clearance. *In vivo* studies with a MPTP mouse model demonstrated that 6-Bio has a neuroprotective activity, enhances autophagy and clearance of toxic protein aggregates and ameliorates MPTP-induced behavioral deficits. The results demonstrated that 6-Bio modulates autophagy in a GSK3B-dependent manner and the induction of autophagy in mammalian cells appears to be mTOR dependent.

Instead of general activation of macroautophagy, targeting selective ALP components including Beclin-1, TFEB, and lysosomes have been tried. Activation of Beclin-1 induces autophagosome formation and initiation of autophagy. Overexpression of Beclin-1 has been shown to reduce accumulation of α-SYN in PC12 cells and mice with overexpressed α-SYN (Spencer et al., 2009; Wang et al., 2016). In addition, the drug-induced activation of Beclin-1 has been demonstrated to increase autophagy and promotes α-SYN clearance in neuronal cell lines and PD animal models (Lu et al.,

2012; Savolainen et al., 2014). One attractive target to stimulate macroautophagy downstream of mTORC1 is modulating transcriptional levels of TFEB. TFEB regulates macroautophagy and lysosomes and acts as a link between upstream signaling pathways (Settembre et al., 2011). Overexpression of TFEB eliminated α-SYN oligomers and rescued midbrain DA neurons from α-SYN toxicity in overexpressing rats (Decressac and Bjorklund, 2013). Another strategy for stimulating ALP in PD is a direct modulation of lysosomes. The potential of targeting the lysosome system has been demonstrated with acidic nanoparticles which were able to stimulate lysosomal degradation and revert the lysosomal dysfunction in genetic PD models (Baltazar et al., 2012; Bourdenx et al., 2016). Ambroxol, AT2101 (isofagomine) and histone deacetylase inhibitors can correct the folding of GCase and therefore increase the GCase and lysosome function (Blanz and Saftig, 2016). The small-molecule chaperones have been demonstrated to enhance GCase activity, improve lysosomal function and enhance α-SYN clearance in preclinical models of PD (Steet et al., 2006; Khanna et al., 2010; Sun et al., 2012; Yang et al., 2013; McNeill et al., 2014; Richter et al., 2014; Ambrosi et al., 2015). Especially ambroxol is widely studied presently. In mice overexpressing α-SYN or heterozygous L444P mutation in *CBA1*, ambroxol treatment increased the GCase activity while decreasing phosphorylated and endogenous levels of α-SYN (Migdalska-Richards et al., 2016, 2017b). In non-human primates, ambroxol increased brain GCase activity (Migdalska-Richards et al., 2017a). In patients with Gaucher disease, ambroxol was able to cross the blood–brain barrier and high-dose oral administration was safe and well-tolerated (Narita et al., 2016).

Currently, ambroxol is in phase II clinical trials tested for treatment of PD and PD with dementia (ClinicalTrials.gov Identifier: NCT02941822 and NCT02914366, respectively) (Silveira et al., 2019).

Downstream targeting of the CMA components presents an alternative approach to develop new strategies for PD. Induced overexpression of LAMP2A in human SH-SY5Y cells, rat primary cortical neurons *in vitro* and nigral DA neurons *in vivo* decreased α-SYN accumulation and protected α-SYN-induced DA degeneration (Xilouri et al., 2013). Retinoic acid alpha receptors have been identified as CMA inhibitors, and synthetic derivatives of all-*trans*-retinoic acid were developed to reverse this effect (Anguiano et al., 2013). These derivates specifically stimulated CMA and LAMP2A was identified as one of the targets.

ROLE OF ER STRESS IN PD AS A RESULT OF DYSFUNCTIONAL CELLULAR PROTEOSTASIS

Endoplasmic reticulum is the first component of the secretory pathway mainly responsible for protein synthesis, post-translational processing and folding of newly synthesized proteins. The proteins are then transported to their final destinations in membrane-bound vesicles. Disturbance in any of these functions including proper folding capacity and disposal of misfolded proteins leads to ER stress and activation of intracellular signal transduction pathway that is essentially intended to re-establish ER homeostasis. These biological processes are collectively called the UPR. Inability to restore ER functions induces cell death via apoptosis. Growing evidence from studies in human PD post-mortem brain, additionally to genetic and neurotoxin models, suggests that ER stress is a common feature in PD that contributes to PD pathology. Recently, the generation of neuronal cultures from iPSCs derived from PD patients indicated that ER stress leads to the accumulation of ER-associated degradation (ERAD) substrates and placed this ER dysfunction as an early component of PD pathogenesis (Chung et al., 2013; Heman-Ackah et al., 2017).

Causes of ER Stress in PD

The ER is crucial for protein folding, trafficking to the Golgi, UPR, and calcium buffering. The imbalance between the load on ER functions and ER capacity leads to ER stress. In PD, the mechanisms leading to ER stress and the actual role of the UPR in degeneration of the DA neurons are the object of intensive research. Oligomeric α-SYN has been shown to accumulate in the ER in animal models and PD patient brains (Colla et al., 2012). The aggregation of α-SYN induces ER stress, which eventually results in inflammation and neurodegeneration.

A number of studies have shown that α-SYN affects Rab1, a protein involved in trafficking components from the ER to the Golgi. Over-expression of Rab1 in animal models of PD reduced stress levels and protected DA neurons against degeneration (Coune et al., 2012). Further, α-SYN directly interacts with nascent activating transcription factor 6 (ATF6), effectively preventing its association with COPII vesicles that generally transfer proteins to the Golgi. This has specific implications: (1) interfering with the Rab1 protein could lead to accumulation of unfolded proteins in the ER, and (2) inhibition of ATF6 would generally stop the ERAD, triggering the cell to signal apoptosis (Credle et al., 2015). Other studies are linking PD genes with alteration of the secretory pathway, including LRRK2, Parkin, DJ-1, ATP13A2 (Mercado et al., 2013), and VPS35 (Zimprich et al., 2011), which may result in pathological levels of ER stress contributing to the etiology of the disease. Furthermore, increase in cytoplasmic Ca^{2+} levels induced by 6-hydroxydopamine (6-OHDA) (a toxin capable to generate, *in vitro* and *in vivo*, some features of PD-associated neurodegeneration) was detected in 6-OHDA-treated rats. Pretreatment with ryanodine or ER stress inhibitor 4-PBA inhibited RyR receptor Ca^{2+} channels and protective midbrain DA neurons from degeneration (Huang et al., 2017).

UPR Response in PD

The primary function of the UPR is a maintenance of ER protein homeostasis. When cells undergo constant ER stress, the UPR is responsible for the elimination of damaged cells through apoptotic mechanisms, some of which appear to be specific to ER stress and others that are included in general apoptotic pathways (Xu et al., 2005). The activation of UPR depends on three ER stress sensors proteins, protein kinase RNA-like endoplasmic reticulum kinase (PERK) receptor, inositol-requiring enzyme 1

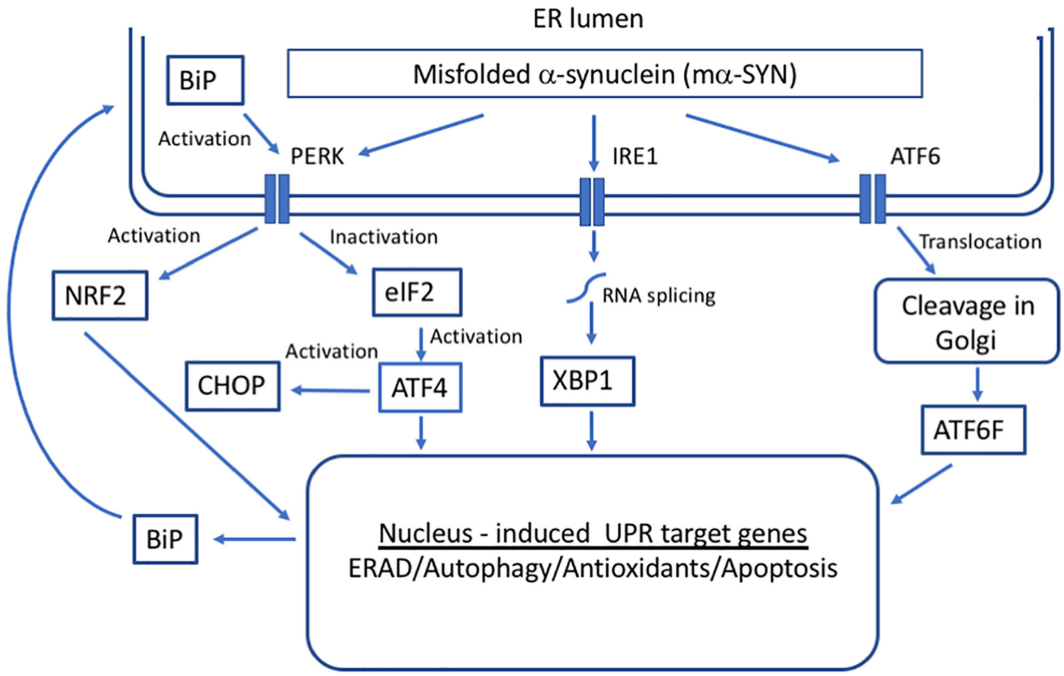

FIGURE 2 | Unfolded protein response (UPR) in response to alpha-synuclein misfolding. Three transmembrane proteins have been identified as sensors of unfolded proteins in the ER in mammalian cells: IRE1 (inositol-requiring protein 1), ATF6 (activating transcription factor 6), and PERK (protein kinase RNA-like ER kinase). Upon the accumulation of mα-SYN, BiP dissociates from UPR sensors inducing their activation that leads to the transcription of genes whose protein products increase the folding capacity of the cell.

(IRE1), and ATF6 (Schröder and Kaufman, 2005) (**Figure 2**). Under normal physiological conditions, all three effectors are negatively regulated by the ER chaperone glucose-regulated protein 78/ binding immunoglobulin protein (GRP78/BiP), which suppresses their activity by binding to their luminal ends (Bertolotti et al., 2000).

Under conditions of ER stress and increase in unfolded proteins, BiP dissociates from UPR sensors inducing their activation. Activation of the ER pathways helps to fight the cellular stress through the combined actions of suppressing the translation of new proteins, inducing ER chaperones that promote protein refolding and activating the proteasome to degrade misfolded/unfolded proteins. There is direct evidence that GRP78/BiP levels are increased in cell as well as animal models of PD forming a complex with α-SYN (Bellucci et al., 2011). Moreover, it has been demonstrated that aging leads to a significant decline in GRP78/BiP expression (up to 40%; Naidoo, 2009). However, under chronic ER stress, UPR sensors shift their signaling toward induction of cell death by apoptosis (Urra et al., 2013).

PERK Signaling

PERK is a type I ER transmembrane protein kinase with a luminal domain and a cytoplasmic domain that has kinase activity (Liu et al., 2002). Upon ER stress, BiP releases the luminal domain of PERK, which then dimerizes and autophosphorylates to become active (Harding et al., 1999). Following trans-autophosphorylation, this kinase phosphorylates the alpha subunit of eukaryotic initiation factor-2 (eIF2), inactivating it by Ser-51 phosphorylation and attenuating protein translation. This inhibitory effect of translation helps to alleviate ER stress by decreasing the overload of misfolded proteins and thereby protecting the cells under conditions where proteins cannot achieve proper folding (Fels and Koumenis, 2006). This event leads to activating transcription factor 4 (ATF4). The UPR-related transcriptional factor ATF4 upregulates a subset of genes that control oxidative stress, metabolism, protein folding, and glutathione biosynthesis (Harding et al., 2000). Important targets of ATF4 include *NRF2*, which regulates the functions of a variety of antioxidant genes (He et al., 2001), and *CHOP*, which conversely is a key in the activation of apoptotic pathways and cell death (Han et al., 2013). In PD patients, the activation of PERK/ATF4 signaling is observed in different brain areas. α-SYN has been shown to accumulate within the ER of nigral DA cells, directly activating the PERK/eIF2α signaling and increasing the expression of ATF4 (Bellucci et al., 2011). The activation of this pathway overlapped with pro-apoptotic changes.

Further evidence supporting this pathway comes from PD-associated gene studies. By inducing the A53T α-SYN mutation to PC12 cells, UPR activates CHOP and GRP78/BiP by increasing their expression and increases the phosphorylation of eIF2α (Smith et al., 2005). Interventions to block ER stress and caspase activity using inhibitors, and to knock down the expression of caspase-12 using siRNA, protected against A53T α-SYN -induced cell death (Smith et al., 2005). In PINK1- and Parkin-associated PD models, mitofusins cause enhanced ER stress

signaling, by interconnecting damaged mitochondria to the ER membranes (Celardo et al., 2016). PERK signaling inhibition, either pharmacological or genetic suppression, was beneficial in these experimental models of PD (Celardo et al., 2016). LRRK2 mutations also cause familial PD by accumulation and aggregation of α-SYN and ubiquitinated proteins over time mainly due to the impairment of protein degradation pathways (Tong et al., 2010). This is likely to result in the build-up of unfolded proteins leading to ER stress, although there is little evidence of UPR activation yet. On the other hand, studies with *GBA1* gene mutations revealed that treatment with chemical chaperones (ambroxol and isofagomine) can combat *GBA*-mediated ER stress by increasing *GBA* levels and activity in fly models and in fibroblasts from PD patients (Sanchez-Martinez et al., 2016). In rodent models of PD, *ATF4* upregulation in DA neurons of SN resulted in severe nigrostriatal degeneration caused by activating caspase 3/7-dependent pathway (Gully et al., 2016). On the other hand, Grp78/BiP over-expression exerted neuroprotective effects in a rat model of PD (Gorbatyuk et al., 2012).

IRE1 Signaling

IRE1 is a type I ER transmembrane sensor and cell fate executor. IRE1 gets activated in response to the accumulation of misfolded proteins by autophosphorylation. The activation induces RNase activity that is consequently needed for unconventional splicing of X-box binding protein 1 (XBP1) (Calfon et al., 2002). Spliced XBP1 translocates to the nucleus where it commands transcription of genes responsible for quality control, protein folding, lipid synthesis and ERAD pathway (Sriburi et al., 2004). In PD, XBP1 controls the survival of DA neurons (Valdés et al., 2014). The developmental ablation of XBP1 preconditions DA neurons against the effect of the neurotoxin, 6-OHDA (Mollereau et al., 2014, 2016). The effect is specific to SNpc, as it is not seen in other brain regions. Contrary, reduction of XBP1 in DA neurons of adult mice caused ER stress with CHOP induction leading to degeneration (Valdés et al., 2014), highlighting the critical role of XBP1 depending on the development stage. In addition, a gene therapy approach using adeno-associated viral vectors to deliver XBP1 active form to the SNpc confers protection of DA neurons against 6-OHDA-mediated toxicity (Valdés et al., 2014). Moreover, XBP1 transgene delivered to mouse striatum using recombinant adenoviral vectors protected DA neurons against MPTP-induced degeneration (Sado et al., 2009). XBP1 is also protective when it is delivered in neural stem cells transfected with this transcription factor resulting in increased survival and improved behavior in a rotenone-induced rat model of PD (Si et al., 2012). Among other functions, XBP1 and ATF6 mediate the transcription of BiP. While the overexpression of this chaperone protects DA neurons and increases motor performance in a rat model of PD, the age-related decline in BiP expression as well as siRNA-mediated downregulation, increases DA neuron vulnerability to α-SYN in the same PD model (Salganik et al., 2015).

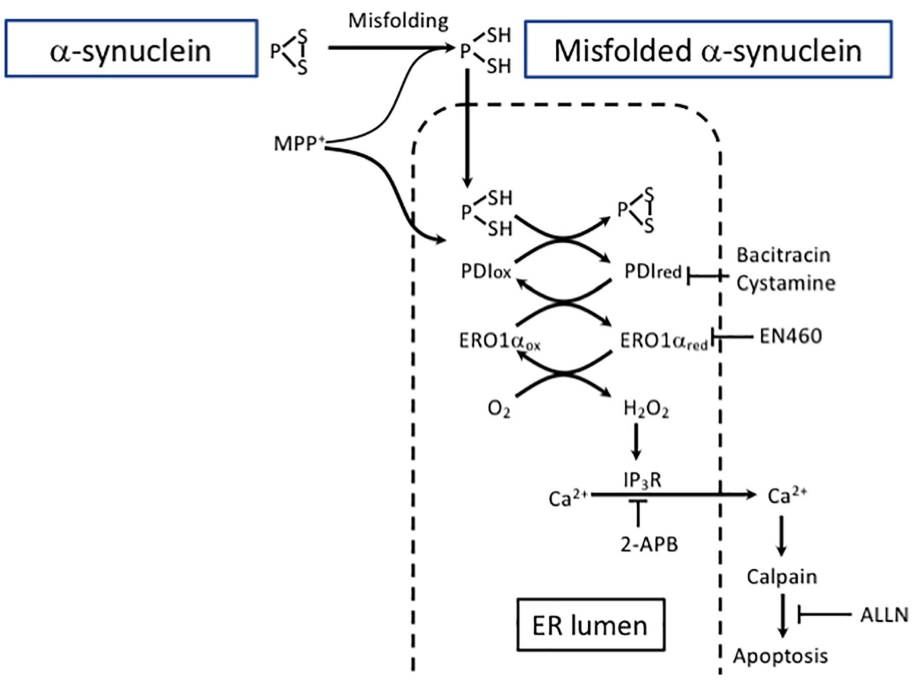

FIGURE 3 | Excessive protein refolding in ER leads to oxidative stress and apoptosis. Depending on the structure, aggregates can be degraded either by macroautophagy or CMA. Alternatively, misfolded α-SYN undergoes refolding in the ER. However, excessive refolding upregulates PDI reduction. Re-oxidation of PDI is linked with an increase in hydrogen peroxide generation causing dysregulation of IP3R permeability and an increase in cytosolic calcium. Calcium release from the ER may activate calpain and eventually lead to apoptosis. The pharmacological inhibition of PDI by bacitracin or cystamine prevents ER redox imbalance and downstream proapoptotic events. The inhibition of the ERO1 catalyzed re-oxidation of PDI by EN460 results in a protective effect similar to PDI inhibitor.

ATF6 Signaling

ATF6 is a type II ER transmembrane protein. Upon the accumulation of misfolded proteins in the ER, ATF6 moves to the Golgi apparatus where it is cleaved by two proteases (Haze et al., 1999). The cytosolic domain of ATF6 is translocated to the nucleus where it activates the transcription of ER chaperones (Gotoh et al., 2002). Contrarily, reduced levels of ATF6 in the nucleus of cells due to the impairment in ATF6 trafficking to the Golgi are likely to trigger apoptosis (Credle et al., 2015). In PD, α-SYN directly targets ATF6 and inhibits ATF6 processing leading to an impaired up-regulation of ERAD genes, which sensitizes cells to apoptosis (Credle et al., 2015). In ATF6α knockout animals, the accumulation of ubiquitin-positive inclusions and enhanced loss of DA neurons induced by MPTP, a PD-triggering neurotoxin, was detected (Egawa et al., 2011). This suggests that activation of the UPR has an important adaptive function to maintain protein homeostasis in this model. ATF6 mainly controls the levels of BiP and ERAD elements rather than development and survival of DA neurons in mice under resting conditions (Egawa et al., 2011).

SUPPRESSION OF EXCESSIVE PROTEIN OXIDATIVE FOLDING AS AN ALTERNATIVE SOLUTION FOR LOWERING ER STRESS

As indicated above, ER stress is increasingly implicated in PD, and emerging evidence highlights the complexity of the UPR, with both protective and detrimental components being described. Mild insults increase the activity of chaperones, such as protein disulfide isomerase (PDI) that is responsible for the oxidative folding through formation of disulfide bonds in proteins (Rao and Bredesen, 2004). To promote correct disulfide bond formation in unfolded/misfolded proteins, the redox environment in the ER is oxidatively maintained (Hwang et al., 1992). In neurons, the increased activity of PDI represents an adaptive response that is induced to protect the cells (Haynes et al., 2004). In contrast, recent studies have revealed alternative roles for PDI in neurodegenerative diseases (Hoffstrom et al., 2010; Lehtonen et al., 2016).

In PD, ER homeostasis is disrupted in DA neurons in SNpc and PDI co-localizes with α-SYN in LBs (Conn et al., 2004). We have recently demonstrated that treatment with 1-methyl-4-phenylpyridinium (MPP+), a neurotoxin associated with PD, upregulated the expression of α-SYN and PDI in human neuroblastoma SH-SY5Y cells and that α-SYN co-localized with PDI. The α-SYN accumulation not only activated PDI but resulted in the accumulation of a reduced form of PDI due to an increasingly reduced ER redox environment (**Figure 3**).

Protein disulfide isomerase inhibitors bacitracin and cystamine prevented the accumulation of α-SYN and MPP+-induced reductive shift in the ER by hindering PDI excessive reduction (Lehtonen et al., 2016). Moreover, the data suggest that redox misbalance and hydrogen peroxide production due to PDI re-oxidation in the ER are the outcome of a severe toxic insult caused by α-SYN accumulation. Calpain is a Ca^{2+} -sensitive non-lysosomal protease reported to be disruptive in SN of PD patients as well as in experimental PD models (Crocker et al., 2003). In the model described by Lehtonen et al. (2016), the release of Ca^{2+} was succesfully blocked not only by 2-aminoethoxydiphenyl borate (2-APB), an inositiol-3-phosphate receptor (IP3R) inhibitor, but also by bacitracin, a PDI inhibitor, and it promoted neuroblastoma cell survival. Additionally, ALLN (*N*-acetyl-leu-leu-norleual, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal), a calpain I inhibitor, protected these cells from MPP+ toxicity. Overall, these results are in line with a previously published study using a PC12 cell model of Huntington's disease showing the potential of PDI inhibitors to suppress the cells' death induced by misfolded proteins (Hoffstrom et al., 2010). Importantly, a beneficial effect of PDI inhibition is coupled with consecutive enhancement of autophagy that is turned on to support cell survival. Furthermore, PDI inhibition also protected against MPP+ -induced DA neurodegeneration in *Caenorhabditis elegans*.

Collectivelly, excessive protein refolding taking place in the ER leads to an increase in the reduced form of PDI and to the activation of the PDI-Ero1 cycle, causing overproduction of hydrogen peroxide and promoting generation of ROS. These events lead to the dysregulation of IP3R that causes an increase in cytosolic calcium followed by calpain activation-induced apoptosis. In contrast, PDI inhibition prevents ER redox imbalance and enhances the autophagic clearance pathway. Therefore, when considering therapeutic approaches, it is necessary to take into account the balance between ER-linked refolding or/and alternative protein clearance by autophagy.

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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REVIEW
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Linking the Endoplasmic Reticulum to Parkinson's Disease and Alpha-Synucleinopathy

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Accumulation of misfolded proteins is a central paradigm in neurodegeneration. Because of the key role of the endoplasmic reticulum (ER) in regulating protein homeostasis, in the last decade multiple reports implicated this organelle in the progression of Parkinson's Disease (PD) and other neurodegenerative illnesses. In PD, dopaminergic neuron loss or more broadly neurodegeneration has been improved by overexpression of genes involved in the ER stress response. In addition, toxic alpha-synuclein (αS), the main constituent of proteinaceous aggregates found in tissue samples of PD patients, has been shown to cause ER stress by altering intracellular protein traffic, synaptic vesicles transport, and Ca^{2+} homeostasis. In this review, we will be summarizing evidence correlating impaired ER functionality to PD pathogenesis, focusing our attention on how toxic, aggregated αS can promote ER stress and cell death.

Keywords: alpha-synuclein, ER stress, UPR, misfolded proteins, Parkinson's disease, alpha-synucleinopathy, alpha-synuclein aggregates

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INTRODUCTION

Misfolded proteins are a common cellular abnormality that is shared among neurodegenerative diseases. Parkinson's Disease (PD), a multifactorial neurodegenerative disorder that affects the motor, cognitive and peripheral system, is characterized by the accumulation of misfolded, aggregated alpha-synuclein (αS) fibrils into proteinaceous intracellular inclusions in neuronal soma or neurites, named Lewy bodies (LB) or Lewy neurites (LN) (Goedert et al., 2013). The aggregation process of αS is a nucleation-type of reaction where αS monomer converts into a β-sheet conformation that elongates into filamentous structures called protofibrils and fibrils, becoming progressively insoluble (Lashuel et al., 2002; Cremades et al., 2012; Tuttle et al., 2016). The presence of αS inclusions is associated with neuronal damage and has been found in other types of neurodegenerative diseases besides PD that are collectively referred as α-synucleinpathies. In PD one of the most affected neuronal populations, but surely not the only one, is represented by the dopaminergic neurons of the substantia nigra pars compacta. Here widespread dopaminergic neuronal death causes depletion of striatal dopamine, whose reduction is responsible for motor and cognitive dysfunction experienced by PD patients. Since its discovery in 1997, many observations have pointed to the aggregation of αS as one of the culprits of neuronal demise (as examples Feany and Bender, 2000; Masliah, 2000; Lee et al., 2002; Lakso et al., 2003). In addition, the prion-like ability of the protein to spread and propagate its toxic template has shown how αS pathology can disseminate between different anatomically connected areas, from the peripheral nervous system to the brain (Braak et al., 2003; Luk et al., 2012; Masuda-Suzukake et al., 2013; Rey et al., 2013; Holmqvist et al., 2014; Recasens et al., 2014; Sacino et al., 2014). At a neuronal level, αS toxicity has been associated with impairment in numerous cellular functional aspects, including mitochondrial, proteasomal and lysosomal abnormalities, axonal transport

deficits and alteration in synaptic transmission (Bendor et al., 2013). More recent evidence has emerged supporting the endoplasmic reticulum (ER) stress, a condition of altered ER functionality, as a mediator of αS toxicity. In this review, we will summarize the link between PD and ER stress, focusing our attention on how pathological αS impairs ER functionality, induces ER stress and ultimately contributes to neuronal death.

ER STRESS AND UNFOLDED PROTEIN RESPONSE

Folding of secreted and transmembrane proteins is one of the main functions of the ER. Membrane and extracellularly targeted proteins are translated on ribosomes localized on the cytosolic surface of the rough ER and promptly inserted into the ER membrane or lumen (Görlach et al., 2006). In the ER, proteins achieve a specific folded conformation, acquire post-translational modifications such as glycosylation and formation of disulfide bonds, and are selectively targeted for secretion or destined for the plasma membrane or other cellular compartments. The ER is also responsible for biosynthesis of lipids and steroid hormones and is a primary site for Ca^{2+} storage. Proteins that failed to fold properly are retro-translocated into the cytosol by the ER-associated degradation (ERAD) pathway and degraded by the proteasomes (Smith et al., 2011). To sustain extensive protein folding capability, cells must promptly maintain an adequate level of ER folding machinery and ERAD proteins. Because of the high concentration of proteins in the ER (estimated at 100 mg/mL), the ER quality control is a fundamental mechanism that maintains and preserves cell metabolism and normal functions. Perturbations of this balance lead to accumulation of aberrant proteins in the ER, a condition called ER stress, that if left unchecked, can have deleterious consequences and can lead to the collapse of the whole secretory pathway and cellular homeostasis. In addition to defects in the protein folding machinery, other conditions culminating directly or indirectly in the accumulation of misfolded proteins (including starvation, infections, changes in ER Ca^{2+} concentration and dysregulation in the redox potential of the ER) are able to trigger ER stress. Because of this fundamental role in protein homeostasis, eukaryotic organisms have developed a concerted and coordinated multi-signaling pathway, named unfolded protein response (UPR), that aims to restore ER functionality through the increase of cellular folding capacity and the transient reduction of the flux of proteins entering the ER (Walter and Ron, 2011). To achieve such a status, a massive transcriptional upregulation of ER chaperons, foldases, glycosylases, ERAD proteins, lipid biosynthesis to facilitate ER membrane expansion and, at the same time, degradation of selective mRNA messengers with attenuation of general protein translation, must be well coordinated in order to protect cells from ER stress and recover ER protein quality control (Harding et al., 1999; Hollien and Weissman, 2006; Hollien et al., 2009). However, when the ER stress is too severe and there is a persistent build-up of misfolded proteins that cannot be efficiently eliminated, the UPR can become cytotoxic and can directly initiate programmed cell death through both caspase-dependent and independent pathways (Lin et al., 2007).

In eukaryotes, the UPR is highly conserved and comprises three parallel branches, each of them initiated by a specific ER stress sensor. Such sensor is represented by an ER resident protein, which is sensitive to ER perturbations and signals this information to the cytosol and the nucleus. There are three ER stress sensors: (1) the inositol-requiring enzyme 1 (IRE1); (2) the double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK); (3) the activating transcription factor-6 (ATF6) (**Figure 1**).

PERK is an ER-resident type I transmembrane protein with a cytosolic kinase domain. Upon ER stress, PERK phosphorylates the α subunit of the eukaryotic translational initiation factor 2α (eIF2α) at residue Ser51 (Shi et al., 1998). Phosphorylation inactivates eIF2, disrupting the formation of GTP·eIF2α·Met-tRNAi ternary complex required for mRNA translation leading to a reduction in general protein synthesis and consequently to a decrease of the protein influx into the ER lumen (Harding et al., 2000a; Scheuner et al., 2001). However, in conditions of limited availability of eIF2, specific mRNAs, that contain inhibitory upstream open reading frame sequences in their 5′-untranslated region, are preferentially translated (a process called attenuation). One of these transcripts encodes for the activating transcription factor 4 (ATF4), that selectively upregulates transcription of genes involved in restoring ER functionality such as enzymes for amino acid biosynthesis and transport, protein folding and anti-oxidant response (Vattem and Wek, 2004; Starck et al., 2016). Within ATF4′s known targets is CHOP, a C/EBP homologous transcription factor that controls the upregulation of components involved in apoptosis (Harding et al., 2000a; Ma et al., 2002). Additionally, CHOP binds and promotes transcription of growth arrest and DNA damage-inducible protein 34 (GADD34), an inducible regulatory subunit of the protein phosphatase PP1C. PP1C dephosphorylates eIF2α, providing a feedback mechanism for tightly regulating the phosphorylation status of eIF2α and, in turn, for controlling inhibition of protein synthesis (Connor et al., 2001; Novoa et al., 2001). Another target of PERK kinase activity is NRF2, a transcription factor that induces the translation of antioxidant proteins and detoxifying enzymes (Cullinan and Diehl, 2004, 2006; Marciniak, 2004).

Similar to PERK, IRE1 is a bifunctional ER type I transmembrane protein, highly conserved through evolution and with a carboxy-terminal cytoplasmic kinase and RNase domains (Wang et al., 1998). Mammalian IRE1 has two homologs, IRE1α, an ubiquitous protein and IRE1β which is expressed specifically in the gastrointestinal and respiratory tracts (Bertolotti et al., 2001; Tsuru et al., 2013). In the presence of ER stress, both IRE1 homologs form homo-oligomers through the association of their ER luminal domain. The oligomer then cleaves a 26-base intron from the mRNA encoding the X-box binding protein-1 (XBP1) (Yoshida et al., 2001). Spliced Xbp1, sXBP1, activates downstream a wide set of genes encoding proteins involved in ER membrane biogenesis, protein folding and ERAD (Lee et al., 2003; Acosta-Alvear et al., 2007). In addition to XBP1 cleavage, IRE1 is also implicated in the degradation of specific mRNAs of membrane and secreted proteins, 28S ribosomal RNA and microRNAs as a part of a Regulated IRE1-Dependent Decay

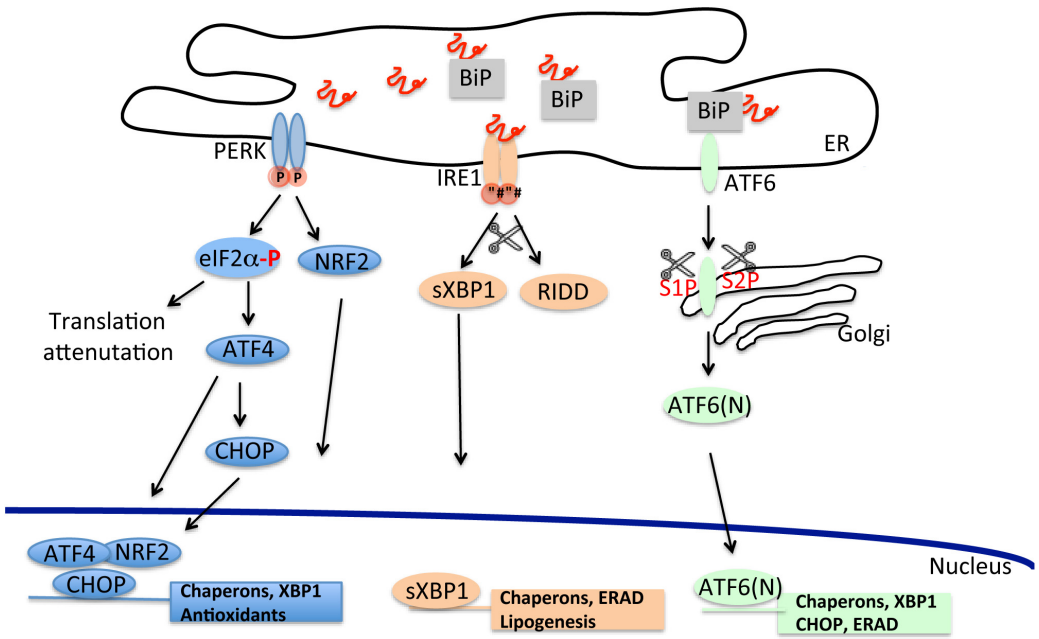

FIGURE 1 | The unfolded protein response cascade. Stressful conditions due to starvation, infections, oxidative damage, and changes in ER Ca^{2+} concentration can lead to accumulation of misfolded proteins in the ER. Induction of the unfolded proteins response through the activation of its three independent arms (PERK, IRE1, and ATF6) counteracts the build-up of misfolded proteins and improves the ER folding capacity.

(RIDD) pathway (Hollien and Weissman, 2006). RIDD may indirectly contribute to the reduction of protein influx in the ER. However, exhaustive RIDD activity, such as in conditions of chronic ER stress, can indiscreetly degrade messengers of protein involved in protein folding, exacerbating the overload of misfolded polypeptides and indirectly contributing to induction of cell death (Han et al., 2009; Upton et al., 2012).

Unlike PERK and IRE1, ATF6 is an ER-associated type 2 transmembrane protein with (carboxy-terminal luminal domain stress-sensing) a basic leucine zipper domain functioning as transcription factor (Haze et al., 1999). In unstressed conditions, ATF6 is an oligomer that upon activation dissociates into a monomeric form and translocates in the Golgi where it is sequentially cleaved by serine protease site-1 (S1P) and metalloprotease site-2 (S2P) to release an active cytosolic form, ATF6 (N) (Schindler and Schekman, 2009). After migration in the nucleus, ATF6 (N) binds to ER stress response element (ERSE) and activates the transcription of genes involved in ERAD, ER homeostasis and folding machinery such as the ER chaperons BiP/grp78 and Grp94 (Yoshida et al., 2000). Cross-talk between the different UPR pathways has been shown for sXBP1 mRNA whose expression can be unregulated also by PERK and ATF6 (Yoshida et al., 2001; Tsuru et al., 2016) and in the case of CHOP, whose expression appears to be stimulated also by ATF6 (N) (Yoshida et al., 2000; Tsuru et al., 2016).

How can PERK, IRE1, and ATF6 sense misfolded proteins? It is thought that each UPR sensor is maintained in an inactive or quiescent state through binding with the ER chaperon BiP/grp78 (Bertolotti et al., 2000). BiP/grp78 is part of the ER translocon pore and is involved in numerous ER-related functions, such as translocation of nascent polypeptides, protein folding, targeting of misfolded proteins to ERAD machinery and ER calcium homeostasis (Hendershot, 2004). During ER stress BiP/grp78 senses and binds misfolded proteins, dissociating from the binding to the luminal domain of UPR sensors, with concomitant activation of these signaling proteins and initiation of the three different UPR cascades. More recent evidence has suggested that IRE1 can directly sense and bind misfolded polypeptides, without the mediation of BiP/grp78, becoming activated and inducing UPR (Credle et al., 2005; Kimata et al., 2007; Gardner and Walter, 2011). It is not clear whether a similar mechanism applies also to PERK or ATF6 although selective deletion of BiP/grp78 binding site on ATF6 does not result in the constitutive activation of ATF6-dependent branch of the UPR in unstressed conditions (Shen et al., 2005).

Besides its protective function, the UPR has been recently implicated in memory and synaptic plasticity as it has been shown that the PERK-eIF2α branch or XBP1 can regulate gene expression of proteins implicated in long-term potentiation, memory formation and synapsis remodeling (Trinh and Klann, 2013; Martínez et al., 2016). Thus it appears that the UPR is not only a rescue mechanism in case of stressful conditions in the ER but also a way to modulate normal cellular function and homeostasis.

ER Stress-Induced Apoptosis

When the initial cellular response fails to restore ER homeostasis and misfolded proteins overload cannot be efficiently removed, the UPR switches from an adaptive response to induce cell death. Although the mechanism and key players have not been entirely identified, it appears that the same UPR branches involved in

the initial prosurvival response have the capacity to induce apoptosis in the case of severe ER stress. Activation of CHOP by PERK/ATF4 or ATF6 or XBP1 (Harding et al., 2000a,b; Scheuner et al., 2001) appears to be central for the induction of ER stress-driven apoptotic signal. Pro-apoptotic activity of CHOP is mediated by both the upregulation of Bim, a protein that belongs to the BH3-only family (Puthalakath et al., 2007) and by the suppression of the anti-apoptotic protein Bcl-2. The BH-3 only family is comprised of proteins able to induce formation of the mitochondrial pore and, consequently, to induce the release of cytochrome c. Instead, the Bcl-2 protein family inhibits the formation of the mitochondrial outer membrane pore. For CHOP to activate the apoptotic cascade, both factors, Bim and Bcl-2, have to be regulated, although in the opposite direction. Release of cytochrome c then activates caspase-9 in the apoptosome with consecutive cleavage of caspase 3 and initiation of the apoptotic process. More recently, CHOP activity has been proposed to be mediated by PUMA, a p53-upregulated modulator of apoptosis (Galehdar et al., 2010; Ghosh et al., 2012).

In addition to CHOP, upon extensive ER stress, IRE1 can also promote apoptosis through binding to the TNF-α receptor-associated factor 2 (TRAF2) and stimulation of apoptosis signal-regulating kinase-1 (ASK1) (Nishitoh et al., 2002). ASK1 in turn, activates JNK that phosphorylates and inhibits antiapoptotic factors Bcl-2 and Bcl-XL. Interestingly, Bax and Bak, which belong to the BH-3 only family, can modulate directly IRE1 activity by relocating to the ER membrane under ER stress conditions (Zong et al., 2003; Hetz et al., 2006; Klee et al., 2009). Moreover, cells lacking Bax and Bak are resistant to apoptosis after treatment with different ER stress stimuli (Scorrano et al., 2003; Buytaert et al., 2006). Thus, exhaustive ER stress can induce cell death through a tight and well controlled cross-talk with the mitochondria.

However, besides induction of apoptosis through the mitochondria, other pathways have been proposed to take part in cellular demise upon UPR activation. Initial observations had suggested how the initiation of the UPR-dependent cell death cascade could be mediated directly by the ER through activation of the ER-resident caspase, caspase 12 (Nakagawa et al., 2000; Yoneda et al., 2001). However, inhibition of caspase 12 expression in MEF cells does not make cells more vulnerable to ER stressors indicating that caspase 12 is not specifically activated in conditions of ER stress (Obeng and Boise, 2005; Saleh et al., 2006). Also caspase 12 in humans does not appear to be functional whereas the pro-inflammatory caspase 4 seems to now be a more suitable candidate in mediating the ER stress-induced apoptosis (Lu et al., 2014). Nevertheless, the PERK–ATF4–CHOP pathway has been shown to promote ER stress-dependent apoptosis bypassing the mitochondria by recruiting cell death receptors such as TRAIL-R1/DR4 and TRAIL-R2/DR5 and their death ligands (Martín-Pérez et al., 2012; Li et al., 2015). Here, activation of such receptors leads to cleavage of pro-caspase 8 that, in turn, can selectively cleave caspase 3. Moreover, activation of the same PERK branch can also lead to autophagy, where transcriptional upregulation of autophagy-related genes such as Atg5, Atg3, Atg7, Atg10, Atg12, Atg16l1, Becn1, p62, and Nbr, is downstream of ATF4 expression (B'chir et al., 2013). In summary, in case of chronic ER dysfunction, it appears that multiple signal pathways involving the ER, mitochondria and the cytosol can contribute to the ER stress-induced cell death.

ER Stress and Inflammation

The UPR is also actively involved in inducing inflammation through the stimulation of the transcriptional activity of NF-κB and JNK, key mediators of the proinflammatory response. Bacterial infections can induce all three branches of the UPR and activation of the UPR is necessary for production of proinflammatory cytokines (Smith et al., 2013). Stimulation of Toll-like receptors (TLRs), innate immune receptors known to sense pathogen invasion, such as TLR4 and TLR2, specifically activate the IRE-1/XBP-1 branch for production of cytokines such as IL-1β, IL-6, TNF-α and interferon in macrophages (Martinon et al., 2010; Shenderov et al., 2014). TLR4 signaling appears mediated by MyD88 and TRAF6 that interact and activate IRE-1 through ubiquitination and by blocking its inactivation by PP2A phosphatase activity (Qiu et al., 2013). More recently the activity of two other immune receptors, NOD1 and NOD2, known to sense bacterial cell wall molecules, was shown to be mediated by IRE1α activation after *Brucella abortus* or *Chlamydia muridarum* infections (Keestra-Gounder et al., 2016). In addition to IRE1, also the PERK/eIF2/CHOP pathway can mediate TLR4 signaling during inflammation (Afrazi et al., 2014). In conditions of ER stress, attenuation of global mRNA translation, mediated by the PERK/eIF2α phosphorylation, reduces the protein level of IκB, an inhibitory protein that sequesters NF-κB in a quiescent state through binding. Without IκB, NF-κB can migrate into the nucleus and can transcriptionally activate the upregulation of proinflammatory genes (Deng et al., 2004). In addition to PERK, IRE-1α can also stimulate NF-κB activity, through the recruitment of TRAF2 and consequent binding and activation of IκB kinase (IKK) (Hu et al., 2006). Phosphorylation of IκB by IKK signals selective degradation of IκB through the proteasome and promotes activation of NF-κB. BesidesNF-κB, the IRE-1α-TRAF2 complex can also induce inflammation by direct recruitment and activation of the JNK signaling and consecutive recruitment of AP-1 and transcription of proinflammatory genes (Urano et al., 2000). In addition, other mechanisms, such as the production of reactive oxygen species (ROS) in the ER, the level of glutathione and the release of intracellular Ca^{2+} can activate NF-κB signaling inducing inflammation. Production of ROS, in the form of oxygen peroxide, occurs normally in the ER during the catalysis of disulfide bonds formation and it is mediated by two ER-resident proteins PDI and ERO1 (Görlach et al., 2015). Similarly, oxidative stress in the ER is also the result of increased consumption of glutathione, employed as reducing agent of improperly formed disulfide bonds. Thus, an increase in the ER protein load may lead to an overproduction of ROS and, in turn, may initiate an inflammatory response. To control the level of oxidative stress the PERK pathway, through NRF2 and ATF4, induces transcription of antioxidant and oxidant-detoxifying enzymes, including genes involved in regulating cellular level of glutathione (Cullinan and Diehl, 2004). Thus, ER stress through activation of the IRE1 and PERK branches can directly initiate neuronal inflammation, a key process in the

pathogenesis of neurodegenerative diseases, providing a direct link between accumulation of misfolded/aggregated protein and pro-inflammatory conditions.

ER STRESS AND PD PATHOGENESIS

Several reports support the link between ER stress and PD pathogenesis. One of the first of these was obtained in pharmacological neurotoxic models of PD where acute treatment with MPTP, 6-hydroxydopamine (6-OHDA) or rotenone, in cell cultures induced, although at different extent, activation of the UPR genes (Ryu et al., 2002; Holtz and O'Malley, 2003). Moreover ablation of CHOP in mice protected dopaminergic neurons against 6-OHDA, indicating that the ER stress response contributes directly to neurodegeneration *in vivo* (Silva et al., 2005). Specific sensitivity of the dopaminergic system to ER stress was also confirmed by more recent evidence and could partly explain how this population is particularly vulnerable to protein misfolding. For instance, inhibition of XBP1 protein expression in the substantia nigra of adult mice triggered chronic ER stress and specific neurodegeneration of dopaminergic neurons, whereas local recovery of XBP1 level through gene therapy increased neuronal survival and reduced striatal denervation after 6-OHDA treatment (Valdes et al., 2014). Similar results were obtained in mice after MPTP administration or in neuroblastoma cell lines treated with MPTP or proteasome inhibitors (Sado et al., 2009). In both cases, overexpression of XBP1 rescued neuronal cells from dying, indicating that the UPR plays a pivotal role in dopaminergic neuronal survival. In the same way knocking down ATF6 expression in mice exacerbated neurotoxicity after MPTP insult (Egawa et al., 2011). Interestingly, treatment with MPTP has been shown to induce UPR by affecting ER Ca^{2+} homeostasis through inhibition of store-operated calcium entry (SOCE), whose activity is fundamental for maintaining ER Ca^{2+} level (Selvaraj et al., 2012). In this context, MPTP would inhibit the expression of transient receptor potential channel 1 (TRPC1), a regulator of SOCE, decreasing Ca^{2+} entry into the cells. Overexpression of TRPC1 protected against MPTP-induced loss of SOCE and UPR, while knocking down the gene in mice increased UPR and cell death of dopaminergic neurons. Thus, at least for MPTP, induction of UPR appears to be directly linked to Ca^{2+} imbalance.

Activation of the ER stress response was also reported in human PD brain. Accumulation of ER chaperons was found in LBs (Conn et al., 2004) while increased PERK/p-eIF2α signaling was demonstrated in dopaminergic neurons of the substantia nigra in post-mortem tissue from PD cases, confirming that PD pathology is intimately associated with activation of ER stress *in vivo* (Hoozemans et al., 2007). Interestingly, at least two protective mechanisms against ER stress have been shown to involve modulation of genes such as Parkin and LRRK2, whose mutated forms have been associated with familiar cases of PD. Parkin, an E3 ubiquitin ligase implicated in the regulation of mitophagy, was found increased after treatment with ER or mitochondria stressors and this increase was mediated directly by ATF4 binding to the parkin promoter (Bouman et al., 2011; Sun et al., 2013). Overexpression of parkin protected cells from ER stress by promoting splicing of XBP-1 and the induction of the UPR prosurvival response (Duplan et al., 2013). Also mutations causing loss of function of LRRK2, a protein involved in maintaining neuronal cellular stability, have shown to abrogate upregulation of BiP/grp78 level after 6OHDA treatment or overexpression of αS, enhancing neuronal death *in vitro* and *in vivo* (Yuan et al., 2011). Thus, additional protective mechanisms may be important in preserving cellular environment from detrimental effects of ER stress whereas alteration in such pathways may contribute to PD progression.

ER Stress and α-Synucleinopathy

In genetic models of PD obtained by overexpression of αS, the association between ER stress and α-synucleinopathy has been studied extensively. In mammalian cell cultures, mice and yeast, toxicity due to overexpression of human wild-type, A53T mutant or C-terminal truncated αS was correlated with ER stress and activation of the UPR (Smith et al., 2005; Cooper et al., 2006; Bellucci et al., 2011; Colla et al., 2012a; Chung et al., 2013; Heman-Ackah et al., 2017). Interestingly, αS-induced dysfunctional ER and ER-stress activated cell death were both attenuated by treatment with L-DOPA, a dopamine analog and the only known treatment for PD at the moment (Song et al., 2017). In pc12 cells, overexpression of mutant αS induced cellular stress in a time-dependent matter that initiated with oxidative and proteasome damage and later culminated with ER stress and activation of ER-stress dependent cell death program (Smith et al., 2005). Blockage of caspases activity with inhibitors, siRNA or treatment with the ER stress inhibitor salubrinal, protected against A53T αS-induced cell death, indicating that the ER stress mediates αS toxicity and contributes to cellular death (Boyce et al., 2005). In addition, since proteasome and mitochondria deficits appeared before UPR activation, this suggested that onset of the ER stress response was the final protective pathway to contain αS damage before apoptosis had to be initiated.

Because αS was not known to be a resident protein of the ER, questions on how αS induces ER stress remained open until multiple observations placed αS in close proximity or within the ER and showed its direct interaction with ER and vesicular traffic components (**Figure 2**). In yeast, overexpression of mutant A53T αS caused toxicity through inhibition ER-Golgi vesicular transport that was completely abrogated by the overexpression of Rab1, a member of the Rab/GTPase family important for intracellular protein traffic modulation (Cooper et al., 2006). Interestingly, Rab1 overexpression was able to rescue dopaminergic neurons from toxicity induced by αS overexpression in other PD animal models, such as *Drosophila, Caenorhabditis elegans* and rat primary cultures. In yeast, vesicular transport deficit was consistent with the inhibition of docking and fusion of vesicles to the Golgi membrane and could be also rescued by overexpression of other members of the Rab family, such as Rab3A and Rab8A (Gitler et al., 2008). Rab3A and Rab8A are responsible for promoting vesicles' transport at other sites such as the presynaptic button and the post-Golgi element. This suggested that αS overexpression caused traffic defects not only at the ER-Golgi step, but also at multiple sites

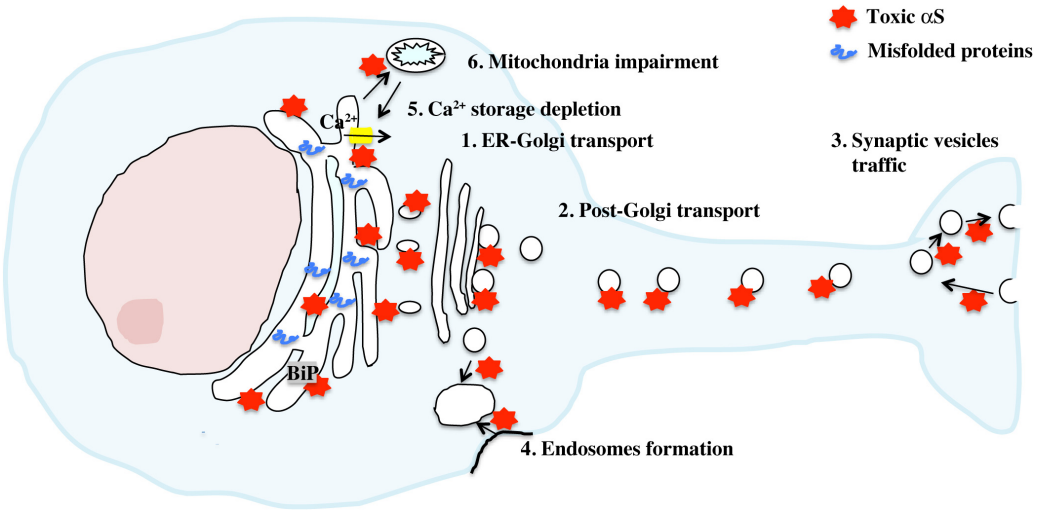

FIGURE 2 | Activation of the UPR by toxic αS. Accumulation of toxic αS in neurons directly affects cellular secretion and proteins traffic at multiple stages thanks to the ability of αS to bind synaptic vesicles and biological membranes. This traffic defect appears to be transferred progressively from the synapse to the Golgi/ER compartments, resulting in a toxic build up of misfolded proteins with concomitant activation of the UPR. Depletion of ER Ca^{2+} level, mediated by direct binding of αS aggregates to SERCA, an ER Ca^{2+} channel, or indirectly through mitochondria impairment increases cytosolic Ca^{2+} and exacerbates ER dysfunction.

in the secretion pathway, including at the plasma membrane, an observation that fits well with the physiological role of αS in promoting neurotransmission. As a matter of fact, at the synapse, αS has been described to interact with the SNARE complex and to promote vesicles docking and fusion to the membrane in the presynaptic button (Burre et al., 2010; Diao et al., 2013; Wang et al., 2014). *In vivo*, overexpression of wild-type αS in mice significantly inhibited neurotransmission by delaying vesicles recycling and reclustering after endocytosis at the synaptic terminal (Nemani et al., 2010). In *Drosophila*, overexpression of αS induced accumulation of synaptic vesicles with a larger size at the neuromuscular junction, a defect that was rescued by Rab11 (Breda et al., 2015). Interestingly, in the yeast model overexpressing αS, the observed traffic defect due to the build-up of clustered vesicles unable to fuse, initiated at the membrane level and later expanded in a retrograde manner to the Golgi and the ER. Other reports pointed out that αS could impair traffic of specific vesicles cargo, such as COPII vesicles loaded with ATF6 protein or vesicles containing lysosomal-targeted hydrolases moving from the ER to the *Cis*-Golgi (Credle et al., 2015; Mazzulli et al., 2016).

Additionally, other reports suggested how protein traffic deficit was not due to αS expression *per se* but related to an acquired toxic function. For instance, formation of aggregates in axonal terminals of primary cultures after exogenous administration of αS pre-formed fibrils did not cause initially a generalized defect in axonal transport but impaired primarily Rab7 and TrkB receptor–containing endosomes and autophagosomes cycling (Volpicelli-Daley et al., 2014). Consistent with this observation, we demonstrated in A53T αS transgenic mice how α-synucleinopathy, intended as accumulation of αS toxic species along the ER and the secretory pathway, but not the overexpression of the protein, was associated with the induction of UPR and ER stress-induced cell death *in vivo* (Colla et al., 2012a). Very importantly, appearance of ER-associated αS oligomers preceded α-synucleinopathy and ER stress whereas treatment with salubrinal, delayed α-synucleinopathy onset in transgenic mice and in the AA2V-A53T αS rat model and reduced the level of αS oligomers and aggregates associated with the ER, but not the total amount of αS, suggesting that accumulation of ER-associated αS species results in ER stress (Colla et al., 2012b). Additionally, αS species that accumulate along the secretory pathway appear to have specific distinct biochemical properties compared to non-membranous associated αS aggregates and can be extremely neurotoxic (Colla et al., 2018). For instance, while mature microsomes-αS aggregates isolated from diseased A53T mice exogenously added to mouse primary neurons induced endogenous aggregation and cell death, same species but isolated from presymptomatic mice, without overt α-synucleinopathy, were still cytotoxic but with lesser extent and were found unable to propagate. Such differences in behavior suggest some sort of toxic maturation of αS species right at the ER/microsomal membrane, pushing forward the hypothesis that the ER, Golgi and synaptic vesicles membranes may be a key site for αS aggregation and toxicity.

In addition, we and others have shown that αS interacts with Bip/Grp78 in physiological conditions (Bellucci et al., 2011; Colla et al., 2012a) suggesting that in case of αS aggregation, accumulation of αS aggregates along the ER membrane might directly signal distress to the ER through its interaction with BiP/grp78. Interestingly overexpression of BiP/Grp78 in rats or XBP-1 in *C. elegans* has been shown to alleviate ER stress and protect dopaminergic neurons from αS neurotoxicity (Gorbatyuk et al., 2012; Ray et al., 2014). On the other hand, a study in mammalian cells and αS transgenic mice reported that mutant

A53T αS induced cell death and UPR by destabilizing ER Ca^{2+} homeostasis. Overexpression of homocysteine-inducible ER stress protein (Herp), a protein that plays a role in maintaining ER Ca^{2+} balance, markedly reduced A53T αS-induced toxicity in mice, whereas knockdown of Herp exacerbated ER stress leading to a significant rise in toxicity (Belal et al., 2012). Similarly, αS aggregates but not the monomer have been shown to bind to and activate SERCA, an ER Ca^{2+} pump, inducing Ca^{2+} release in the cytosol (Betzer et al., 2018). Treatment with CPA, a SERCA inhibitor, normalized Ca^{2+} level and was neuroprotective against αS aggregates toxicity in *C. elegans*. Ultimately, because ER Ca^{2+} level is particularly sensitive to an increase in ROS, including radical species derived from dysfunctional mitochondria, toxic αS could indirectly contribute and exacerbate ER stress by impairing mitochondria metabolism and the respiratory chain (Görlach et al., 2015).

Thus, because of its preference to bind biological membranes that puts αS in direct contact with the ER/Golgi membrane and synaptic vesicles, toxic, aggregated αS is able to promote ER stress by destabilizing Ca^{2+} homeostasis and inhibiting intracellular protein trafficking and vesicles release, affecting the whole secretory pathway and contributing to the build up of misfolded proteins in the ER with consequent impairment in ER functionality.

CONCLUSION

In recent years because of its importance in regulating protein homeostasis, the ER has emerged as a central organelle in the pathogenesis of neurodegenerative diseases. Accumulating evidence support a key role of the UPR cascade in PD progression, correlated specifically to dopaminergic neuronal death and αS toxicity. Specifically, it appears that because of αS's cellular function and its ability to interact with membranes of organelles and vesicles of the secretory pathway and intracellular protein trafficking, toxicity of αS mediated by aggregation might directly or indirectly affect ER functionality and induce ER stress. In addition because ER stress contributes to promote neuroinflammation, a central process in neurodegeneration, regulating the UPR cascade through therapy may become an efficient cellular target that can lower misfolded protein overload as well as improve inflammation. Thus, future genetic or pharmacology-based approaches tackling the UPR in order to recover ER functionality could potentially lead to disease-modifying therapy in PD and possibly other neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

EC conceived the idea, wrote, edited, and reviewed the manuscript before submission.

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REVIEW
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Manganese-Induced Neurotoxicity: New Insights Into the Triad of Protein Misfolding, Mitochondrial Impairment, and Neuroinflammation

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Occupational or environmental exposure to manganese (Mn) can lead to the development of "Manganism," a neurological condition showing certain motor symptoms similar to Parkinson's disease (PD). Like PD, Mn toxicity is seen in the central nervous system mainly affecting nigrostriatal neuronal circuitry and subsequent behavioral and motor impairments. Since the first report of Mn-induced toxicity in 1837, various experimental and epidemiological studies have been conducted to understand this disorder. While early investigations focused on the impact of high concentrations of Mn on the mitochondria and subsequent oxidative stress, current studies have attempted to elucidate the cellular and molecular pathways involved in Mn toxicity. In fact, recent reports suggest the involvement of Mn in the misfolding of proteins such as α-synuclein and amyloid, thus providing credence to the theory that environmental exposure to toxicants can either initiate or propagate neurodegenerative processes by interfering with disease-specific proteins. Besides manganism and PD, Mn has also been implicated in other neurological diseases such as Huntington's and prion diseases. While many reviews have focused on Mn homeostasis, the aim of this review is to concisely synthesize what we know about its effect primarily on the nervous system with respect to its role in protein misfolding, mitochondrial dysfunction, and consequently, neuroinflammation and neurodegeneration. Based on the current evidence, we propose a 'Mn Mechanistic Neurotoxic Triad' comprising (1) mitochondrial dysfunction and oxidative stress, (2) protein trafficking and misfolding, and (3) neuroinflammation.

Keywords: manganese neurotoxicity, Parkinson's disease, protein aggregation, exosome, cell-to-cell transmission and neuroinflammation

METALS IN BIOLOGY

At least 13 metals have been identified as essential for life, and four of these (sodium, potassium, magnesium, and calcium) occur in large amounts. The remaining nine trace metals (manganese, iron, cobalt, vanadium, chromium, molybdenum, nickel, copper, and zinc) assume vital roles in building organic biomolecules as well as in regulating biological functions. In the last couple of decades, the importance of metal ions in protein biology has been an increasingly attractive

research subject given their association with many human diseases, for which metals have been identified as a causative or stimulatory agent. Metals are essential because of their integral role in enzymes that catalyze the basic metabolic or biochemical processes shared by all forms of life on earth. About one-third of all proteins depend on metal ions to carry out their biological functions (Holm et al., 1996). When considering all six classes of enzymes – oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases – over 40% of all enzymes contain metals (Andreini et al., 2008). Moreover, the chemistry of metals allows for a broader set of protein-metal reactions. For instance, redox-active metal ions are often interchangeable depending on the metal concentration and their affinities to protein. Protein affinities for trace metals are substantially determined by universal series, which for divalent metals is the Irving-Williams series ($Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$), wherein Cu^{2+} is highly competitive and can replace lower order metals (Tottey et al., 2008).

These "metalloproteins" are involved in many key biological processes, such as gas transport, cell respiration, antioxidant defense, photosynthesis, and many other vital redox reactions driven by their interaction with metals. Well-characterized examples for redox-active metalloprotein systems are blue-copper proteins, heme-binding proteins and iron-sulfur-cluster proteins. Moreover, recent advances in synthetic chemistry have focused on the study of metal sites in metalloproteins and metalloenzymes to influence biological processes in the battle against many daunting human diseases. Advanced medicinal chemistry approaches have given us new, innovative medicinal applications of metal complexes and organometallic agents. Prime examples for such uses of metals include platinum-containing anticancer drugs (e.g., Cisplatin), lithium-containing depression drugs (e.g., Camcolit), and manganese (Mn)-containing anticancer drugs (e.g., SOD mimics) (Farrell, 2003).

Presumably, all metalloproteins would bind to their desired metal ligands, and this binding can regulate their folding. However, despite the wealth of structural information, the coupled protein-folding, metal-binding pathways for metalloproteins remain largely unknown (Wittung-Stafshede, 2002). Proper protein folding is critical to the conformational integrity and function of proteins. However, metal ligand binding can also induce undesirable structural transitions in proteins that eventually lead to the formation of pathological protein aggregates. Indeed, the pathologies of Alzheimer's disease (AD), PD, and prion diseases are linked to abnormal misfolding of otherwise harmless neural proteins. For example, in AD, increased levels of metals, such as Cu^{2+} and Zn^{2+}, are linked to the aggregation of Aβ protein *in vitro* (Kenche and Barnham, 2011). The theory of metal-induced aggregation is supported by numerous studies tying metal concentrations in the brain with AD, PD, and amyotrophic lateral sclerosis (ALS) in *in vivo* and *in vitro* studies employing recombinant proteins (Brown et al., 2005; Brown, 2011).

In this review, we will focus on α-synuclein, one of the major proteins implicated in PD, and its interactions with metals, specifically, its interaction with Mn in oxidative stress, protein aggregation and neurodegeneration.

PARKINSON'S DISEASE

Parkinson's disease is recognized as the second most prevalent neurodegenerative disorder after AD, affecting roughly 1% of the population over the age of 65. It is also the most common movement disorder in the elderly, resulting in bradykinesia, resting tremor, and rigidity (Lotharius and Brundin, 2002). Several non-motor symptoms involving the autonomic nervous system have also been gaining attention (Pfeiffer, 2009; Schapira et al., 2017). PD is characterized histopathologically by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), leading to the progressive loss of the neurotransmitter dopamine and hence the above-mentioned cardinal motor deficits. Even though PD is also often associated with the abnormal accumulation of misfolded proteins, primarily α-synuclein, in cytoplasmic inclusions called Lewy bodies (LB) and Lewy neurites, the pathophysiological association between Lewy pathology and disease pathogenesis is not well understood. Similar neuropathological lesions involving the deposition of abnormal proteins also characterize other neurological disorders (Ross and Poirier, 2004), including AD (Kotzbauer et al., 2001; Uchikado et al., 2006), Lewy body dementia (LBD) (McKeith et al., 2004), Huntington's disease (HD) (Davis et al., 2014), multiple system atrophy (MSA) (Shoji et al., 2000), and some prion diseases (Aguzzi and Calella, 2009; Aguzzi and O'Connor, 2010).

Although aging remains the greatest risk factor for idiopathic PD, a small fraction of patients were identified with familial PD, which is caused by mutations in several genes associated with protein metabolism, ion transport and mitochondrial function. Genes associated with early-onset PD include α-*synuclein* (PARK1), *parkin* (PARK-2), *PINK1* (PARK6), *DJ-1* (PARK7) and *ATP13A2* (PARK9), while those linked with late-onset PD include *LRRK2* (PARK8) and *VPS35* (PARK-17) (Dawson et al., 2010; Roth, 2014). A growing number of epidemiological and clinical studies have identified environmental risk factors for PD, including repeated head trauma, heavy metal toxicity, pesticide toxicity, obesity, and some surrogate measures such as rural living, contaminated well water, substance abuse, and farming (Priyadarshi et al., 2001; Dick et al., 2007). Interestingly, some of these environmental triggers and toxins induce pathophysiological features that mimic PD when they are administered in experimental animal settings. One such toxin is MPTP (methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a compound produced as an impurity during synthesis of the illicit narcotic desmethylprodine. MPTP causes chronic and severe Parkinsonism by selectively damaging the SN, resulting in PD-related motor deficits (Langston et al., 1983; Ballard et al., 1985; Appendino et al., 2014). Other compounds widely used in experimental models to study the etiopathogenesis of PD include the narcotic methamphetamine, the dopamine derivative 6-hydroxydopamine, and pesticides such as rotenone, paraquat, and dieldrin. These neurotoxins cause nigrostriatal cell death by interfering with mitochondrial function, inducing oxidative stress, protein aggregation, and modifying proteasomal function (Kanthasamy et al., 2008; Latchoumycandane et al., 2011; Ghosh et al., 2013; Jin et al., 2015b). In addition, exposure to

heavy metals (e.g., iron, lead, mercury, cadmium, arsenic, and Mn) and metal-based nanoparticles increases the risk of PD through the neurotoxic accumulation of metals in the SNpc and by increasing oxidative stress-induced apoptosis (Afeseh Ngwa et al., 2009; Milatovic et al., 2009; Afeseh Ngwa et al., 2011; Kanthasamy et al., 2012; Aboud et al., 2014; Harischandra et al., 2015a).

MANGANESE

Manganese is considered to be a key inhaled environmental pollutant as well as a putative risk factor for environmentally linked PD and related neurodegenerative disorders. Being the 12th most abundant element and composing approximately 0.1% of the earth's crust, Mn is ubiquitously present in the environment (Martinez-Finley et al., 2012). Besides the earth's crust, other Mn sources include direct atmospheric deposition, wash-off from plant and other surfaces, leaching from plant tissues, ocean spray, and volcanic activity. Mn occurs in trace amounts in all body tissues as it is essential for many ubiquitous enzymatic reactions, including the synthesis of amino acids (AA), lipids, proteins, and carbohydrates. It also plays a key nutritional role in bone growth, fat and carbohydrate metabolism, blood sugar regulation, and calcium absorption (Bowman et al., 2011). Being present in whole grains, rice, nuts, tea, leafy green vegetables, and Mn-containing nutritional supplements, the primary route of Mn exposure in humans is through dietary intake. The abundance of Mn-enriched food in the typical daily diet makes it relatively easy to accrue the daily reference intake (DRI) of 2.3 mg/day for men and 1.8 mg/day for women (Aschner and Aschner, 2005), thereby minimizing the risk of Mn deficiency-related birth defects, impaired fertility, osteoporosis, and enhanced susceptibility to seizures (Dendle, 2001; Aschner and Aschner, 2005; Sarban et al., 2007).

Despite its nutritional benefits, prenatal and postnatal overexposure to Mn affects infant neurodevelopment, exemplifying its role as both an essential nutrient and a toxicant (Zota et al., 2009; Claus Henn et al., 2010). High Mn exposure in early life is associated with poor cognitive performance, especially in the verbal domain of children (Menezes-Filho et al., 2011). In older cohorts, chronic excessive exposure to occupational or environmental sources of Mn causes manganism, which is characterized by a severe neurological deficit that often resembles the involuntary extrapyramidal symptoms associated with PD (Kwakye et al., 2015). Couper (1837), at the University of Glasgow, reported the first case of Mn-induced neurotoxicity, which was discovered in employees of Charles Tennant and Co., a manufacturer of bleaching powder. Later, public awareness of Mn neurotoxicity arose as more clinical studies identified a PD-like syndrome in workers employed at a Mn ore-crushing plant and a ferromanganese factory (Cook et al., 1974; Huang et al., 1989). In addition, Rodier (1955) detailed clinical features of manganese neurotoxicity in Moroccan miners. Since then, the commercial applications for Mn have broadened considerably so that now Mn exposure also occurs through its use as an additive in gasoline (methylcyclopentadienyl manganese tricarbonyl, MMT) and fertilizers, and as manganese violet in paint and cosmetics (Martinez-Finley et al., 2012). Mn neurotoxicity occurs often in agricultural workers exposed to organic Mn-containing pesticides, such as manganese ethylene-bis-dithiocarbamate (Maneb) and in chronic abusers of the street drug 'Bazooka', a cocaine-based drug contaminated with manganese carbonate (Ensing, 1985). The other major anthropogenic sources of environmental Mn include municipal wastewater discharge, welding, mining and mineral processing, metal (alloy, steel, and iron) manufacturing emissions, fossil fuel combustion, and dry-cell manufacturing. Although the precise mechanisms through which Mn is absorbed into the body are not fully understood, it is known to accumulate predominantly in the brain's basal ganglia region. Beyond the many commonalities shared between manganism and PD, it is also worth pointing out their differences. Behaviorally, manganism is mainly characterized by milder and less frequent resting tremor that tends to be postural or actional, a propensity to fall backward, excessive salivation, and frequent dystonia consisting of facial grimacing, hand dystonia, and/or plantar flexion (Calne et al., 1994). Manganism patients were also reported to have symptoms of irritability, emotional lability, and hallucinations and psychoses referred to as "manganese madness" (Huang, 2007). Pathologically, Mn neurotoxicity affects primarily neurons in both the globus pallidus and striatum, whereas PD predominantly affects dopaminergic neurons in the SNpc (Roth, 2014). Therefore, in fact, the PD-like behavior deficits in manganism result from Mn's capability to suppress dopamine release from the striatum, thus generating behavioral dysfunctions common to both PD and manganism (Kim et al., 2002; Racette et al., 2005; Fitsanakis et al., 2006; Roth et al., 2013).

MANGANESE HOMEOSTASIS

The homeostasis of Mn and other metal ions is maintained through tightly regulated mechanisms of uptake, storage, and secretion that strictly limit their abundance in the cellular compartment. The distribution and neurotoxicity of Mn is governed largely by the routes of exposure, which are primarily ingestion and inhalation. In humans, the primary route of exposure is through Mn-enriched food and well water. However, the molecular mechanisms of oral Mn absorption are not well understood. Roughly 3–5% of the Mn ingested gets absorbed into the body from the gastrointestinal tract (GIT) (Finley et al., 1994). Under homeostatic conditions, Mn enters the portal circulation through either passive diffusion (Bell et al., 1989) or active transport via divalent metal transporter 1 (DMT1) (Erikson and Aschner, 2006; Fitsanakis et al., 2007), which was the first mammalian transmembrane iron transporter to be identified. Formerly known as Nramp2 or DCT1, DMT1 is a 12-transmembrane domain protein responsible for the uptake of various divalent metals including Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}, and it transfers iron across the apical surface of intestinal cells and out via transferrin (Tf)-cycle endosomes (Andrews, 1999). Besides using a mechanism similar to that for iron, there are no known metal transporters specific for transporting Mn

into cells. In plasma, approximately 80% of Mn^{2+} is bound to α-macroglobulin or albumin, while only a small fraction (<1%) of Mn^{3+} is bound to Tf. It has been proposed that, like iron, Mn in plasma is oxidized from Mn^{2+} to the Mn^{3+} valence state by the ferroxidase enzyme ceruloplasmin and loaded onto plasma Tf for circulating into tissues (Davidsson et al., 1989). Circulating Mn diffuses throughout the body, including bone, kidney, pancreas, liver, and brain (Martinez-Finley et al., 2012).

Once in the brain, Mn^{3+} entry into neurons occurs by the Tf-Mn^{3+} complex binding to the transferring receptor (TfR) and it becomes localized in endosomes. Subsequent recruitment of v-ATPases acidifies endosomes and dissociates Mn^{3+} from the Tf/TfR complex, reducing it to Mn^{2+}, which is quite stable at physiological pH, and thereafter, neuronal transport occurs via DMT1 independent of the Tf pathway. In the brain, DMT1 is highly expressed in the DA-rich basal ganglia, putamen, cortex, and SN (Huang et al., 2004; Salazar et al., 2008), which may account for Mn's pattern of accumulation and neurotoxicity. Other primary transport mechanisms for Mn are through capillary endothelial cells of the blood-brain-barrier (BBB) (Crossgrove et al., 2003) or through the CSF via the choroid plexus (Murphy et al., 1991). Since Mn neurotoxicity primarily occurs through occupational exposure, such as inhalation of Mn fumes or dust in welding, dry-cell battery manufacturing, and the smelting industry, the nasal passage through the olfactory epithelium to the olfactory nerve is another major Mn transport mechanism into the brain (Tjalve et al., 1996). In fact, DMT1 is highly expressed in the olfactory epithelium and is required for Mn transport across the olfactory epithelium, as has been shown in the rat (Thompson et al., 2007). Evidence also exists for Mn transport into the central nervous system (CNS) through store-operated calcium channels (Crossgrove and Yokel, 2005), ionotropic glutamate receptor calcium channels (Kannurpatti et al., 2000), and Mn citrate transporters (Crossgrove et al., 2003).

Another mechanism regulating Mn homeostasis in the brain involves Mn being transferred with high affinity into cells by the Zinc transporters ZIP-8 and ZIP-14, which are Zrt-/Irt-related protein (ZIP) family metal transporters encoded by SLC39A8 and SLC39A14, respectively. These transporters are highly expressed in the liver, duodenum, kidney, and testis, and are localized on apical surfaces of brain capillaries (Girijashanker et al., 2008; Wang et al., 2012). Taking advantage of its particular magnetic properties, Aoki et al. (2004) employed magnetic resonance imaging (MRI) to show that Mn uptake also occurs through the choroid plexus. One day after they systemically administered Mn^{2+} to rats, the distribution of Mn in the brain extended to the olfactory bulb, cortex, basal forebrain, and basal ganglia, overlapping specific brain structures vulnerable to Mn-induced neurotoxicity (Aoki et al., 2004). In cells such as neurons and astrocytes, toxic accumulations of Mn are found primarily in the mitochondria, heterochromatin, and nucleoli (Lai et al., 1999; Morello et al., 2008).

Mn also shares the Ca^{2+} uniporter mechanism and the rapid mode (RaM) of Ca^{2+} uptake of mitochondrial calcium influx, resulting in Mn sequestration in mitochondria, which gets removed only very slowly from the brain (Gavin et al., 1990). This Mn accumulation inhibits the efflux of calcium, decreases MAO activity, and inhibits the respiratory chain and ATP production (Zhang et al., 2003), which may partly explain the role of mitochondrial dysfunction in Mn neurotoxicity. Previously, Mn detoxification and efflux from cells was thought to be primarily regulated by ferroportin (Fpn), also known as HFE4, MTP1, and IREG1, which are proteins encoded by the SLC40A1 gene. Although Fpn was initially identified as the iron exporter, more recent findings suggest that Fpn also interacts with Mn, zinc, and cobalt to export them from the cell (Troadec et al., 2010; Yin et al., 2010; Madejczyk and Ballatori, 2012). Furthermore, Mn exposure increases Fpn mRNA levels in mouse bone marrow macrophages (Troadec et al., 2010) and it significantly increases Fpn protein levels in HEK293T cells (Yin et al., 2010). Increasing Fpn levels were linked to reduced Mn accumulation in both the cerebellum and cortex of mice treated with Mn (Yin et al., 2010), further confirming that Fpn removes Mn and reduces Mn-induced neurotoxicity.

Recently, the secretory pathway of the Ca^{2+}/Mn^{2+} ATPases SPCA1 and SPCA2, which are localized at the Golgi, was suggested as an alternative way of cytosolic Mn detoxification by sequestering into the Golgi lumen (Sepulveda et al., 2009). Overexpressing SPCA1 in HEK293T cells conferred tolerance of manganese (Mn^{2+}) toxicity by facilitating Mn^{2+} accumulation in the Golgi, thereby increasing cell viability (Leitch et al., 2011). However, the degree to which SPCA1 and SPCA2 regulate Mn homeostasis has yet to be determined. Another mode for Mn egress through Golgi has been attributed to SLC30A10 in humans (Tuschl et al., 2012). Recently, SLC30A10 was shown to be localized on the cell surface where it acted as a Mn efflux transporter to reduce cellular Mn levels and protect against Mn-induced toxicity (Leyva-Illades et al., 2014). Mutations in the SLC30A10 gene have been associated with hepatic cirrhosis, dystonia, polycythemia, Parkinsonian-like gait disturbances, and hypermanganesemia in cases unrelated to environmental Mn exposure (Tuschl et al., 2012). A genome-wide association study mapping genes involved in regulating Mn homeostasis mapped serum Mn levels to SLC30A10 (Ng et al., 2015). Along with its expression in the liver and CNS, SLC30A10 is also expressed in the GIT. Interestingly, this transporter is present mainly on the apical surface of enterocytes that line the GIT and presumably help transport Mn to the lumen. In fact, it is the liver and GIT that are primarily responsible for maintaining Mn homeostasis in the body as indicated by whole body as well as endoderm-specific SLC30A10 knockouts (KOs) resulting in hypermanganesemia, while pan neuronal/glial SLC30A10 KOs produce normal levels of Mn in the CNS (Taylor et al., 2019). The authors also found that a lack of SLC30A10 in the CNS led to an increased accumulation of Mn in the basal ganglia and thalamus when these mice were exposed to elevated Mn levels. Importantly, these recent discoveries involving SLC30A10 and its mutations reinforce its crucial role in humans as a Mn transporter, broadening our understanding of familial Parkinsonism as a result of *SLC30A10* mutations.

The p-type transmembrane ATPase protein ATP13A2 (or PARK9) located at the lysosome also protects cells from Mn-induced toxicity (Tan et al., 2011). Although ATP13A2's

function in mammalian cells remains elusive, loss-of-function mutations in ATP13A2 cause Kufor-Rakeb Syndrome (KRS), an autosomal recessive form of early-onset Parkinsonism with pyramidal degeneration and dementia (Ramirez et al., 2006). Overexpression of wild-type (WT) ATP13A2, but not KRS pathogenic ATP13A2 mutants, protects mammalian cell lines and primary rat neuronal cultures from Mn^{2+}-induced cell death by reducing intracellular Mn concentrations and cytochrome c release, suggesting a role of ATP13A2 in Mn detoxification and homeostasis (Tan et al., 2011). A summary of the above-mentioned receptors and channels involved in cellular Mn homeostasis appears in **Figure 1**.

MANGANESE AND α-SYNUCLEIN PROTEIN MISFOLDING

Belonging to a family that includes β- and γ-synuclein, α-synuclein (αSyn) is a small 140-AA, highly conserved vertebrate protein encoded by a single 7-exon gene located on chromosome 4. It is predominantly a neuronal protein expressed in presynaptic terminals throughout the mammalian brain and CSF where it is estimated to account for as much as 1% of total protein in soluble cytosolic brain fractions. Functionally, αSyn remains poorly understood, but emerging evidence points to roles in membrane trafficking, dopamine regulation, and synaptic plasticity. The link between αSyn and PD pathogenesis is based on case studies of familial and sporadic PD patients presenting with misfolded αSyn-rich Lewy pathology during autopsy (Poulopoulos et al., 2012). Also, compelling evidence demonstrates that mutations in the gene encoding αSyn are directly linked to the onset of PD (Liu et al., 2012). Furthermore, rare familial forms of PD also have been linked to the overexpression of αSyn due to *SNCA* gene duplication and triplication.

The aggregation and fibrillation of αSyn, forming intracellular proteinaceous aggregates, have been implicated in several other neurodegenerative disorders besides PD, including LBD, Lewy body variant of AD, MSA, and Hallervorden–Spatz disease. The idea that extracellular αSyn species can accelerate the spread of PD pathology throughout the brain gained much consideration with the findings of host-to-graft propagation of αSyn-positive Lewy pathology in fetal ventral mesencephalic and embryonic nigral neurons transplanted in human PD patients (Kordower et al., 2008; Li et al., 2008) and misfolded αSyn species in human CSF and plasma (El-Agnaf et al., 2003; Kordower et al., 2008). Although multiple studies have hypothesized the intercellular transmission of pathological αSyn species in PD (Lee et al., 2008; Desplats et al., 2009; Dunning et al., 2013), its exact mechanistic role in disease pathogenesis and related synucleinopathies largely remains unknown. Available *in vitro* evidence thus far postulates that extracellular αSyn induces pathogenic actions by multiple mechanisms including, but not limited to, the triggering of neuroinflammatory responses and mitochondrial dysfunction leading to neurodegenerative processes (Su et al., 2008; Emmanouilidou et al., 2010).

As a member of the family of intrinsically unstructured proteins, αSyn is natively unfolded and lacks a defined secondary protein structure. However, upon interaction with lipid membranes, it adopts an α-helical conformational change, and under conditions that trigger aggregation, αSyn undertakes the characteristic crossed β-conformation and self-aggregates into soluble oligomers, which gradually form insoluble amyloid-like fibrils. The αSyn protein comprises three main structural domains (**Figure 2**): (1) an N-terminal amphipathic region, (2) an amyloid-binding central domain (NAC), and (3) a C-terminal acidic tail. The N-terminus (residues 1-60) contains four series of 11-AA repeats containing the highly conserved consensus sequence KTKEGV, which also is important for α-helix conformation upon binding to phospholipid membranes. The core NAC region (residues 61–91) is important in protein aggregation and it also contains two additional KTKEGV repeats. Within the NAC, a hydrophobic GAV peptide motif (residues 66–74), consisting of Ala, Val, and Gly AA residues, has been identified as the required core for human αSyn protein fibrillization and cytotoxicity (Du et al., 2006). Finally, the proline-rich C-terminus (residues 91–140) is highly acidic and accounts for the intrinsically disordered properties of αSyn (Harischandra et al., 2015a). The N-terminal and NAC regions form αSyn's membrane binding domain, whereas the C-terminal region is believed to contain protein–protein and protein–small molecule interaction sites.

Importantly, αSyn wields its metalloprotein properties through its three metal-binding sites: two each at the N-terminus and one at the C-terminus. A systematic analysis of mono-, di-, and trivalent metal ligands (Li^+, K^+, Na^+, Cs^+, Ca^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{3+}, Al^{3+}, and Fe^{3+}) revealed that metal binding induces conformational changes that cause normally benign αSyn protein to aggregate (Uversky et al., 2001). Of the 15 metal cations studied, Uversky et al. (2001) determined Al^{3+} to be the most effective stimulator of protein fibril formation followed by Cu^{2+}, Fe^{2+}, Co^{3+}, and Mn^{2+}, with each causing conformational changes detectable by intrinsic protein fluorescence and far UV-circular dichroism. Furthermore, Uversky's team also showed that Mn^{3+} induced immediate di-tyrosine formation, suggesting that Mn is responsible for the metal-induced oxidation of αSyn. Among the three metal-binding sites, those located at the N-terminus, specifically the ^{1}MDVFMKGLS^{9} and ^{48}VAHGV^{52} regions, demonstrated high-affinity binding for Cu^{2+} (K_d $\sim$ 0.1 μM) (Rasia et al., 2005), whereas metal-interaction sites near residues 49–52 and residues 110–140 are known to bind with divalent metals like Mn (Uversky et al., 2001; Binolfi et al., 2006, 2008). In a detailed study, the metal ions Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} bound preferentially to the ^{119}DPDNEA^{124} motif, in which Asp121 acted as the main anchoring site with low affinity (mM) to metal ligands (Binolfi et al., 2006). These discoveries on the structural components of αSyn's interaction with metals strengthen the link between metal neurotoxicity and PD, further suggesting that metal dyshomeostasis plays an even more important role in the development of neurodegenerative disorders than previously acknowledged (Binolfi et al., 2006).

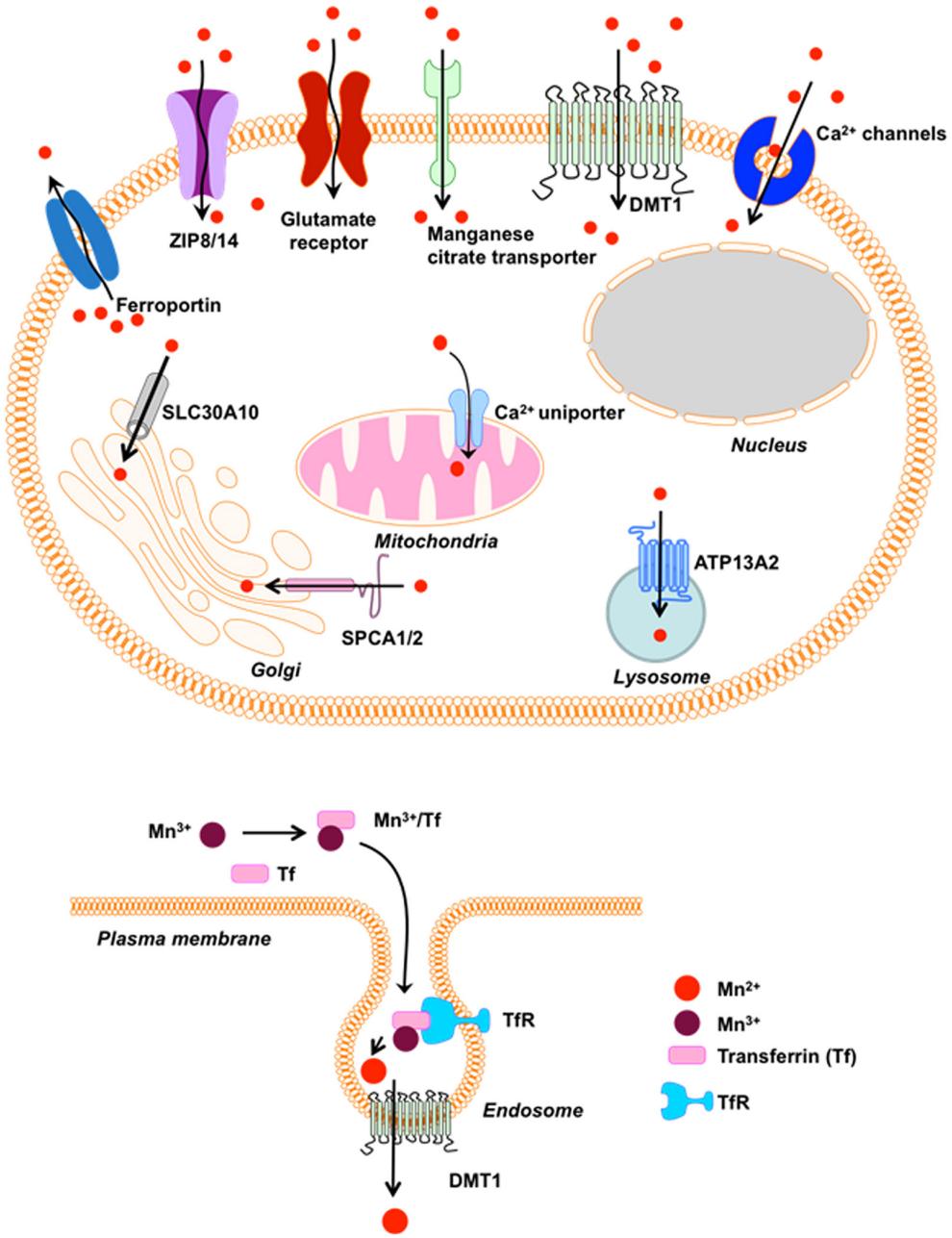

FIGURE 1 | Receptors/Channels involved in Mn homeostasis. Various cellular receptors such as divalent metal transporter 1 (DMT1) and transferrin receptor (TfR), as well as ion channels, store-operated Ca^{+2} channels (SOCC) or voltage-gated Ca^{+2} channels (VGCC) that facilitate the entry of divalent Mn into cells, whereas SLC40A (ferroportin) and Ca^{+2} facilitate its expulsion from cells and mitochondria, respectively. Mn^{+2} is passively transported via VGCC and glutamate-activated ion channels while Mn^{+3} entry is facilitated via transferrin.

Our *in vitro* studies show that physiological levels of human WT αSyn attenuate acute Mn-induced dopaminergic neuronal degeneration. However, this neuroprotective effect is diminished by chronic exposure to Mn toxicity, which accelerates αSyn misfolding (Harischandra et al., 2015a). Furthermore, using a genetically modified *Caenorhabditis elegans* model system, Bornhorst et al. (2014) reported enhanced Mn accumulation and oxidative stress in pdr1 and djr1.1 mutants, which were reduced by αSyn expression. This protective role of αSyn in Mn-induced neurotoxicity was further validated using αSyn transgenic animals (Yan et al., 2019). By treating αSyn KO ($\alpha Syn^{-/-}$) and WT ($\alpha Syn^{+/+}$) mice with different Mn concentrations, this study demonstrated that the presence of αSyn ameliorates high-dose Mn-induced neurotoxicity. Taken together, these findings point to a novel, neuroprotective role of WT αSyn in attenuating acute Mn

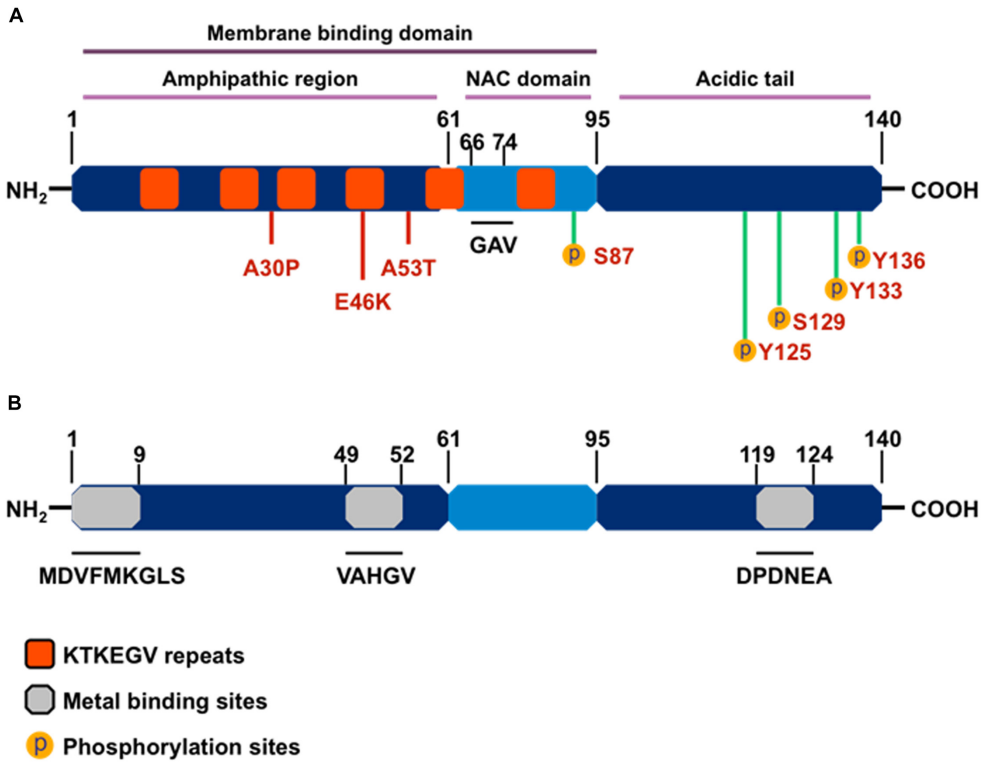

FIGURE 2 | The structure of α-synuclein. **(A)** Orange boxes denote the characteristic KTKEGV repeat and red lines denote amino acid mutations seen in familial PD patients. The central hydrophobic core (middle light blue), or NAC domain, promotes α-synuclein aggregation. The C-terminal domain (right dark blue) is the acidic tail and contains most known phosphorylation sites. **(B)** Gray boxes denote known metal-binding sites in the α-synuclein structure.

toxicity, an effect which may stem directly from its metal-binding capability (**Figure 3**).

Although the physiological role of αSyn with respect to Mn toxicity still needs to be fully validated, the effects of Mn on αSyn expression, aggregation, and subsequent cytotoxicity have been studied in *in vitro*, *in vivo,* and *ex vivo* models of PD (Gitler et al., 2009; Verina et al., 2013; Xu et al., 2013). Studies conducted with neuronal cell culture models show that Mn treatment upregulates cellular αSyn levels and leads to αSyn aggregation (Cai et al., 2010). In contrast, knocking down αSyn using antisense αSyn treatment (Li et al., 2010) or siRNA (Cai et al., 2010) can reverse Mn-induced cytotoxicity. In parallel studies, overexpressing αSyn in rat mesencephalic cells (MES 23.5) not only enhanced their susceptibility to Mn exposure (Prabhakaran et al., 2011), but also attenuated Mn release from Mn-treated cells without significantly attenuating the major Mn transporter proteins DMT1, VGCC, and Fpn1 (Ducic et al., 2015). Thus, these studies further suggest that αSyn's metal-binding capacity serves as an intracellular Mn store that helps to regulate free-roaming Mn cations.

MANGANESE AND ENDOSOMAL TRAFFICKING

Accumulating evidence indicates that secretion and cell-to-cell trafficking of pathological forms of αSyn may explain the typical progression of PD. In particular, vesicular trafficking has attracted considerable attention as an initiator or enhancer of the neurodegenerative process underlying PD. Dysfunction of the cellular trafficking pathway can compromise synaptic function and lead to the accumulation of misfolded αSyn. Similarly, changes in endosomal sorting and degradation greatly influence the intracellular trafficking of misfolded proteins, thereby enabling the cell-to-cell transmission of toxic αSyn species in a prion-like manner. Recent genetic studies also suggest that defects of endolysosomal function could disrupt αSyn homeostasis and mitochondrial function, causing neurotoxicity through unknown mechanisms (Kett and Dauer, 2016). Indeed, several PD-linked gene mutations or polymorphisms (DNAJC13/RME-8, VPS35, ATP13A2, ATP6AP2, RAB7L1, GBA, GAK, LRRK2) interrupt protein trafficking and degradation via the endosomal pathway (Perrett et al., 2015), highlighting the importance of the endosomal pathway in the progression of neurodegenerative disease.

It has been shown that αSyn overexpression blocks endoplasmic reticulum (ER)-to-Golgi vesicular trafficking (Cooper et al., 2006) and that αSyn is functionally associated with endocytic vesicular trafficking, retromer complex proteins, phosphatases, and Rab GTPases (Breda et al., 2015; Chung et al., 2017). In this regard, recent attempts to identify molecular regulators of αSyn oligomerization have identified several Rab proteins, including Rab8b, Rab11a, Rab13, Slp5

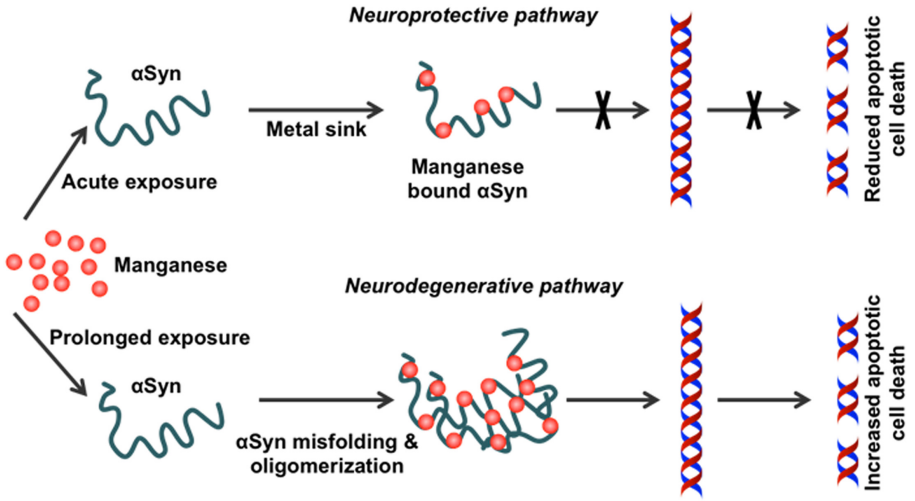

FIGURE 3 | Effect of chronic Mn overexposure on α-synuclein misfolding. Metal-binding sites on α-synuclein (αSyn) allow it to become a metal sink for free-roaming metals in cells. During an acute exposure to Mn, free-roaming Mn binds to the metal-binding sites on the C-terminus of this protein. In this way, αSyn effectively works as a chelator for different metal ions, including Mn. However, continued exposure to Mn can saturate this chelating property. Once saturation occurs following additional binding of Mn to the C-terminus, the natively conformed protein misfolds. Misfolded αSyn leads to the production of pro-inflammatory factors. Thus, Mn overexposure leads to progressive protein misfolding in the neurons and induces inflammation and finally neurodegeneration.

(Goncalves et al., 2016), and Rab1, which (Cooper et al., 2006) promote the clearance of αSyn inclusions and attenuate αSyn-induced toxicity. Furthermore, Rab11a and Rab13 expression enhanced the endocytic recycling and secretion in cells accumulating αSyn inclusions (Goncalves et al., 2016). In contrast, Rab11 regulates the recycling of extracellular αSyn (Liu et al., 2009) and modulates αSyn-mediated defects in synaptic transmission and locomotor behavior in experimental PD models (Breda et al., 2015). This is particularly interesting as Rab11 has been identified as a major regulator of endosomal recycling (Grant and Donaldson, 2009) and controls the secretion of smaller αSyn oligomers by exosomes (Poehler et al., 2014).

Exosomes are nano-sized vesicles (50–150 nm) that are released from cells into the extracellular space (Thery et al., 2002). Exosomes circulate throughout the body and readily cross the blood–brain and other barriers. Great interest in exosomes is emerging because of their potential role in disease progression as well as their possible use in early biomarker discovery (Sarko and McKinney, 2017) and drug delivery (Luan et al., 2017). Toxicology researchers are building upon the discovery that environmental toxicants change the exosome signature of human health conditions such as cancer and neurodegenerative diseases (Harischandra et al., 2017; Munson et al., 2018; Ngalame et al., 2018). In this regard, the impact that Mn exposure has on the neuronal exosome signature and its subsequent effect on neuroinflammation and neurodegeneration have been studied in great detail in our laboratory (Harischandra et al., 2015a,b, 2017, 2018, 2019). We have shown that Mn exposure significantly upregulates the small GTPase Rab27a, which mediates the membrane fusion of multivesicular bodies (Pfeffer, 2010) that subsequently release exosomes into the extracellular environment (Harischandra et al., 2018). Furthermore, our miRNA profiling analysis of Mn-induced neuronal exosomes indicates increased expression of certain miRNAs (e.g., miR-210, miR-325, miR-125b, miR-450b) known to control key biological mechanisms, including inflammation, autophagy, protein aggregation, and hypoxia (Harischandra et al., 2018). In subsequent studies, we show how Mn exposure promotes the exosomal secretion of aggregated αSyn into the extracellular medium. These exosomes were endocytosed through caveolae-mediated endocytosis, thereby inducing neuroinflammation that subsequently evoked neurodegenerative processes in both cell culture and animal models (Harischandra et al., 2019). Interestingly, serum exosome samples collected from welders chronically exposed to Mn-containing welding fumes show increased misfolded αSyn in their exosomes, further implicating environmental Mn exposure in developing Parkinsonism (Harischandra et al., 2019). In parallel studies, we revealed Mn's role in inflammasome activation in microglial cells. We found that Mn acts as signal 2 for NLRP3 inflammasome activation in LPS-primed microglial cells, triggering the exosomal release of ACS "prionoids," resulting in inflammasome propagation (Sarkar et al., 2019). Together, our results highlight Mn's role in modulating endosomal trafficking through the exosomal release of cargo capable of triggering neuroinflammation and progressive neurodegeneration.

MANGANESE AND NEUROINFLAMMATION

In addition to the importance of oxidative stress in the Mn-induced dysfunction of dopaminergic neurons, glial cell activation also plays an important role in potentiating Mn neurotoxicity by inducing the release of non-neuronal-derived ROS and inflammatory mediators such as proinflammatory

cytokines. The state of glial activation is defined by its morphology and by the proliferation, migration and expression of immune modulatory molecules. The two major types of glial cells in the CNS are astrocytes and microglia, with the latter constituting about 10% of all glial cells in the CNS.

It is now well documented that glial activation is prominent in the brains of humans exposed to Mn, as well as in non-human primate and rodent models of Mn neurotoxicity (Erikson and Aschner, 2006; Huang, 2007; Perl and Olanow, 2007; Cordova et al., 2013). Neuroinflammation is regarded as a key mediator in mechanisms underlying the loss of dopaminergic neurons in PD. The activation of microglia plays a major role in the response to environmental stress and immunological challenges by scavenging excess neurotoxins, removing dying cells and cellular debris, and releasing proinflammatory cytokines (Carson et al., 2007; Tansey et al., 2008). Inducible nitric oxide synthase (iNOS), which produces large amounts of nitric oxide (NO), is released by microglia in response to inflammatory mediators such as LPS and cytokines. The levels of NO are reported to be elevated in the CNS of human PD cases and in animal models of PD (Mogi et al., 1994). Consistent with this finding, iNOS KO animals are resistant to MPTP-induced dopaminergic neuronal loss in the SN (Przedborski and Vila, 2003). The transcription factor NF-κB, required for transcribing proinflammatory molecules, is also activated in the SN of PD patients and MPTP-treated mice (Ghosh et al., 2007). In contrast to microglia, astrocytes do not attack any pathological targets, but instead produce factors that mediate inflammatory reactions seen in the SN of PD brains (Miklossy et al., 2006). Activated astroglial cells were recently found in human PD brains and in the MPTP mouse model of PD (Ghosh et al., 2007; Ghosh et al., 2009).

Astrocytes play a major role in Mn-induced neuroinflammation as they represent a "hub" for brain Mn homeostasis (Wedler and Denman, 1984). The transferrin receptors found on astrocytes readily bind to Tf-Mn^{3+}, so it is not surprising to find more Mn in astrocytes than in any other neural cell types. Indeed, astrocytes can exhibit Mn concentrations 10- to 50-fold greater than those measured in neurons, making them more susceptible to Mn toxicity than other cell types. During glutamate-induced excitotoxicity, excess glutamate abruptly increases intracellular Ca^{2+} to levels that block Mn^{2+} uptake, prompting a release of mitochondrial Mn^{2+} into the cytosol. High levels of cytosolic Mn^{2+} in astrocytes activate glutamine synthetase, which removes excess glutamate (Wedler et al., 1994). However, excessive extracellular Mn^{2+} can disrupt intracellular Ca^{2+} signaling in astrocytes by competitively occupying Ca^{2+}-binding sites, thus interfering with mitochondrial Ca^{2+} homeostasis (Farina et al., 2013), which triggers astrogliosis. In addition, Mn^{3+} causes astrocyte swelling via oxidative/nitrosative pathways (Rama Rao et al., 2007). Increased Mn levels in astrocytes elevate the expression of proinflammatory signals such as iNOS and IL-6 (Moreno et al., 2008). *In vitro* studies show that Mn-treated astrocytes use larger amounts of L-arginine, which is a substrate for NO (Hazell and Norenberg, 1998). While timely expression of these signals is necessary in response to neuronal stress or cellular damage, excessive production is counter-productive, often exacerbating the toxic insult. Microarray gene expression profiling of primary human astrocytes exposed to Mn reveals an upregulation of genes encoding proinflammatory cytokines with a concurrent downregulation of genes involved in cell cycle regulation and DNA replication and repair (Sengupta et al., 2007).

The glutamate-GABA cycle (GGC) is important especially in the context of astrocyte-neuron metabolism. The AA glutamine is a precursor for the production of both glutamate and GABA (Bak et al., 2006). Deamidation of neuronal glutamine to glutamate produces ammonia, which is then transferred to astrocytes and utilized in the amidation of glutamate. Glutamine released by astrocytes is taken up by glutamatergic and GABAergic neurons that incidentally show projections in the basal ganglia and help regulate voluntary movements (Sidoryk-Wegrzynowicz and Aschner, 2013). However, in response to excessive Mn in the brain, Mn rapidly enters astrocytic mitochondria. As mentioned in the previous section, high levels of mitochondrial Mn impair cellular respiration and prevent the production and activation of glutathione peroxidase (GPx). Taken together, astrocytes appear to be particularly affected by a disruption of Mn homeostasis in the brain. This in turn could negatively affect GABAergic and glutamatergic projections in the basal ganglia, leading to the motor deficits characterizing Mn neurotoxicity.

MANGANESE IN OXIDATIVE STRESS AND NEURODEGENERATION

Although the mechanisms of Mn-induced nigrostriatal cell death are not well characterized, Mn neurotoxicity appears to be regulated by multiple factors, including oxidative injury, mitochondrial dysfunction, protein misfolding, and neuroinflammation.

Mn is a redox-active metal whose high reduction potential aids the removal of harmful byproducts of oxygen metabolism, such as superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), when as a cofactor it forms manganese superoxide dismutase (MnSOD). However, when allowed to accumulate, Mn exacerbates oxidative damage. At just 2% of body weight while consuming 20% of the total oxygen and calories, the brain is highly metabolically active and hence highly susceptible to oxidative damage. Since Mn is known to accumulate in the globus pallidus and striatum, these regions are especially vulnerable to oxidative injury because of their intense oxygen consumption, significant dopamine content, and their high content of non-heme iron. A recent study evaluating the effect of Mn on dopamine transporter (DAT)-transfected and non-transfected HEK cells shows that Mn prevents dopamine reuptake in transfected cells and also mobilizes DAT receptors from the cell surface to intracellular compartments. Consequently, dopamine-induced cell toxicity is observed (Roth et al., 2013). Our laboratory systematically characterized the cell signaling mechanisms underlying Mn-induced oxidative stress. We showed that Mn treatment in rat-derived mesencephalic dopaminergic neuronal (N27) cells increases reactive oxygen species (ROS) production (Harischandra et al., 2015a) that can sequentially activate proapoptotic processes like mitochondrial cytochrome

c release, caspase-3 activation, and DNA fragmentation. This mitochondria-dependent apoptotic cascade did not involve caspase-8 activation, but was triggered by the Mn treatment (**Figure 4**) (Latchoumycandane et al., 2005). Moreover, the redox-sensitive protein kinase C delta (PKCδ), involved in neurodegenerative disorders such as AD, prion disease, and PD (Kanthasamy et al., 2006; Ciccocioppo et al., 2008; Jin et al., 2011; Harischandra et al., 2014), is reported to be a key mediator in Mn-induced apoptosis (Anantharam et al., 2002; Latchoumycandane et al., 2005). Later studies in differentiated N27 cells also demonstrate that chronic low-dose Mn exposure impairs tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, through activation of PKCδ and protein phosphatase-2A (PP2A) activity (Zhang et al., 2011). Notably, *in vitro* and *in vivo* administration of the hydrophilic antioxidant vitamin E analog trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) reverses Mn-induced neurotoxicity and rescues dysfunctional dopaminergic transmission and Mn-induced motor coordination deficits (Milatovic et al., 2011; Cordova et al., 2013), further emphasizing the relationship between oxidative stress and Mn-related neurodegeneration.

The neurotransmitter dopamine belongs to the catecholamine and phenethylamine families. The chemical structure of catecholamines predisposes them to oxidation, and their well-characterized metabolic routes can yield quinones and free radicals, suggesting that dopamine may also serve as a neurotoxin contributing to the neurodegenerative process through oxidative metabolism. By promoting dopamine auto-oxidation, Mn potentiates dopamine toxicity in high Mn-accumulating areas of the brain (e.g., globus pallidus and striatum). Under homeostatic conditions, monoamine oxidases (MAO) enzymatically oxidize dopamine to produce dihydroxyphenylacetic acid (DOPAC), which catechol-*O*-methyltransferase (COMT) methylates to homovanillic acid (HVA). Alternatively, COMT can convert dopamine to 3-methoxytyramine (3-MT), which MAO then oxidizes to HVA. H_2O_2 is another byproduct of this dopamine turnover or deamination, generating inherent oxidative stress conditions in the nigrostriatal system. Dopamine can also be non-enzymatically oxidized by molecular oxygen, yielding H_2O_2 and quinones. These quinones also undergo intramolecular cyclization and oxidative reactions to produce neuromelanin (Graham, 1978; Hermida-Ameijeiras et al., 2004). In dopaminergic SN neurons, neuromelanin augments dopamine's vulnerability to auto-oxidation through quinone modification (Graham, 1978). Therefore, the degradation of dopamine, either enzymatically or non-enzymatically, produces H_2O_2. Two prominent Mn valence states, Mn^{2+} and Mn^{3+}, are found in biological systems. In the presence of high levels of divalent Mn^{2+}, H_2O_2 can convert to highly toxic hydroxyl radicals ($^{\cdot}OH$) via the Fenton reaction. But because of its higher oxidative state, Ali et al. (1995) found Mn^{3+} to be an order of magnitude more cytotoxic than Mn^{2+} in Mn-dosed rats. In fact, Mn^{3+}-induced dopamine oxidation, generating quinones and H_2O_2, appears to be independent of oxygen and far more rapid than that mediated by Mn^{2+} (Archibald and Tyree, 1987). Since Mn^{2+} can readily oxidize to Mn^{3+} in the human brain via superoxides, the auto-oxidation of catecholamines can only further potentiate oxidative stress.

Impairment of the cellular antioxidant machinery, causing an imbalance between ROS generation and its elimination, plays a major role in the development of certain neurodegenerative processes. The antioxidant glutathione (GSH), present in both neurons and astrocytes, provides the first line of cellular defense against ROS. GSH actively disposes of exogenous peroxides by acting as a co-substrate in reactions catalyzed by GPx, thus playing important functional roles in the CNS. Altered striatal concentrations of GSH, glutathione disulfide (GSSG), ascorbic acid, malondialdehyde (MDA), and the activities of glutathione reductase (GR) and GPx have been previously reported with Mn neurotoxicity, suggesting that an impaired neuronal antioxidant system renders the brain susceptible to Mn-induced neurotoxicity (Chen and Liao, 2002; Dukhande et al., 2006; Maddirala et al., 2015). Moreover, inhibiting GSH synthesis potentiates the Mn-induced increase in inosine, hypoxanthine, xanthine, and uric acid levels in the striatum and brainstem of aged rats (Desole et al., 2000), indicating that Mn-induced cytotoxicity is mediated through mitochondrial dysfunction. Therefore, the specific vulnerability of dopamine neurons to Mn plays a pivotal role in impairing cellular antioxidant defenses, wherein breakdown of the mitochondrial oxidative energy metabolism cascade leads to dopaminergic cell death. Excess ROS fuels the oxidation of membrane polyunsaturated fatty acids (PUFA), yielding numerous arachidonic acid (ARA) peroxidation products, including reactive aldehydes such as 4-hydroxy-*trans*-2-nonenal (4-HNE), 4-oxo-*trans*-2-nonenal (4-ONE), MDA, acrolein, F_2-isoprostanes (F_2-IsoPs), and isofurans (Esterbauer et al., 1991; Aluru et al., 2015). The lipid ARA had been released from neural membrane glycerophospholipids through the activation of cytosolic phospholipases A_2 ($cPLA_2$), which are enzymes coupled to NMDA receptors (Farooqui and Horrocks, 2007; Farooqui and Farooqui, 2011). Since most biological membranes of cells and organelles are composed of PUFA, lipid peroxidation is the main molecular mechanism involved in the oxidative damage to cell structures and in toxicity-mediated cell death. Consistent with these observations, primary rat cortical neurons exposed to a very high Mn dose (500 μM) for 6 h show structural damage to neurons and a roughly 50% increase in F_2-IsoPs levels compared to controls (Milatovic and Aschner, 2009). Likewise, in primary astrocyte cultures exposed to the same experimental conditions, F_2-IsoPs levels increased 51% compared to control cultures (Milatovic et al., 2007). However, the direct role of Mn in CNS toxicity associated with lipid peroxidation remains debatable as some investigators argue that *in vivo* administration of Mn alters cellular Ferrous (Fe^{2+}), which plays a permissive role in increasing lipid peroxidation and augmenting neuronal vulnerability (Shukla and Chandra, 1981; Chen et al., 2000, 2006).

Moreover, dopamine-derived quinones are known to bind and modify several PD-related proteins such as αSyn, DJ-1, and parkin (Conway et al., 2001; LaVoie et al., 2005; Girotto et al., 2012). However, of all the cellular macromolecules prone to oxidative damage, damaged nucleic acids are particularly hazardous due to the elevated risk of potentially irreparable

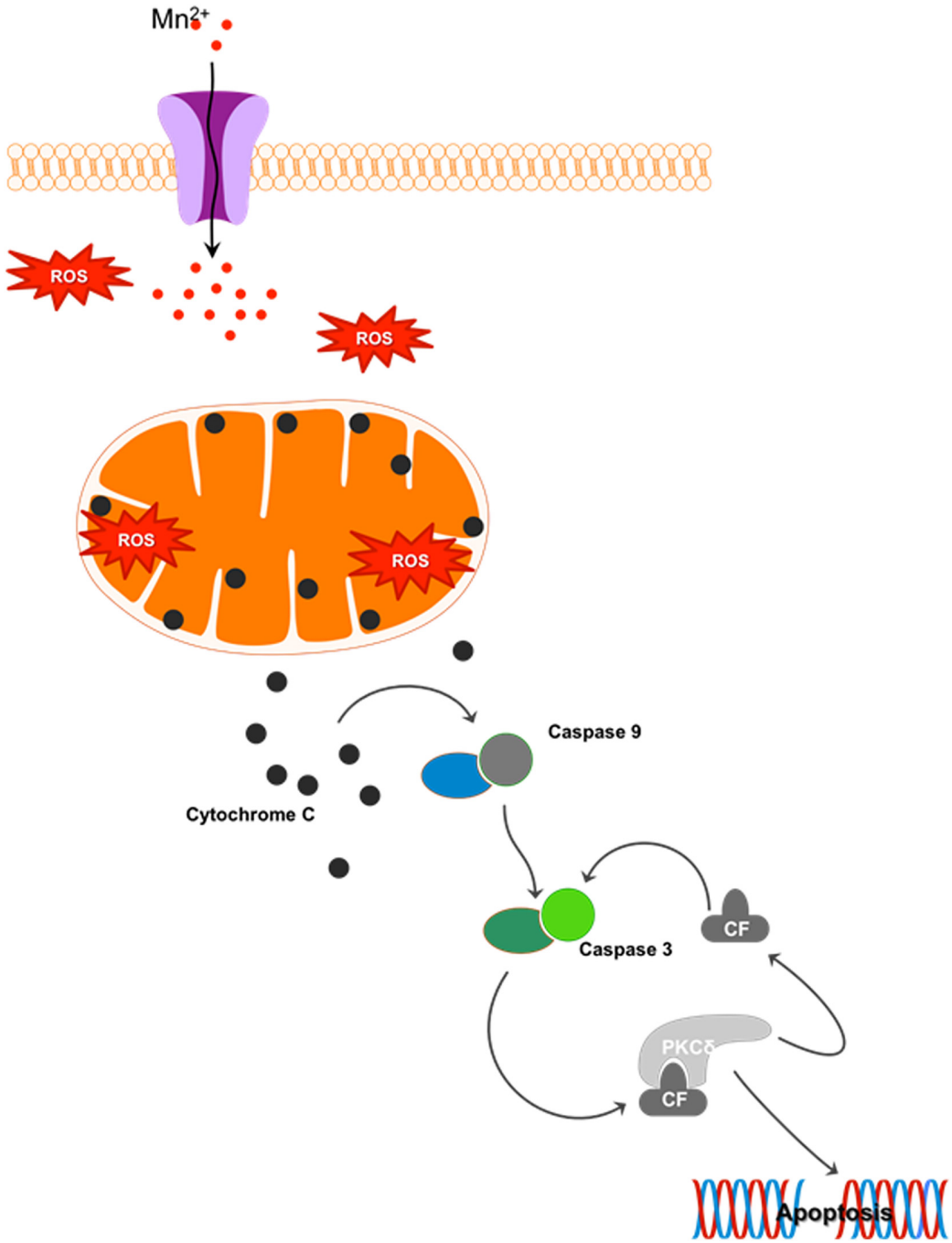

FIGURE 4 | Mn-induced cell death: Cellular Mn homeostasis is dependent upon efficient uptake, retention and excretion by various cell receptors and/or ion channels. In the presence of excess Mn, homeostatic mechanisms will down-regulate the receptors involved in the uptake of this metal, while up-regulating those involved in its release from the cell. However, during chronic exposure to high concentrations of Mn, these system checks are not maintained. Continued uptake of Mn under these conditions increases the production of reactive oxygen species (ROS), leading to mitochondrial dysfunction. Impaired mitochondrial function leads to the release of cytochrome c, activating the apoptosis initiator caspase-9, which in turn cleaves caspase-3. The cleaved fragment of caspase-3 interacts with protein kinase C delta (PKCδ), a pro-apoptotic protein. Caspase-3-mediated proteolytic cleavage of PKCδ leads to DNA fragmentation and apoptosis.

genetic base mutations. Among the five nucleobases, guanine is the most susceptible to hydroxyl radical-mediated oxidation (Cooke et al., 2003; Cerchiaro et al., 2009), which produces the well-studied oxidized DNA product 8-hydroxyguanosine (8-OHG). Interestingly, elevated 8-OHG as well as reduced 8-hydroxyl-2-deoxyguanosine (8-OHdG) have been observed in the SN and cerebrospinal fluid (CSF) of PD patients (Zhang et al., 1999; Isobe et al., 2010). In contrast, *in vitro*

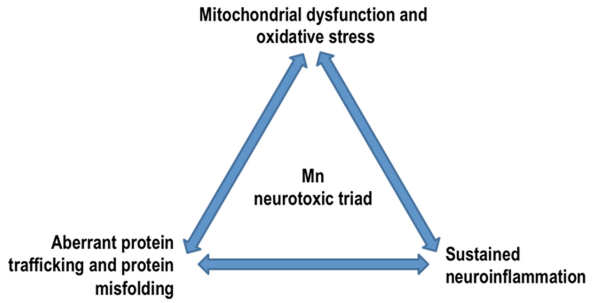

FIGURE 5 | Mn neurotoxic triad: by dysregulating key protein degradative and trafficking pathways, Mn overexposure results in a 'neurotoxic triad' comprising mitochondrial dysfunction and oxidative stress, protein misfolding and trafficking, and neuroinflammation.

studies of Mn toxicity reported increased 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) content in the DNA of dopamine-treated PC12 cells (Oikawa et al., 2006). Stephenson et al. (2013) have also shown that Mn catalyzes the auto-oxidation of catecholamines in SH-SY5Y cells with the ensuing oxidative damage to thymine and guanine DNA bases, further indicating the damaging effect of Mn-induced semi-quinone radical ions and ROS production on DNA.

Mn preferentially accumulates in mitochondria, through the mitochondrial Ca^{2+} uniporter, where it is mainly bound to mitochondrial membrane or matrix proteins (Gavin et al., 1999). Succinate, malate, and glutamate are important substrates for mitochondrial respiration, but at high concentrations, Mn^{2+} binds to these substrates effectively inhibiting mitochondrial respiration (Gavin et al., 1999). Interference in oxidative phosphorylation triggers the downstream release of inflammatory signals, leading ultimately to apoptosis. Recent evidence sheds light on Mn-induced ER stress and ER-mediated cellular apoptosis. Rats given three different doses of Mn for 4 weeks showed a dose-dependent increase in apoptotic cells in the striatum, as evidenced by chromatin condensation, as well as up-regulation of markers of mitochondrial and ER stress-mediated apoptosis (Wang et al., 2015). Furthermore, Mn induces the transcriptional and translational up-regulation of αSyn (Li et al., 2010), promoting susceptibility to Mn-induced neurotoxicity through ERK1/2 MAPK activation, NF-κB nuclear translocation, and activation of apoptotic signaling cascades leading to dopaminergic cell death (Li et al., 2010; Prabhakaran et al., 2011).

Mn affects not only cellular viability, but also various factors involved in neurotransmitter regulation. Acetylcholine esterase (AChE) is an enzyme that hydrolyses acetylcholine (ACh), thus regulating its availability in the synaptic cleft (Whittaker, 1990; Pohanka, 2012). Chronic exposure to high levels of Mn can inhibit the activity of AChE, thereby allowing ACh to accumulate in the synaptic cleft and subsequently overstimulating muscarinic and nicotinic ACh receptors. While the precise mechanism has not been determined, inhibiting AChE increases levels of ROS and RNS (Milatovic et al., 2006; Santos et al., 2012), which further leads to lipid peroxidation as well as production of citrulline, a marker of RNS activity. Ali et al. (1983) reported that Mn overexposure in rats on a low-protein diet reduces the level of GABA in the brain while increasing the animals' susceptibility to seizures. However, the effect depended on the treatment regime and age of rats. For instance, low-dose Mn given thrice weekly for 5 weeks increased GABA levels (Takagi et al., 1990). Additional mechanistic studies are needed to better understand Mn's role in GABA dysregulation. In the case of glutamate, high levels of Mn in the brain may trigger constitutive NMDA activation leading to excitotoxic-related neuronal death. Once released into the synaptic cleft, most glutamate is removed by astrocytes via the glutamate-aspartate transporter (GLAST). However, high levels of extracellular Mn^{2+} decrease the expression of GLAST and induce astrocyte apoptosis (Erikson et al., 2002). Chronic exposure to Mn can also increase the amplitude of excitatory postsynaptic potentials (EPSPs) in striatal neurons. With respect to the neurotransmitter dopamine, Ingersoll et al. (1999) demonstrated Mn transport to dopaminergic neurons via DAT. Another study done on $DAT^{-/-}$ mice receiving high doses of Mn reported a lower amount of striatal Mn compared to WT mice given the same dose. Interestingly, only the normally DAT-rich region of the striatum showed this contrasting pattern, which was not seen in areas not expressing DAT (Erikson et al., 2005). Young non-human primates exposed to a low dose of Mn twice weekly for about 9 weeks show retracted microglial processes even while dopaminergic neurons remained unchanged (Verina et al., 2011). More information is needed on the effect of this microglial disturbance on nigrostriatal neurons.

To conclude, Mn influx and efflux are tightly controlled in the body by various receptors and ion channels. However, overexposure to Mn can lead to the toxic accumulation of Mn in the brain, especially in the basal ganglia, causing hyperactivity of cortico-striatal neurons. While contradictory evidence arises from different dose regimens, in general Mn also impairs the regulation of neurotransmitters, such as dopamine, glutamate, and GABA by inhibiting the enzyme activity that regulates optimum neurotransmitter levels. High levels of glutamate and/or acetylcholine in the synaptic cleft overstimulate NMDA receptors leading to excitotoxic neuronal death. Mn may get transported into dopaminergic neurons via DAT. Excess cellular Mn^{2+} disrupts Ca^{2+} homeostasis in cells, leading to decreased dopamine production and neuronal death. Mn also causes ER and mitochondrial stress leading to neuronal apoptosis and/or gliosis. In light of the mounting evidence pointing to the deleterious effects of Mn on neurons and glia, researchers are examining the use of metal chelators and antioxidants as therapeutic interventions against manganism.

MANGANESE IN OTHER DISEASES

Until the last decade, Mn neurotoxicity was mainly associated with Parkinsonism, and very little attention had been given to its potential role in other neurodegenerative diseases. However,

with growing interest in the neurobiology of heavy metals, Mn has now been linked to other major neurodegenerative diseases such as HD and prion diseases (Choi et al., 2010; Martin et al., 2011; Kumar et al., 2015). Furthermore, gene expression in the frontal cortex of *cynomolgus macaques* exposed to various Mn doses indicates that the amyloid-β (Aβ) precursor-like protein 1 (APLP1) of the amyloid precursor family was highly up-regulated, thereby linking Mn exposure to AD (Guilarte et al., 2008). Along with this gene array analysis, immunochemistry revealed the presence of Aβ plaques and αSyn aggregates, which have been linked to PD as well as AD, and which have also been seen in the gray and white matter of Mn-exposed animals (Guilarte, 2010).

In contrast to Mn-induced Parkinsonism, the pathogenesis of HD, an autosomal dominant disorder characterized by the neurodegeneration of medium spiny neurons in the striatum, appears to involve a Mn transport deficiency (Kumar et al., 2015). Recent experiments carried out with immortalized mutant HD cell lines ($STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$) show reduced TfR levels and substantial deficits in Mn uptake (Williams et al., 2010). In follow-up studies, YAC128 HD transgenic mice accumulated less Mn in their basal ganglia, including their striata, which are focal regions for both HD neuropathology and Mn accumulation (Madison et al., 2012). Furthermore, transition metal analysis of HD patients has shown significantly increased iron together with significantly decreased cortical copper and Mn (Rosas et al., 2012), further supporting the role of Mn in HD.

Prion protein (PrP) is widely known for its association with transmissible spongiform encephalopathies (TSE), a class of neurodegenerative diseases caused by the accumulation of an abnormal isoform of the prion protein known as PrP^{Sc} (Jin et al., 2015a). The cellular prion protein PrP^{C} has a high-binding affinity for divalent metals. In fact, above-normal Mn content has been detected in the blood and brains of humans infected with Creutzfeldt–Jakob disease (CJD), in scrapie-infected mice, and in bovines infected with bovine spongiform encephalopathy (BSE) (Wong et al., 2001; Hesketh et al., 2007; Hesketh et al., 2008). The binding of Mn to prion protein mitigates Mn's neurotoxicity during the early acute phase of Mn exposure (Choi et al., 2007). However, prolonged Mn exposure alters the stability of prion proteins without changing gene transcription (Choi et al., 2010), suggesting that Mn contributes to prion protein misfolding and prion disease pathogenesis. Interestingly, prion proteins survive significantly longer in a Mn-enriched soil matrix (Davies and Brown, 2009), a finding with important implications for the environmental transmissibility of PrP^{Sc}. The role of Mn in TSE was further validated by our lab's discovery that it enhances the ability of the pathogenic PrP^{Sc} isoform to regulate Mn homeostasis (Martin et al., 2011) and by Davies and Brown (2009) reporting that Mn increases the infectivity of scrapie-infected cells. Therefore, deepening our understanding of how metals interact with disease-specific proteins will provide further insight into the pathogenesis and potential treatment of neurodegenerative diseases.

FUTURE DIRECTIONS

Our review of existing literature related to Mn overexposure and associated health issues has revealed genetic, sex- and age-related susceptibilities, signaling cascades involved in Mn neurotoxicity, and comparisons between PD and other motor disorders. Yet, many aspects of Mn overexposure and homeostasis remain largely understudied. For example, early childhood exposure to drinking water containing elevated Mn levels has been conclusively shown to compromise certain aspects of memory and learning; however, how absorption of excessive levels of Mn via the GIT leads to cognitive deficits (a CNS component) is still largely unknown. Secondly, given that the nasal tract and GIT are two well-known microbial environments, does overexposure to Mn via inhalation or ingestion alter the community composition of nasal or gut microbes or otherwise cause dysbiosis? Are changes in microbial populations offset by other lines of host defense against Mn toxicity or do these changes exacerbate the neuropathology? Thirdly, MRI and positron emission tomography (PET) on Mn-exposed individuals have shown changes in brain Mn accumulation and dopamine and GABA neurotransmitter levels. Yet despite the strong links between manganism and Parkinsonism, no case control study has examined the extent of elevated Mn accumulation in the brain and its associated neuropathology, including the presence of Lewy bodies/neurites and elevated phospho-α-synuclein expression. Additionally, longitudinal studies examining the immediate, intermediate and long-term effects of elevated Mn exposure on children and adults are needed to identify age- and sex-specific susceptibilities, and potential biological, psychological and cognitive biomarkers. Lastly, studies systematically identifying Mn exposure limits based on age, sex, and exposure duration as well as changes in signaling cascades associated with metal homeostasis and protein aggregation need to be conducted.

CONCLUSION

Chronic exposure to excessive Mn induces various neurological and psychiatric symptoms. While the body can efficiently remove excess Mn, primarily through the gut and liver, the brain cannot. Because of direct passage via the nasal neuroepithelium, inhaling large doses of Mn can lead to its accumulation in the brain's basal ganglia. Astrocytes are particularly sensitive to Mn toxicity and may compound neuroinflammation by releasing pro-inflammatory cytokines in response to excess Mn. Mn can also bind to substrates of oxidative phosphorylation, thus inhibiting mitochondrial respiration and thereby augmenting oxidative stress. Chronic exposure to Mn causes benign α-synuclein monomers, present in all neurons, to undergo a conformational change to the oligomeric structures that are toxic to neurons. Additionally, Mn dysregulates key protein degradative and trafficking pathways including proteasomes, autophagy, and endosomal trafficking. Taken together, we entertain the notion

that a 'neurotoxic triad,' comprising mitochondrial dysfunction and oxidative stress, protein misfolding and trafficking, and neuroinflammation, plays a major pathogenic role in Mn neurotoxicity (**Figure 5**). Beyond its effects on the CNS, excess Mn also interferes with the body's iron metabolism and can cause kidney failure. Early detection and chelation therapy can effectively reverse the harmful effects caused by this metal; however, if it progresses untreated, it can cause severe neurological and physiological defects. As with any metal, the bioaccumulation and teratogenic effects of Mn remain a risk, yet this aspect has not been studied in detail. Similarly, an in-depth study of Mn's role in protein misfolding and the upregulation of genetic markers for various neurological diseases in humans must be conducted. By combining the results of epidemiological surveys with human case studies as well as mechanistic studies done in *in vitro* and in animal models of Mn toxicity, we will eventually decipher the causes and symptoms of neurodegeneration caused by Mn toxicity well enough to develop effective therapeutic strategies that can be readily used against environmentally linked PD and related chronic neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

DSH and SG conceived and wrote the article. GZ, HJ, AK, VA, and AGK provided intellectual input for review content and edited the manuscript. All authors read and approved the manuscript.

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PERK-Mediated Unfolded Protein Response Activation and Oxidative Stress in PARK20 Fibroblasts

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PARK20, an early onset autosomal recessive parkinsonism is due to mutations in the phosphatidylinositol-phosphatase Synaptojanin 1 (Synj1). We have recently shown that the early endosomal compartments are profoundly altered in PARK20 fibroblasts as well as the endosomal trafficking. Here, we report that PARK20 fibroblasts also display a drastic alteration of the architecture and function of the early secretory compartments. Our results show that the exit machinery from the Endoplasmic Reticulum (ER) and the ER-to-Golgi trafficking are markedly compromised in patient cells. As a consequence, PARK20 fibroblasts accumulate large amounts of cargo proteins within the ER, leading to the induction of ER stress. Interestingly, this stressful state is coupled to the activation of the PERK/eIF2α/ATF4/CHOP pathway of the Unfolded Protein Response (UPR). In addition, PARK20 fibroblasts reveal upregulation of oxidative stress markers and total ROS production with concomitant alteration of the morphology of the mitochondrial network. Interestingly, treatment of PARK20 cells with GSK2606414 (GSK), a specific inhibitor of PERK activity, restores the level of ROS, signaling a direct correlation between ER stress and the induction of oxidative stress in the PARK20 cells. All together, these findings suggest that dysfunction of early secretory pathway might contribute to the pathogenesis of the disease.

Keywords: PARK20, PERK (PKR-like endoplasmic reticulum kinase), oxydative stress, ER stress, Synaptojanin 1, membrane trafficking, mitochondrial dysfunction, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (Gao and Hong, 2011; Cannon and Greenamyre, 2013; Beitz, 2014; Feng et al., 2015). A combination of environmental and genetic factors has been considered to concur to the neuronal death. However, the exact molecular mechanisms are still unknown. Notwithstanding, the alteration of

mitochondrial function (Winklhofer and Haass, 2010; Pilsl and Winklhofer, 2012), of reactive oxygen species (ROS) homeostasis (Gaki and Papavassiliou, 2014; Al Shahrani et al., 2017; Guo et al., 2018; Paladino et al., 2018) as well as the dysregulation of protein folding control and/or autophagic flux (McNaught and Olanow, 2006; Malkus et al., 2009; Karabiyik et al., 2017; Remondelli and Renna, 2017) have been implicated in PD pathogenesis.

Among genetic PD, PARK20 is a rare autosomal recessive juvenile Parkinson's form due to mutations in Synaptojanin1 (Synj1), a phosphatidylinositol phosphatase (PtdInsPP) (Krebs et al., 2013; Quadri et al., 2013; Olgiati et al., 2014). The homozygous R258Q mutation was almost simultaneously reported in three unrelated families from Iran and Italy (Krebs et al., 2013; Quadri et al., 2013; Olgiati et al., 2014). Subsequently, the p.R459P mutation was found in an Indian family (Kirola et al., 2016); and, more recently, another Iranian kindred has been described with the p.R839C mutation (Taghavi et al., 2018). Finally, a frameshift mutation (p.S552Ffs*5) in heterozygous state with the benign p.T1236M missense variant has been identified in one late onset PD patient from Moroccan consanguineous parents (Bouhouche et al., 2017), correlating Synj1 lesions to the risk of PD development.

Synj1 is a highly conserved PtdInsPP existing in two isoforms: the 145-kDa neuronal isoform and the ubiquitous 170-kDa isoform (Ramjaun and McPherson, 1996). Synj1 function relies on two sequential PtdInsPP domains: the N-terminal Sac1 and the central 5-phosphatase domains (5′-PP) (Di Paolo and De Camilli, 2006). The Sac1 domain of Synj1 acts on PtdIns 3- and 4-monophosphate, which are enriched in the endosomal and Golgi membranes respectively (Guo et al., 1999). Instead, the 5′-PP domain of Synj1 dephosphorylates phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] located in the plasma membranes (McPherson et al., 1996; Cremona et al., 1999). Additionally, the Synj1 protein also contains a COOH-terminal proline rich region that retains the ability to interact with SH3 domains of a variety of proteins that regulate its subcellular localization and function (McPherson et al., 1996; Dittman and Ryan, 2009).

Thanks to its double enzymatic activity, Synj1 exerts multiple roles in dependence on the cell context. In nerve terminals, Synj1 participates to the control of synaptic vesicles retrieval (McPherson et al., 1996; Song and Zinsmaier, 2003; Mani et al., 2007) and cooperates with DNAJC6, another PD-causative gene (PARK19), in the process of clathrin disassembly from synaptic vesicles during endocytosis (Chang-Ileto et al., 2011; Edvardson et al., 2012). Proper Synj1 activity is essential to control homeostasis and function of early endocytic pathways in different cell types, including neuronal cells (Fasano et al., 2018). Consistently, early endosomes of PARK20 fibroblasts resulted enlarged and the recycling trafficking impaired (Fasano et al., 2018). On the other hand, unbalanced Synj1 expression is significantly involved in a number of neurological and psychiatric disorders, such as: Bipolar Disorder (Saito et al., 2001; Stopkova et al., 2004), Down's Syndrome and Alzheimer's Disease (Berman et al., 2008; Voronov et al., 2008; Chang and Min, 2009; Cossec et al., 2012; Martin et al., 2014), unraveling a critical role in neurons.

The p.R258Q mutation into the Synj1 Sac1 domain was shown to abolish either the 3- or 4- phosphatase activity, while it does not affect the 5-phosphatase activity (Krebs et al., 2013). Therefore, the loss of Sac1 function could alter the rate of PtdIns3P and PtdIns4P, two crucial PtdInsPs for the control of structure and function of endosomal (Efe et al., 2005; Di Paolo and De Camilli, 2006) and ER or Golgi complex membranes (De Matteis and D'Angelo, 2007; D'Angelo et al., 2008), respectively. Moreover, as we have recently shown, Sac1 domain is necessary for proper endosomal trafficking and at least 50% of its activity is required to ensure correct functionality (Fasano et al., 2018).

Here, we investigated whether p.R258Q mutation in the Sac1 domain of Synj1 could also influence vesicular trafficking at the early stages of the secretory pathway. Our experiments show that the ER exit machinery and the ER-to-Golgi trafficking are markedly compromised in p.R258Q mutated cells. As a consequence, PARK20 fibroblasts accumulate larger amounts of cargo proteins within the ER. This condition, referred to as ER stress, activates the PERK/eIF2α/ATF4/CHOP pathway of the Unfolded Protein Response (UPR) and induces oxidative stress and mitochondrial damage.

MATERIALS AND METHODS

Cell Cultures

Fibroblasts were derived directly from the skin punch biopsies of the two Italian patients carrying the p.R258Q mutation at homozygous state (Quadri et al., 2013; Olgiati et al., 2014). A written informed consent was obtained from each patient. As control cells, primary adult Human Dermal Fibroblasts (HDF) were purchased from Sigma-Aldrich. PARK20 fibroblasts and HDF were grown in one ready-to-use Fibroblast Growth Medium (FGM from Sigma-Aldrich) at 37°C and 5% CO_2 in humidified atmosphere. Experiments were performed on both cell lines at similar culture passages (P5-P6). When indicated, cells were starved in Fibroblasts Basal Medium (FBM from Sigma-Aldrich), which does not contain FBS and growth factors supplement. Drug treatments were performed with 1 μM GSK2606414 (Calbiochem) or 500 nM Thapsigargin (Sigma-Aldrich) for the indicated time.

Immunofluorescence

Cells seeded on glass cover slips were washed in phosphate-buffered saline (PBS), fixed in PBS-4 % paraformaldehyde and permeabilized 30 min in PBS containing 0.5% BSA, 0.005% saponin and 50 mM NH_4Cl. Cells were immunostained with the following primary antibodies: rabbit polyclonal anti-ERGIC-53 (α-CT) (Spatuzza et al., 2004), mouse monoclonal anti-GM130 (BD Biosciences), rabbit polyclonal anti-Giantin (Abcam), rabbit plyclonal anti-KIAA0310 (Bethyl Laboratories), rabbit polyclonal anti-Sec31a, rabbit plyclonal anti-Sar1 (Millipore), rabbit polyclonal anti-collagen IV (Rockland immunochemicals), mouse monoclonal anti-KDEL (StressGen). Primary antibodies were detected with Alexa 488- and Cy3-conjugated antibodies (Jackson Immuno Research Laboratories).

For mitochondria staining, cells were incubated for 30 min at 37°C with 200 nM Mitotracker Red CMXRos (Invitrogen-Molecular Probes) in FBM before fixing in cold acetone for 5 min on ice. Images were acquired on a laser scanning confocal microscope (TCS SP5; Leica MicroSystems or LSM 510 Meta; Zeiss MicroSystems) equipped with a plan Apo 63X, NA 1.4 oil immersion objective lens. Quantitative analysis was performed on a minimum of 30 cells by setting the same threshold of fluorescence intensity in all the samples analyzed.

Co-localization analyses and the mean intensity fluorescence quantification were carried out by using either the Leica SP5 or Zeiss software or the ImageJ program as previously described (Paladino et al., 2008; Gorrasi et al., 2014; Iorio et al., 2018; Ranieri et al., 2018). Briefly, the number of co-localized pixels was normalized for the total fluorescent pixels in the image. The degree of colocalization was assessed by calculating the Pearson's correlation coefficient. Mean fluorescence intensity was measured in Region of Interest (ROI) of equal area in control and PARK20 samples. The number and size of SEC31a and SEC16a fluorescent spot was measured by using the ImageJ program. The distance from the nucleus of ERGIC-53 fluorescent spots was measured by using the scale bar drawing tool of Leica SP5 software.

Electron Microscopy

Cells were fixed in 1% glutaraldehyde dissolved in 0.2 M HEPES buffer (pH 7.4) for 30 min at room temperature and then post-fixed with a mixture of 2% OsO4 and 100 mM phosphate buffer (pH 6.8) (1 part 2% OsO4 plus 1 part 100 mM phosphate buffer) for 25–30 min on ice. Then, the cells were washed three times with water and incubated with 1% thiocarbohydrizide diluted in H2O for 5 min, incubated in a mixture of 2% OsO 4 and 3% potassium ferrocyanide (1 part 2% OsO4 plus 1 part 3% potassium ferrocyanide) for 25 min on ice and overnight at 4°C in 0.5% uranyl acetate diluted in H2O. After dehydration in graded series of ethanol, the cells were embedded in epoxy resin and polymerized at 60°C for 72 hr. Thin 60 nm sections were cut at the Leica EM UC7 microtome. EM images were acquired from thin sections using a FEI Tecnai-12 electron microscope equipped with a VELETTA CCD digital camera (FEI, Eindhoven, Netherlands).

Western Blotting

Actively growing cells seeded on 60 mm dishes were starved in FBM for 18 h prior to be subjected to the indicated treatments. Cells were then harvested in lysis buffer (10 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100) supplemented with protease and phosphatase inhibitor cocktail (Roche). Equal amounts of protein extracts were analyzed by 8 or 10% SDS-PAGE and transferred on Protran nitro-cellulose membranes (Schleicher and Schuell). Membranes were blocked either in PBS containing 10% non-fat dry milk and 0.1% Tween-20, or in TBS containing 5% BSA and 0.1% Tween-20, depending on the antibody used.

Membranes were cut in stripes according to the molecular weight expected for the single proteins analyzed, incubated with the primary followed by secondary antibodies and then visualized by ECL reaction (Amersham International) (see **Supplementary Figures S3**, **S4**). The following primary antibodies were used: rabbit monoclonal anti-PERK (Cell Signalling Technology), rabbit polyclonal anti-eIF2α and anti-phospho-eIF2α (Cell Signalling Technology), rabbit monoclonal anti-ATF4 (Abcam), mouse monoclonal anti-GADD153 (Santa Cruz Biotechnology), mouse monoclonal anti-HO1 (Santa Cruz Biotechnology) and mouse monoclonal anti-α Tubulin (Santa Cruz Biotechnology). HRP-conjugated IgG (Jackson Immuno Research Laboratories) were used as secondary antibodies. Filters were exposed to ChemiDoc MP System (Bio-Rad Laboratories Inc.) and the densitometry analysis of autoradiographs was performed by the ImageJ program on three independent experiments.

Oxidative Stress Assays

10^6 cells for each treatment were disposed in a well of BD Falcon 96-well black plates and starved in FBM for 18 h prior to be subjected to the indicated treatments. Cytosolic ROS were quantified by a fluorescence microplate reader [Tecan Infinite 200 Pro] using dihydrorhodamine 123 (DHR 123) probe (Santa Cruz Biotechnology), a cell-permeable non-fluorescent substance that undergoes intracellular oxidation in the presence of ROS. In detail, cells were incubated for 1 h with 50 μM of DHR123/HBSS and then washed two times with freshly prepared Hank's balanced salt solution. Subsequently, formation of DHR 123 has been monitored by fluorescence spectroscopy using excitation and emission respectively of 500 ʎ nm and 536 ʎ nm. In some experiments, cells were pre-incubated with 1 μM GSK2606414 for 2 h, before measurements. Fluorescence signals have been recorded using Tecan i-control software and expressed as arbitrary units.

In another approach, we measured the ROS on single-cells. To this purpose, cells grown on glass bottom dishes were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μM) for 10 min at 37°C in culture medium without serum and, then, imaged *in vivo* in CO_2 independent medium as previously described (Piccoli et al., 2013). Images were collected by a Zeiss confocal LSM510 using Ar–Kr laser beam (λex 488 nm); same laser power and same settings were used for control and patient fibroblasts in all experimental conditions. Data are expressed as arbitrary units of fluorescence and reported as mean ± SD from three independent experimental conditions.

For NADPH oxidase activity measurement, the lucigenin-enhanced chemioluminescence assay was used to determine NADPH oxidase-mediated superoxide radical (O_2^-) production as previously described (Carrizzo et al., 2017; Schiattarella et al., 2018). Cells, cultured in 100 mm dishes, were detached using 0.25% trypsin/EDTA (1 mmol/l), washed with PBS, and resuspended in modified HEPES buffer containing (mmol/l) NaCl 140, KCl 5, MgCl2 0.8, CaCl2 1.8, Na2HPO4 1, HEPES 25 and 1% glucose, pH 7. Subsequently, cells were homogenated using VWR pellet mixer [#431-0100] and 100 μg of extract were distributed on a 96-well microplate. The reaction was started by the addition of NADPH (0.1 mmol/l) to each well (250 μl final volume) and lucigenin (5 μmol/l). The luminescence was measured using Tecan Infinite M200 multimode microplate fluorometer at 37°C every 10 s for 60 min. Each experiment

was performed in triplicate. In some experiments, cells were pre-incubated with 1 μM GSK2606414 for 2 h, before measurement of luminescence.

RT-PCR and XBPI Splicing Assay

One microgram of DNAse-treated total RNA was retro-transcribed with the Easy-script plus cDNA synthesis Kit (abm) according to manufacturer instructions. Semi-quantitative PCR was performed on 3 μl of cDNA with the following primers Bip/Grp78-forward: 5′-CTG GGT ACA TTT GAT CTG ACT GG-3′; Bip/Grp78-reverse: 5′-GCA TCC TGG TGG CTT TCC AGCCAT TC-3′; GAPDH-forward: 5′-GAA GGT GAA GGT CGGAGT C-3′; GAPDH-reverse: 5′-GAA GATGGT GAT GGG ATTTC-3′ (Amodio et al., 2011). XBPI splicing assay was performed as previously described (Eletto et al., 2016) by using the following primers: 5′-A AAC AGA GTA GCA GCT CAG ACT GC-3′ and 5′-C CTT CTG GGT AGA CCT CTG GGA G-3′. The resulted un-spliced and spliced XBP1 mRNA were separated by gel electrophoresis on 3% agarose gel. Ethidium bromide-stained amplicons were quantified by densitometry with ImageJ software.

Statistical Analysis

Data are expresses as mean ± SD. All statistical analyses using Student's *t*-test and histograms were completed with Prism statistical software (Graphpad, La Jolla, CA, United States) and differences were considered statistically significant when $P < 0.05$.

RESULTS

PARK20 Fibroblasts Show Unbalanced ER-to-Golgi Trafficking and Abnormal Structure of Golgi Membranes

To test whether membrane trafficking from the ER to the Golgi complex was affected by the p.R258Q mutation, we analyzed fibroblasts derived from homozygous R258Q/R258Q PARK20 patients and from healthy individuals.

Dynamics of membrane trafficking at the early steps of the secretory pathway were analyzed by looking at the intracellular distribution of vesicles carrying the cargo receptor ERGIC-53 (Appenzeller et al., 1999). Normally, the ERGIC-53 protein cycles between the ER and the Golgi complex (Appenzeller et al., 1999) and ERGIC-53 containing vesicles show their typical punctuate distribution depicted by higher concentration in the region closed to the *cis*-Golgi membranes, which in turn are labeled with the resident protein GM130 in wild-type cells (**Figure 1A**, wt). Instead, in patient fibroblasts ERGIC-53 vesicles were reduced both in size and fluorescence intensity (**Figure 1A**, PARK20). In addition, they are delocalized throughout the cytoplasm at higher distance from the perinuclear region with a mean value of 12.3 ± 3.4 μm in the PARK20 cells vs. 31.8 ± 4.3 μm in the control cells (**Figure 1C**). Interestingly, ERGIC53 vesicles redistribution pattern in the PARK20 cells overlapped with the membrane network of the ER, as shown by the fluorescence detected by the anti-KDEL antibody, which label ER resident proteins bearing the KDEL retrieval sequence (**Figure 1B**). In addition, we also detected dramatic changes in the organization of Golgi membranes of PARK20 fibroblasts (**Figures 1A,D**). Both the *cis*-Golgi membranes labeled by the resident protein GM130 (**Figure 1A**) and the overall Golgi architecture revealed by the structural Golgi protein giantin (**Figure 1D**) were more dispersed and relocated in tubular structures extending from the nucleus to the cell edge in the PARK20 cells with respect to control (**Figures 1A,D**). The ultrastructural analysis further showed that the Golgi complex is scattered throughout the cell in PARK20 fibroblasts (**Figure 1E**, arrows). Moreover, while GM130 co-localized almost completely with giantin in control cells, they resulted partially co-distributed in patient cells (**Figure 1D**).

Since both GM130 and giantin are involved in the ER-to-Golgi trafficking (Alvarez et al., 2001), these results further suggest that PARK20 cells undergo unbalanced trafficking at the early steps of the secretory pathway.

PARK20 Fibroblasts Show Reduced Formation of COPII Carrier Vesicles

The results described above prompted us to test whether the abnormal organization of post-ER compartments observed in the PARK20 cells was the result of reduced flow of carrier vesicles budding from the ER. Formation of transport vesicles from the ER membranes requires the assembly of the vesicular coat (COPII) at specific ER membrane domains defined ER exit Sites (ERESs), recognized by the presence of the endogenous Sec16 protein (isoform A). As a rule, Sec16 recruits COPII components for their assembly at the ERESs (Watson et al., 2006). As expected, these latter are visible as punctuate structures dispersed throughout the cytoplasm (**Figure 2A**, Sec16/wt). Instead, in PARK20 cells the number of puncta of Sec16 fluorescence are reduced (**Figures 2A,B**; Sec16/PARK20), indicating that the number of ERESs is considerably decreased in PARK20 cells. As a consequence, the number of COPII vesicles, revealed by antibody recognizing Sec31 (**Figure 2A**, Sec31/wt), a component of the outer layer of the COPII vesicles, was also reduced (**Figures 2A,B**; Sec31/PARK20). Moreover, Sec16 co-localized with Sec31 at the same extent as in control cells (**Figure 2A**, merge), suggesting that Sec16 still organizes COPII assembly at ERESs, but with less efficiency.

Thus, our results strongly indicate that the Synj1 activity localized in the Sac1 domain of the protein is essential for the proper function of COPII. In particular, the reduced number of ERES found in the PARK20 cells indicates that altered phosphatase activity of the Synj1 Sac1 domain reduces the amount of ER exit sites, thus biasing the assembling and/or the stability of COPII vesicles.

The Secretion of Collagen IV Is Impaired in PARK20 Fibroblasts

To test whether reduced formation of ERESs could have an effect on the rate of cargo proteins transport from the ER to the Golgi complex, we examined the level of distribution of endogenous collagen IV (COLIV) along the secretory pathway in PARK20

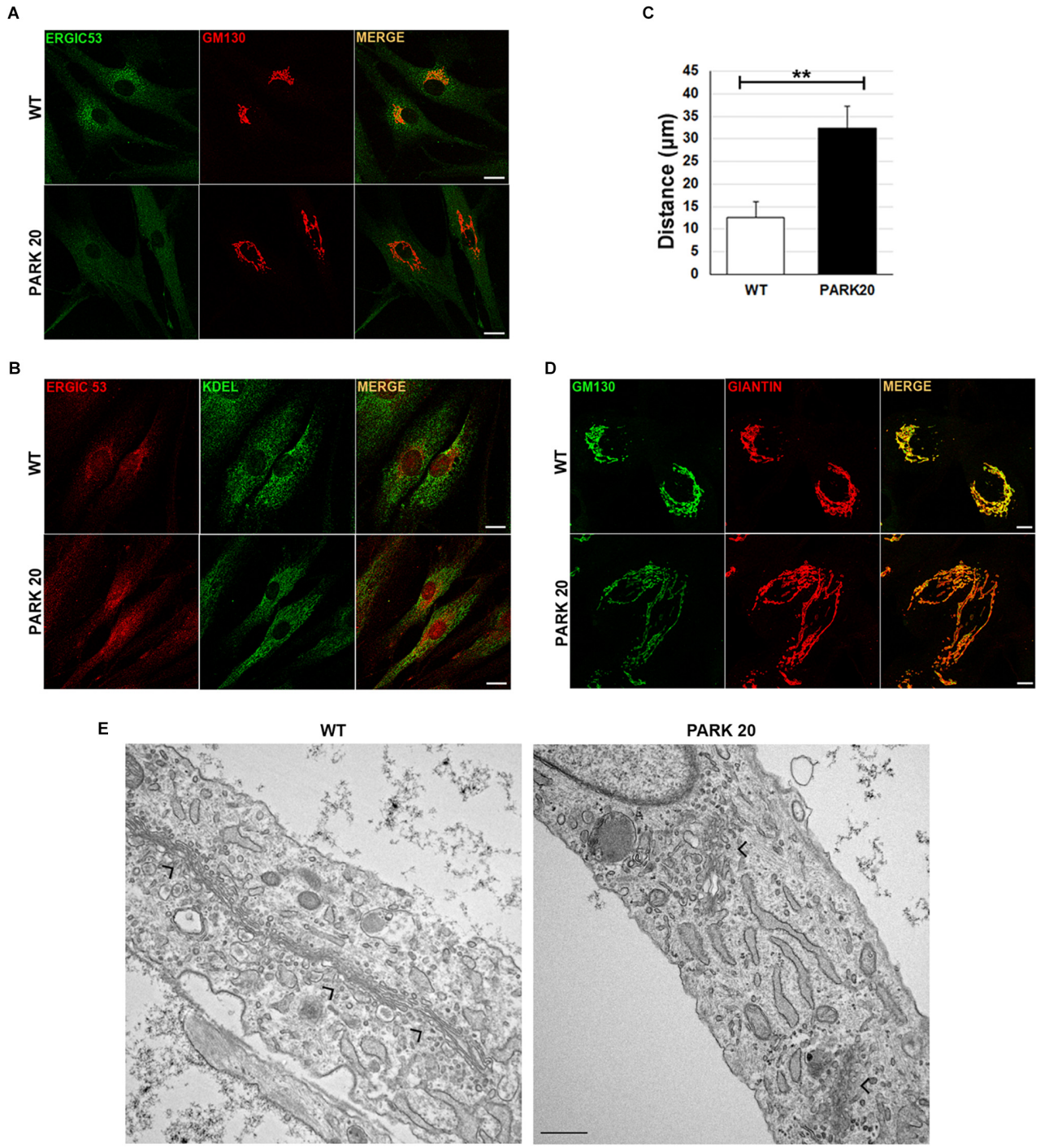

FIGURE 1 | Protein markers of the early secretory pathway are differently distributed in WT and PARK20 fibroblasts. **(A,B,D)** HDF (WT) and PARK20 cells were seeded on glass coverslips, fixed and processed for immunofluorescence with the indicated antibodies. Images were collected by confocal microscope. Scale bars: 10 μm. **(C)** Histogram shows the distance (mean ± SD in μM) from the nucleus of ERGIC-53 fluorescent spots. $N \geq 30$. $^{**}p \leq 0.01$, Student's *t*-test. **(E)** Representative ultrastructural images of WT and PARK20 cells. Scale bar: 1 μm; arrows indicate the position of Golgi complex.

cells vs. control cells (**Figure 3A**). Typically, in normal fibroblasts COLIV is secreted from the cell and accumulates in structures located in the interstitial space out of the cell (**Figure 3A**, COLIV/wt) (Amodio et al., 2016). In PARK20 fibroblasts, COLIV was mainly found within intracellular membranes resembling the ER network stained by the KDEL antibody (**Figure 3A**: compare PARK20 pro-COLIV to KDEL), indicating that COLIV secretion is reduced. In PARK20 fibroblasts, COLIV did not accumulate

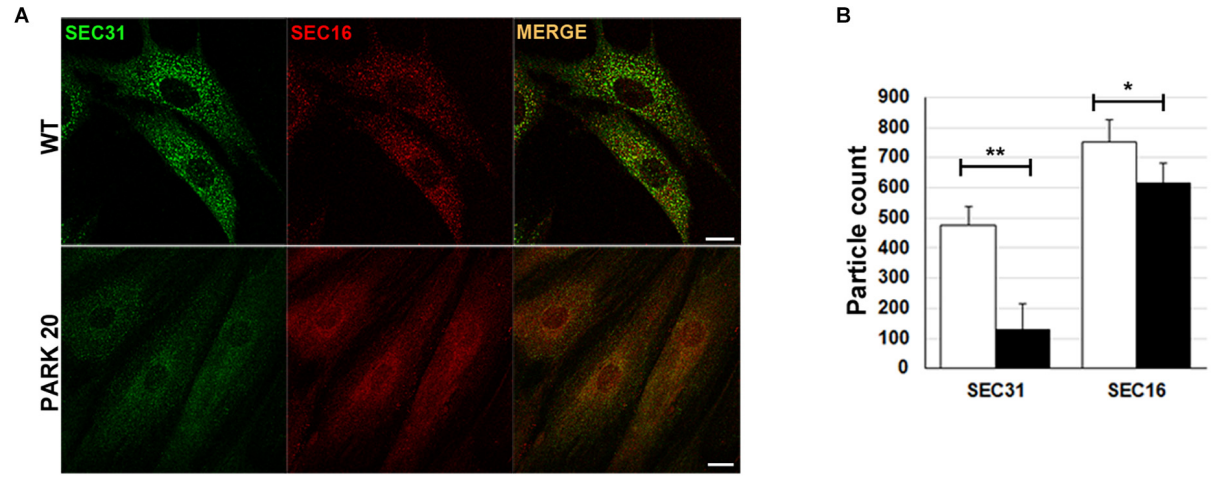

FIGURE 2 | The formation of COPII-coated vesicles are reduced in PARK20 fibroblasts. **(A)** HDF (WT) and PARK20 cells seeded on glass coverslips were fixed, stained with the indicated antibodies and analyzed by confocal immunofluorescence. Scale bars: 10 μm. **(B)** Histogram shows particle count (mean ± SD) of Sec31 (green) and Sec16 (red) fluorescent spots analyzed by Image J. $N \geq 30$. *$p \leq 0.05$, **$p \leq 0.01$, Student's *t*-test.

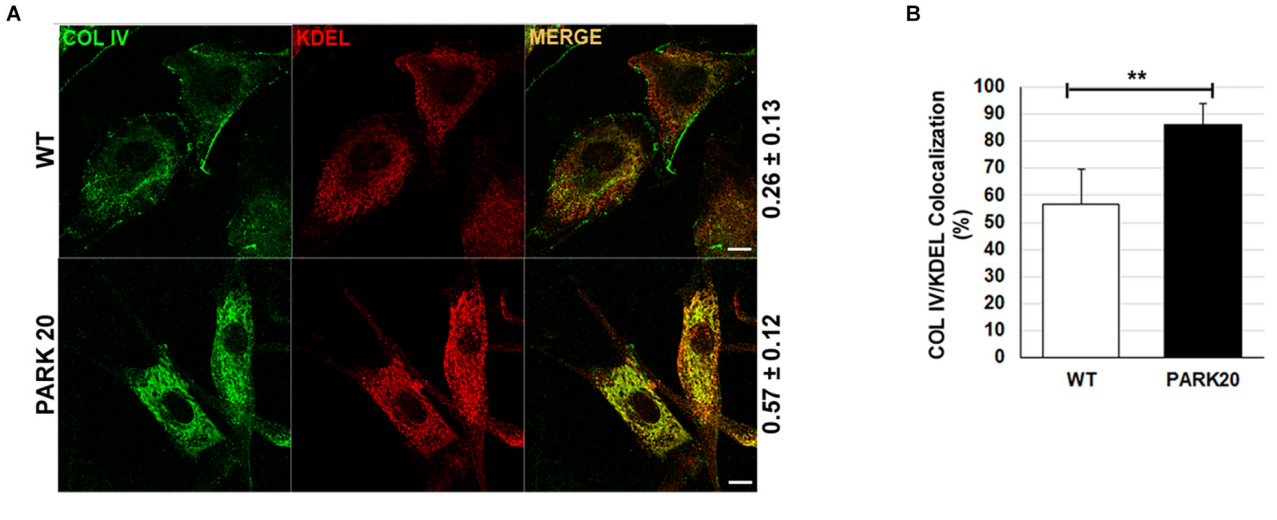

FIGURE 3 | The secretion of collagen IV is impaired in PARK20 fibroblasts. **(A)** HDF (WT) and PARK20 cells were seeded on glass coverslips, processed for immunofluorescence and analyzed by confocal imaging. Numbers on the right refers to Pearson's correlation coefficient, quantified with Leica SP5 software (mean ± SD). $p \leq 0.01$, Student's *t*-test; $N \geq 30$; scale bars: 10 μm. **(B)** Histogram shows the percent of collagen IV pixels colocalizing with KDEL pixels (mean ± SD) analyzed by Leica SP5 software. **$p \leq 0.01$, Student's *t*-test.

outside the cell (**Figure 3A**, PARK20), but it was mainly found within intracellular membranes, in structures that resemble the ER network stained by the KDEL antibody (**Figure 3A**: compare PARK20 pro-COLIV to KDEL). Consistently, by analyzing the rate of colocalization with ER resident proteins labeled by the anti-KDEL antibody, we found an increase in procollagen IV (pro-COLIV) colocalization with ER membranes: 87.1 ± 7.7% in the Synj1 mutated cells compared to 56.6 ± 13.3% in control cells (**Figure 3B**). In line with the immunofluorescence assays, we found that secretion of COLIV by PARK20 fibroblasts into culture medium was strongly reduced in comparison to control fibroblasts (**Supplementary Figure S1A**). All these data indicate that secretion of COLIV in the PARK20 cells was almost completely inhibited presumably as a consequence of the reduced function of the ER exit machinery. Moreover, a slight but appreciable reduction of total protein secretion is observed in PARK20 fibroblasts as well as a reduction of total delivery to the surface (**Supplementary Figure S1**), all suggesting an impairment of secretory trafficking.

The PERK/eIF2a/ATF4 Branch of the Unfolded Protein Response Is Activated in PARK20 Fibroblasts

The presence of higher amounts of proteins retained into the ER, prompted our investigation into whether such accumulation

could activate the ER stress and, as a consequence, the UPR (Walter and Ron, 2011). Therefore, we analyzed the activation state of components of the UPR signaling, such as PERK and IRE1, and the expression level of typical marker of ER stress (Franceschelli et al., 2011; Hiramatsu et al., 2011). To determine PERK activation, we analyzed by western blotting the phosphorylated PERK form (p-PERK) and eIF2α form (p-eIF2a) expressed in the cell extracts obtained from control and PARK20 fibroblasts (**Figure 4**). In particular, PERK phosphorylation was recognized throughout immunoblots (**Figure 4A**) showing the band-shift of p-PERK in western blot analyses as a consequence of the higher molecular weight acquired by the auto-phosphorylation (Harding et al., 1999; Eletto et al., 2016). As expected, in control cells we did not detect p-PERK form in basal conditions, but only after treatment with the UPR inducer thapsigargin (TG) (**Figure 4A**). Strikingly, p-PERK was highly detectable in uninduced PARK20 cells, suggesting that the PERK branch of the UPR was constitutively turned on in PARK20 fibroblasts (**Figure 4A**). As in control fibroblasts, cell exposure to the PERK inhibitor GSK abolished kinase activity of the PERK protein (**Figure 4A**), confirming that this pathway is activated in Synj1 mutated cells.

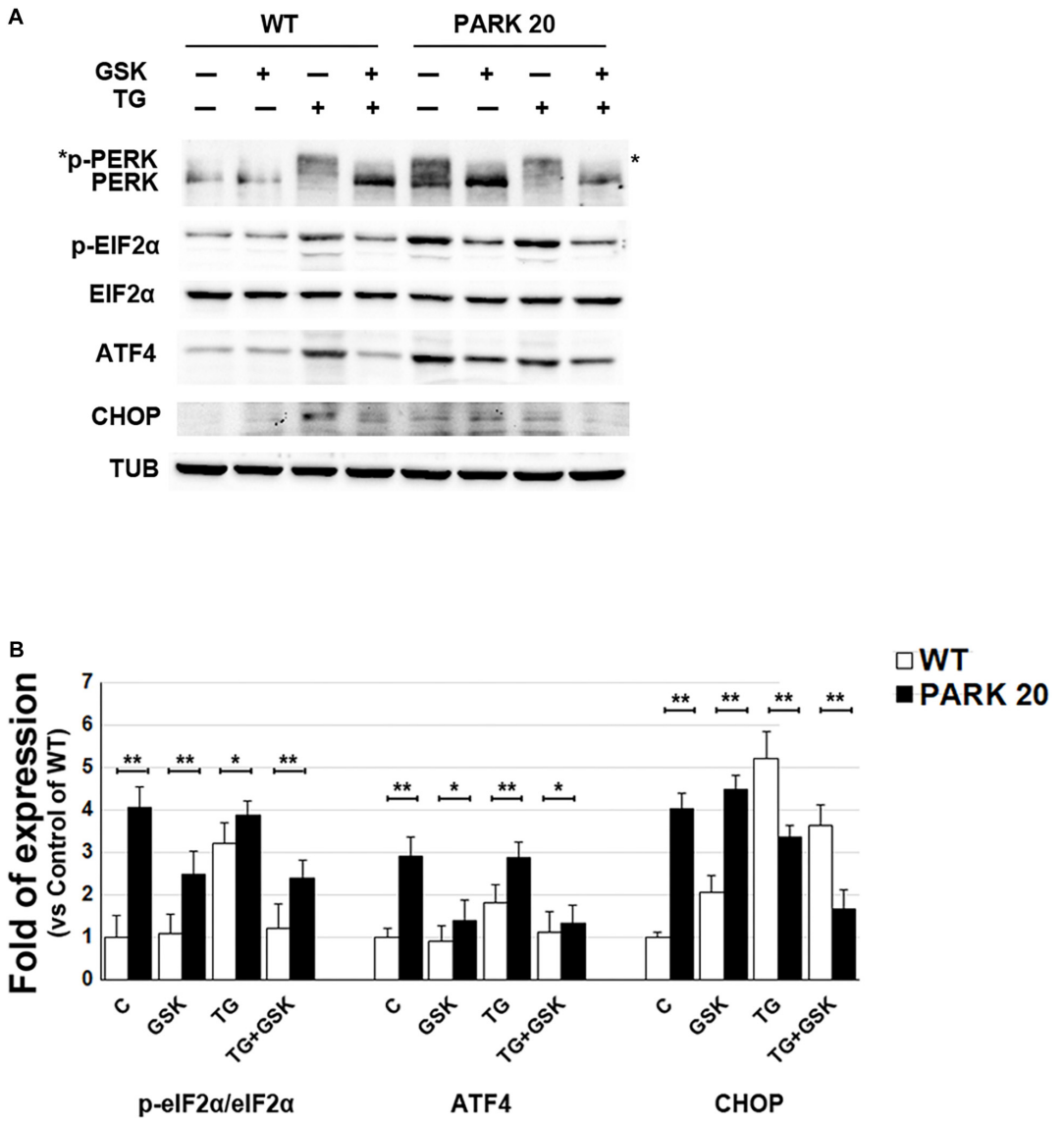

FIGURE 4 | The PERK-dependent pathway of the UPR is up-regulated in PARK20 fibroblasts. **(A,B)** Western blot analysis of the relative expression of markers of the PERK-dependent pathway of UPR. Starved HDF (WT) and PARK20 cells were either untreated (C) or treated for 2 h with 1 μM GSK2606414 (GSK) or 500 nM Thapsigargin (TG) alone (GSK and TG respectively) or in combination (TG + GSK). For TG + GSK samples, a pre-treatment of 30 min with GSK2606414 was performed prior to the addition of TG. α-Tubulin (TUB) was used as loading control. **(B)** Densitometry analyses of three independent experiments are shown. Histogram shows the relative fold induction of the expression of the indicated proteins in the treated samples compared to the control samples. Results are expressed as mean values ± SD; $*p \leq 0.05$, $**p \leq 0.01$, Student's *t*-test.

Then, we analyzed the phosphorylation status of elF2α by using a p-eIF2α antibody, which specifically detects the phosphorylated form of the protein (**Figure 4A**) and we found higher levels of p-eIF2α in PARK20 fibroblasts with respect control cells (**Figures 4A,B**). Moreover, PERK inhibitor GSK was able to reduce p-eIF2α amount in the Synj1 mutated cells (**Figures 4A,B**).

Finally, since increased eIF2α phosphorylation induces the ATF4/CHOP pathway of the ER stress, we analyzed the expression of ATF4 and CHOP in the PARK20 cells and found that both proteins enhanced with respect to control cells (**Figures 4A,B**). Moreover, after PERK inhibitor incubation ATF4 significantly reduced. Conversely, CHOP was not influenced by GSK, suggesting the involvement of positive feedback loops activated downstream to PERK induction (**Figures 4A,B**).

We also tested whether in the Synj1 mutated cells, the IRE1 arm of the UPR and/or the expression of genes under the transcriptional control of the ATF6 pathway of the UPR were equally activated. We found that either the IRE1 endonuclease activity or expression levels of ATF6-controlled genes in the PARK20 cells were similar to those detected in control cells (**Supplementary Figure S2**).

In summary, our results reveal that in the PARK20 cells the PERK/eIF2α/ATF4 pathway of the UPR is constantly activated, presumably as a result of the persistent activation of ER stress induced by the overload of cargo protein within the ER.

Persistent Activation of the PERK Pathway of the UPR Induces Oxidative Stress in PARK20 Cells

The alteration of ER proteostasis and the consequent accumulation of misfolded proteins within the ER is associated with the increment of ER protein folding that strongly induces ROS production (Tu and Weissman, 2004; Santos et al., 2009). Since NADPH oxidase is one of the key sources of cytosolic ROS (Lambeth, 2004), we measured the activity of the NADPH oxidase (NOX) through a quantitative lucigenin-based luminescence assay. Higher NOX activity was observed in PARK20 cells compared to control cells at each time point (**Figure 5A**), demonstrating that PARK20 fibroblasts exhibited pronounced oxidative stress.

It is well documented that the UPR could modulate the oxidative state of the cell, in particular through the PERK/eIF2α/ATF4 pathway (Amodio et al., 2018). Therefore, given the activation of the PERK pathway found in PARK20 fibroblasts, we measured the levels of cytosolic Reactive Oxygen Species (ROS) by dihydroethidium (DHE) fluorescent probe, in absence or in presence of the PERK inhibitor GSK (**Figure 5B**). Upon basal conditions, a significant higher amount of ROS production was detected in PARK20 cells with respect to control cells (24000 vs. 38000 a. u.; 1.6 Fold) (**Figure 5B**). Interestingly, GSK treatment reduced drastically ROS-derived DHE fluorescence, retrieving it to the values found in the control cells (**Figure 5B**). Alternatively, ROS levels were assessed by confocal microscopy imaging cells with the redox-sensitive fluorescent probe 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA) obtaining similar results (**Figure 5C**).

All these data indicate that PERK inhibition significantly reduces cytosolic ROS generation, thus providing evidence that the activation of the PERK pathway of UPR is responsible for the induction of oxidative stress in PARK20 fibroblasts.

Because the PERK pathway of the UPR can also activate antioxidant factors, such as the neuroprotective haemeoxygenase-1 (HO-1) enzyme (Alam et al., 1999; Cullinan et al., 2003; Kensler et al., 2007), we analyzed the expression level of the HO-1 protein in control and PARK20 fibroblasts (**Figure 5D**). Our results clearly show a consistent up-regulation and PERK-dependant expression of the HO-1 protein in PARK20 cells, suggesting that PERK activation could also induce an antioxidant response to oxidative stress in PARK20 cells (**Figure 5D**).

Furthermore, because mitochondria represent the major source of intracellular ROS and their dysfunction is widely reported in PD (Balaban et al., 2005; Lin and Beal, 2006), we investigated whether the observed cytosolic ROS overproduction is associated with mitochondria impairment. Because mitochondrial function is linked to the overall organization of the mitochondrial network (Chan, 2012), we examined the mitochondrial morphology by using the Mitotracker Red CMXRos probe (**Figure 6**). The morphology of mitochondrial network is profoundly altered in PARK20 fibroblasts compared to control cells (**Figure 6**). As evident by 3D reconstructions in the patient cells, mitochondrial network lost the typical interconnected tubular structure and exhibited thinner and shorter tubules with many fewer branch points (**Figure 6B**). Moreover, we also observed a reduced Mitotracker staining in PARK20 compared to control cells (**Figure 6A**). Considering that Mitotracker probe accumulates in mitochondria depending on its membrane potential (MMP), the reduced staining suggests a loss in MPP.

All these data indicate that, in addition to the increase in cytosolic ROS, in the PARK20 cells mitochondrial alteration was dependent on PERK activation and quite reversed by the PERK inhibitor GSK2606414 (**Figure 6B**).

DISCUSSION

In the present work we investigated further into the molecular events underlying the biogenesis of the juvenile parkinsonism PARK20, highlighting a role of Synj1 in regulating the early steps of secretory pathway.

Previous studies have well established that Synj1 is a crucial player for synaptic vesicle endocytosis and renewal at the nerve terminals, thanks to its 5-phospatase activity (Cremona et al., 1999; Kim et al., 2002; Mani et al., 2007; Cao et al., 2017). Recently, it has been demonstrated that Synj1 controls endosomal trafficking in different cell types including neuronal cells, presumably by regulating the of PtdIns3P levels within the endocytic pathways (Efe et al., 2005; Di Paolo and De Camilli, 2006; Krebs et al., 2013; Fasano et al., 2018).

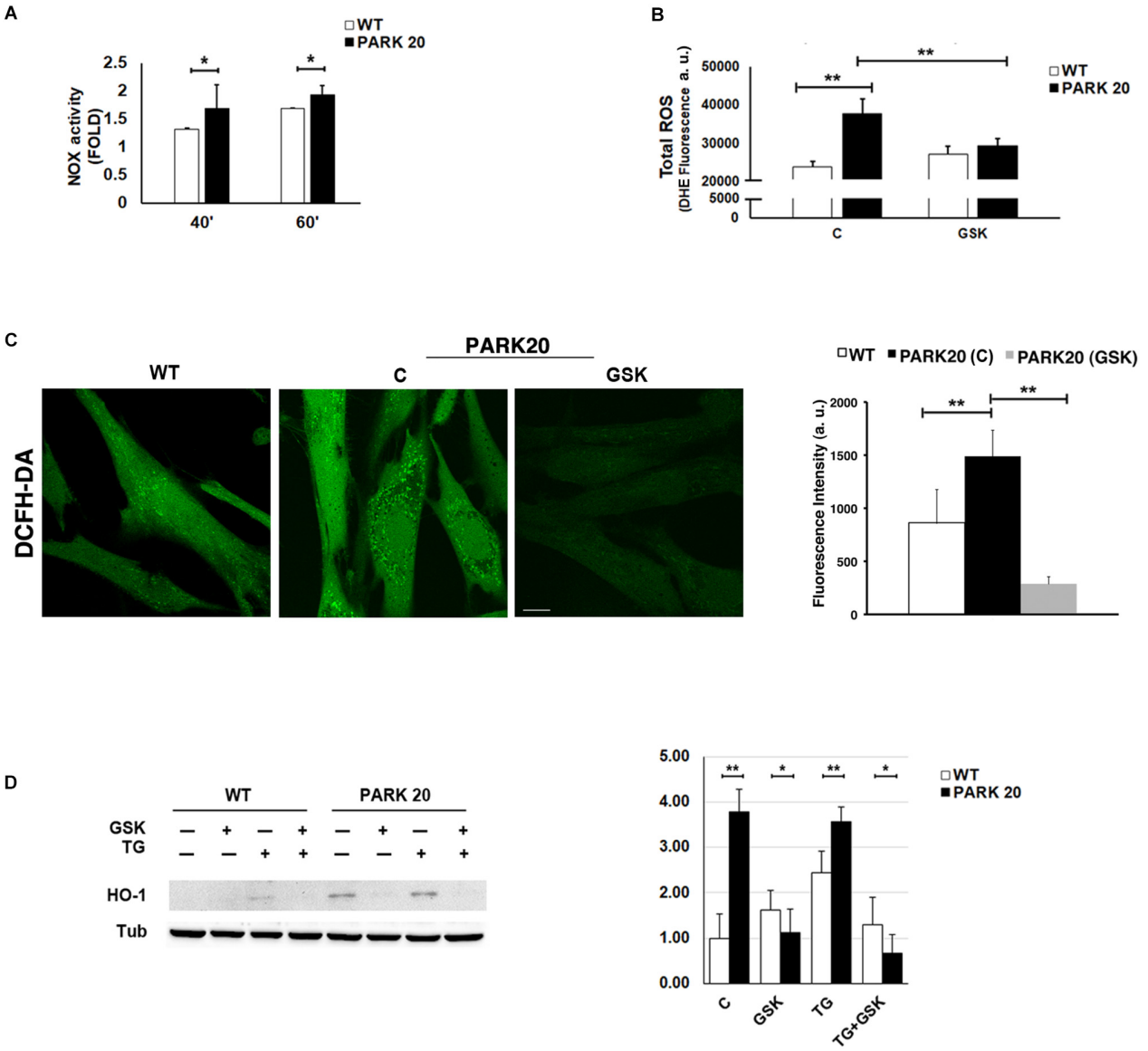

FIGURE 5 | Oxidative stress is increased in PARK20 fibroblasts. **(A)** NADPH oxidase (NOX) activity assay performed in HDF cells (WT) and PARK20 fibroblasts as reported in the methods. Histogram shows the relative fold change of NOX activity expressed as mean ± SD. Results were obtained from three independent experiments. **(B,C)** ROS quantification was carried out by using DHE **(B)** or DCFH-DA **(C)** fluorescent probes. HDF cells (WT) and PARK20 fibroblasts were untreated (C) or treated for 2 h with 1μM GSK2606414 (GSK) and then processed for DHE or DCFH-DA fluorescence quantification as detailed in the methods section. Histograms show mean values ± SD of DHE or DCFH-DA fluorescence expressed as arbitrary unit (a. u.) and calculated on three independent experiments. Scale bar 10 μm. **(D)** Immunoblot detection of HO-1 and densitometric analysis of three independent experiments. Starved HDF (WT) and PARK20 cells were either untreated (C) or treated for 2 h with 1 μM GSK2606414 (GSK) or 500 nM Thapsigargin (TG) alone (GSK and TG respectively) or in combination (TG + GSK) as aforementioned. α-Tubulin (TUB) was used as loading control. Representative immunoblotting is shown. Histogram shows the relative fold induction of HO-1 expression in the treated samples compared to the control samples. Results are expressed as mean values ± SD. *$p \leq 0.05$, **$p \leq 0.01$, Student's *t*-test.

Our results reveal a novel role for Synj1, consisting in the regulation of membrane trafficking at the ER-to-Golgi boundaries. It is likely that this regulatory function might be associated to its PtdIns4P phosphatase activity since ER and Golgi membranes are enriched in this phosphoinositide and the PtdIns4P phosphatase activity of Synj1 is affected by R258Q mutation (Krebs et al., 2013). Consistently, similar effect can be observed when persistent decrease of PtdIns4P hydrolysis is generated by deletion of the PtdIns4P phosphatase Sac1, the major lipid 4-phosphatase in yeast and mammals (Foti et al., 2001; Tahirovic et al., 2005; Liu et al., 2008).

Interestingly, by the use of a newly developed high-resolution imaging technology Sac1, which has been shown to shuttle between ER and Golgi in response to different stimuli (Blagoveshchenskaya and Mayinger, 2009), has been recently found to reside at the ER-Trans Golgi Network (TGN) contacts

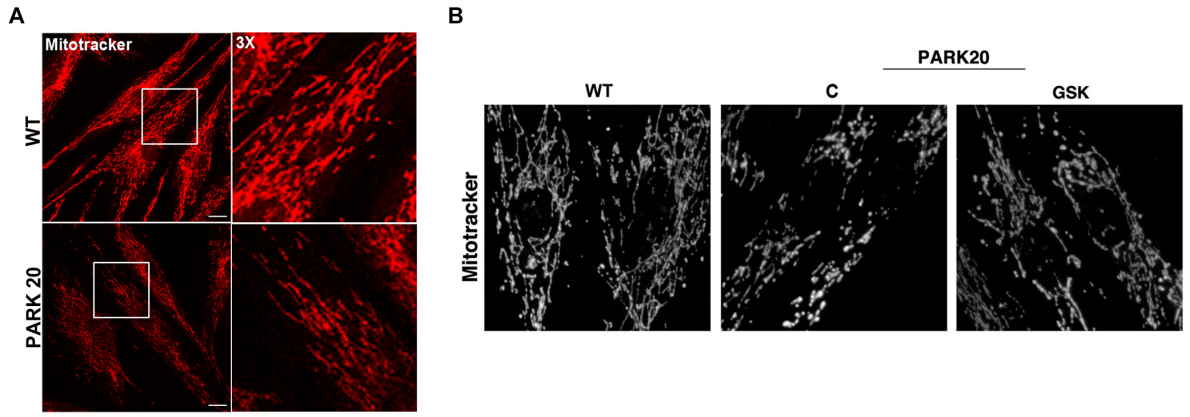

FIGURE 6 | Mitochondrial network is altered in PARK20 fibroblasts. HDF (WT) and PARK20 cells were seeded on glass coverslips and stained with the mitotracker red CMXRos probe for 20 min at 37°C prior to be fixed and processed for immunofluorescence. Serial confocal sections were collected from top and bottom of the cells. Representative images of single confocal sections **(A)** and 3D reconstructions of Z-stack acquired to higher magnification **(B)** are shown. Scale bars: 20 μm.

sites, where its phosphatase activity controls either ER or Golgi PtdIns4P membrane content (Venditti et al., 2019a,b). ER can establish membrane contacts with TGN as well as with endosomal membranes, referred to as ER-endo-lysosomal contact sites (EELCS) (Eden, 2016; Henne, 2017).

Given the interconnection of Synj1 with the endolysosomal pathway, an interesting hypothesis, to be tested in future work, would be to establish, whether Synj1 resides at EELCS and how EELCS helps Synj1/Sac1 activity to maintain PtdIns4P homeostasis.

Another finding supporting a role of Synj1 in controlling the early steps of secretory pathway is the altered distribution of GM130 and giantin, two Golgi factors also involved in the ER-to-Golgi trafficking (Alvarez et al., 2001). Furthermore, PARK20 cells display dramatic changes in the organization of Golgi membranes. The loss of intact Golgi populations with concomitant increase in vesiculated and dispersed Golgi membranes might be due to the unbalanced trafficking or defective membrane tethering and fusion events, consequent to the loss of Synj1 PtdIns4P phosphatase activity (Foti et al., 2001; Tahirovic et al., 2005; D'Agostino et al., 2014, 2018).

Central finding of our work is the discovery that formation of carrier vesicles at the endoplasmic reticulum exit sites (ERESs) is largely inhibited. The consequent traffic jam of secretory proteins within the ER membranes generates the activation of the PERK pathway of the ER stress/UPR, which in turn induces oxidative stress and mitochondrial damage. Therefore, the primary event causing the ER stress activation might rely on the altered control of the PtdIns4P content at ER membranes with consequent impairment of carrier vesicles formation. Indeed, the dynamic control of PtdIns4P level is necessary to coordinate the progression of both ERES formation and COPII assembly (Nagaya et al., 2002; Pathre et al., 2003; Blumental-Perry et al., 2006; Farhan et al., 2008). In these events, a specific role is played by p125A that, upon PtdIns4P recognition, promotes the recruitment of Sec16 at the ERESs, which in turn favors COPII assembly and cargo export from the ER (Shimoi et al., 2005; Iinuma et al., 2007; Ong et al., 2010).

Our results indicate that loss of Sac1/Synj1 activity in the R258Q/R258Q PARK20 cells could profoundly alter these dynamic events, leading to defective export of secretory proteins from the ER.

In PARK20 cells the PERK/eIF2α/ATF4 pathway of the UPR is hyperactive in response to the persistent state of ER stress induced by the ER overload of cargo proteins. This finding opens a novel perspective in the understanding of the molecular events leading to the PARK20 phenotype. The activation of the PERK pathway of the UPR is a common hallmark of various neurodegenerative diseases. In particular, as shown from postmortem analyses in PD patients as well as in animal models of PD, the activation of the PERK pathway represents a common cause of death of dopaminergic neuron (Scheper and Hoozemans, 2015; Gully et al., 2016). In our case, we show that, in PARK20 cells, prolonged PERK activation generates a pronounced production of cytosolic ROS, whereas GSK-mediated inhibition of PERK drastically reduces ROS production.

In this regard, it is worth noting that UPR, through multiple different pathways, can give rise to either pro-oxidant or antioxidant response (Malhotra and Kaufman, 2007; Amodio et al., 2018). In particular, during ER stress the increment of ER protein folding demand strongly induces ROS production (Tu and Weissman, 2004; Santos et al., 2009). In this context, PERK can play the role of a double-edged sword. In fact, in first instance, the PERK-eIF2α-ATF4 axis operates to restore ER proteostasis by reducing ER protein load and by inducing antioxidant pathways through the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) (Alam et al., 1999; Cullinan et al., 2003; Kensler et al., 2007). On this line, we found a significant up-regulation of HO-1 expression in PARK20 cells. On the other hand, the persistent activation of PERK in condition of unsolved protein misfolding can boost up ROS production and induce factors of the pro-oxidant signaling pathway. Among these, the transcription factor CHOP, activated

downstream to the PERK/eIF2α/ATF4/CHOP pathway, plays an important role. CHOP promotes the expression of both Ero1 and NOX that are responsible for ROS production during the oxidative protein folding and ER stress (Li et al., 2010; Anelli et al., 2012). Accordingly, we found a significant increased expression of CHOP in PARK20 cells besides the reported increase of cytosolic ROS. In this regard, it is worth remarking that normal CHOP expression is not recovered after GSK treatment. This finding is not surprising, since after its PERK-dependent activation, CHOP activates other downstream pathways that positively feedback on CHOP expression. Additionally, it is important to consider that CHOP triggers the ERo1-IP3R1-Ca2+/calmodulin-dependent protein kinase II (CaMKII)-NOX2 cascade, in which NOX2 finally induces CHOP expression in a manner independent by PERK (Li et al., 2010; Anelli et al., 2012). All in all, our data support co-existence of a PERK-dependent pro-oxidant and anti-oxidant response. However, we do not know whether one of them may prevail in the etiopathological onset of PARK20 and further investigation is needed in this direction.

Mitochondrial dysfunction is a common hallmark of both sporadic and genetic PD and is often associated with neuronal cell death in a number of neurodegenerative diseases (Subramaniam and Chesselet, 2013). Mitochondria are strictly connected to the ER via the mitochondrial-associated ER membranes (MAMs) through which Ca^{2+}, lipids and ROS are transmitted from the ER to mitochondria (Rainbolt et al., 2014). Our findings support that the activation of PERK-CHOP pathway during chronic ER stress can even potentiate MAMs altering the mitochondrial function. Accordingly, in PARK20 cells we observed that mitochondrial alteration of MMP and morphology is dependent on PERK activation and reversed by the PERK inhibitor GSK2606414. Similar results were obtained in pink1/parkin PD models, where mitofusin contacts with damaged mitochondria sustain PERK signaling, while suppression of PERK signaling by using GSK2606414 or by genetic inhibition has a neuroprotective effect (Celardo et al., 2016), suggesting common molecular features between PARK20 and other PD types.

CONCLUSION

In the present work we show that PARK20 fibroblasts display alteration of the early secretory compartments and impairment of the ER-to Golgi trafficking leading to PERK activation, OS induction and mitochondrial dysfunction. Thus, these results indicate that, beside the role of endosomal system previously shown (Fasano et al., 2018), defects of early secretory pathway could contribute to the PARK20 pathogenesis. Together with our previous findings, our data emphasize the link between membrane trafficking defects and PD. Moreover, although the correlation between mitochondrial dysfunction, OS and PERK activation in PARK20 cells needs to be further investigated, our current findings open a new lead in studying PD and phosphoinositide metabolism, providing possible novel biomarkers that can be used as diagnostic and prognostic tools for the disease.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

GA, OM, DF, and LZ performed and quantified the immunofluorescence data. MO, PDP, and RF carried out the biochemical quantitative analyses of oxidative stress. PB, MP, GDM, VB, CC, and ADR identified the patients, conducted the biopsies and provided primary cultures of fibroblasts. GA, OM, and MR analyzed UPR signaling. GA, OM, LN, and GP carried out the mitochondrial imaging analyses. EP performed electron microscopy experiments. EP and RP carried out the EM analyses. SP and PR contributed to the conception and design of the work, and wrote sections of the 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/fnins.2019.00673/full#supplementary-material

FIGURE S1 | The exocytic trafficking is impaired in PARK20 fibroblasts. **(A)** Wild-type and PARK20 fibroblasts, grown on 60 mm dishes, were incubated in culture medium containing 0.75% serum for 7 h. Then, equal volumes of corresponding culture mediums were collected, TCA-precipitated, separated by 7.5% SDS-PAGE and revealed by western blotting with collagen IV (COL IV) antibody (right panel). 1/20 of total was analyzed by 10% SDS-PAGE and total protein secretion was revealed by Coomassie-Blue staining (left panel). **(B)** Wild-type and PARK20 fibroblasts, grown on 60 mm dishes, were biotinylated to label selectively surface proteins and cell lysates were revealed by western blotting using HRP-conjugated streptavidin.

FIGURE S2 | Activation of UPR-dependent signaling pathways. **(A)** Semi-quantitative RT-PCR of Bip/Grp78 mRNA in HDF (WT) and PARK20 fibroblasts either untreated (0) or treated with 2 μg/ml Tunicamycin (TM) or 500 nM Thapsigargin (TG) for the indicated times. GAPDH mRNA was used as reference. One out of three independent experiment is shown. **(C)** Histogram shows the relative fold expression of Bip/Grp78 mRNA amplified as in **(A)** and calculated by densitometry analysis with ImageJ software. Values are expressed as mean ± SD. Controls (C) refers to untreated samples. $N = 3$. **(B)** XbpI splicing assay performed on samples treated as in **(A)**. GAPDH mRNA was used as reference. Amplicons derived from unspliced-XbpI (u) and spliced-XbpI (s) are shown. Numbers refers to the percent of spliced-XbpI to total XbpI (mean values), quantified by densitometry analysis with Image J from three independent experiments.

FIGURE S3 | The figure represents the uncut or partially cut filters used to mount **Figure 4A**. Predicted MW of proteins are reported on the left of the panels. # Indicates filters of the same experiment stripped and re-probed with antibodies as indicated. * Indicates the phosphorylated form of PERK.

FIGURE S4 | The figure represents the uncut filter used to mount **Figure 5D**. Predicted MW of the HO-1 protein is reported on the left of the panel.

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frontiers in Neuroscience

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GRP78 Level Is Altered in the Brain, but Not in Plasma or Cerebrospinal Fluid in Parkinson's Disease Patients

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Accumulation of misfolded proteins results in cellular stress, and is detected by specific sensors in the endoplasmic reticulum, collectively known as the unfolded protein response (UPR). It has been prominently proposed that the UPR is involved in the pathophysiology of Parkinson's disease (PD). In the present study, the levels of the UPR proteins and mRNA transcripts were quantified in *post mortem* brain tissue from PD patients and matched controls. The level of a key mediator of the UPR pathway, glucose-regulated protein 78 (GRP78), was significantly decreased in temporal cortex and cingulate gyrus, whereas there were no significant changes in the caudate nucleus, prefrontal, or parietal cortex regions. On the other hand, GRP78 mRNA level was significantly increased in caudate nucleus, cingulate gyrus, prefrontal, and parietal cortex regions. GRP78 protein level was also measured in plasma and cerebrospinal fluid, but there were no differences in these levels between PD patients and control subjects. Furthermore, immunofluorescence labeling of the $CD4^+$ T cells from PD patients showed that GRP78 protein is found in the cytoplasm. However, GRP78 level in PD patients was not significantly different from control subjects. Unlike the previous Lewy body dementia study, the present investigation reports reduced cortical protein, but increased transcript levels of GPR78 in PD. In summary, these data provide further evidence that GRP78 regulation is dysfunctional in the brains of PD patients.

Keywords: Parkinson's disease, unfolded protein response, glucose-regulated protein 78, endoplasmic reticulum stress, neurodegenerative diseases

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INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative disease affecting 1–2% of the population over 60 years of age (Dorsey and Bloem, 2018). PD is diagnosed based on the presence of bradykinesia, resting tremor, and postural rigidity. PD is characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta and deposits of intracellular protein inclusions called Lewy bodies, where aggregates of misfolded α-synuclein (α-syn) are the major components (Spillantini et al., 1997). Although these causative factors for PD have been known for many years and extensive research have been done to halt the disease progression, at present, there are no disease modifying therapies available for PD. Current treatments only restore dopamine neurotransmission and reduce symptoms, but do not stop or slow down the disease progression.

Accumulation of specific misfolded proteins is a salient feature of many neurodegenerative diseases, including PD. The build-up of misfolded proteins gives rise to cellular stress, and is detected by specific sensors in the endoplasmic reticulum (ER). To overcome ER stress, mammalian cells activate a specific signaling pathway in the ER called the unfolded protein response (UPR), which is initiated by the binding of the glucose-regulated protein 78 (GRP78, also known as binding immunoglobulin protein, BiP), an ER chaperone, to misfolded proteins (Harding et al., 1999; Rutkowski and Kaufman, 2004). The UPR consists of three pathways, in which includes ER-resident transmembrane proteins, known as protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). UPR serves as a protective mechanism against the buildup of toxic misfolded proteins, in which first inhibits the protein synthesis, then up-regulate genes that are involved in protein folding or disposal in order to stabilize the disturbed ER homeostasis (Walter and Ron, 2011; Hetz, 2012). The early response of the UPR pathway is activated by PERK (Nakka et al., 2016), which leads to the phosphorylation of the eukaryotic translation initiator factor, eIF2α. This event inhibits general protein synthesis in order to reduce the load on the ER, having an important pro-survival role (Harding et al., 2000; Fernandez et al., 2002). However, under prolonged or chronic stress, phosphorylation of eIF2α increases translation of activating of transcription-4 (ATF4) mRNA, which encodes a transcription factor that induces the expression of pro-apoptotic genes such as the C/EBP-homologous protein (CHOP; also known as DDIT3/GADD153) (Zinszner et al., 1998). Although the initial intention of the UPR activation is to overcome ER stress, if a cell fails to reach proteostasis due to chronic or irreversible ER stress, then the UPR signals to cell death by apoptosis (Tabas and Ron, 2011; Urra et al., 2013).

It has been shown that the UPR is activated in *post mortem* human brains of PD patients (Hoozemans et al., 2007; Selvaraj et al., 2012) as well as in animal and cell models of parkinsonism (Mercado et al., 2016), implying that the neurons are prone to ER stress, and that the UPR may have a role in the degeneration of dopaminergic neuron. Numerous studies have demonstrated that the pathological aggregation/accumulation of α-syn activates the UPR pathway, consequently inducing pro-apoptotic changes (Cooper et al., 2006; Sugeno et al., 2008; Bellucci et al., 2011). Despite the fact that there is a general acceptance of the UPR activation in PD, previous human *post mortem* studies have only focused on demonstrating the presence/existence of UPR activation in PD through semi-quantitative immunohistochemical approach. Therefore, the primary aim of the current study was to accurately quantify the changes in the level of UPR proteins and mRNA transcripts in PD patients in various brain regions by using western blot and quantitative real-time PCR.

The cerebrospinal fluid (CSF) has been extensively investigated as a source of robust biomarkers for neurodegenerative diseases, particularly for Alzheimer's disease (AD), but also for atypical parkinsonian disorders (Lleo et al., 2015). CSF is the biological fluid closest to the brain as it is not separated from the brain by the blood brain barrier, unlike plasma. However, at the moment, there is no CSF biomarker available to diagnose PD in clinics. The UPR proteins have never been investigated as a potential biomarker for PD. Therefore, the second aim of the present study was to investigate the possibility of the UPR proteins, specifically GRP78 protein, as a novel biomarker for PD.

MATERIALS AND METHODS

Post Mortem Human Brain Tissues

Post mortem brain tissue was obtained from the MRC London Neurodegenerative Diseases Brain Bank, United Kingdom. All participants gave informed consent for their tissue to be used in research and the study had ethics approval from the UK National Research Ethics Service (08/H1010/4 and KI IRB) and from the Regional Ethics Review Board of Stockholm (2014/1366-31). The demographic details of the patients and control subjects are shown in **Table 1**. It is worth to declare that authors did not have any control over the sample collection, and hence, long *post mortem* delays of these brain samples could not be avoided. Biochemical analyses were undertaken on five different brain regions: caudate nucleus (n = 36), prefrontal cortex (n = 40), temporal cortex (n = 41), anterior cingulate gyrus (n = 38), and parietal cortex (n = 39). Temporal cortex tissues were not available for RNA analysis due to technical reasons. Caudate nucleus was selected for its involvement in motor function in PD; prefrontal cortex was selected for its proposed role in executive function and cognition; cingulate gyrus was selected for the early development of pathology encountered in this region, while parietal cortex was selected because of its pathological predominance in AD as opposed to PD; temporal cortex was chosen due to its suggested role in auditory processing and language.

Participants for CSF and Plasma Collection

Consents from participants were collected according to the Declaration of Helsinki, which was approved by the regional ethical committees. CSF and plasma samples were collected as described previously (Bjorkhem et al., 2013). All participants fulfilled the clinical diagnostic criteria for PD (Gelb et al., 1999), and PD severity was scored with the Unified Parkinson's disease rating scale (UPDRS) and Hoehn and Yahr scale. The Montreal Cognitive Assessment (MoCA) scores were also obtained. Control subjects were healthy volunteers or had mild symptoms without any severe neurological diagnosis (e.g., temporary tension headache or sensory symptoms). Control subjects were age- and gender-matched to PD patients (**Table 1**).

CSF and Plasma Collection

The standardized lumbar puncture procedure was performed according to the Alzheimer's Disease Neuroimaging Initiative recommended protocol. CSF was collected into sterile polypropylene tubes, in which the first 2 mL was discarded, and approximately 10–12 mL of CSF from the first portion

TABLE 1 | Demographical and clinical characteristics of subjects in this study.

Post mortem brain			
Group	**Gender (M/F) (%)**	**Age at death**	**Post mortem delay (h)**
Caudate nucleus			
Control (*n* = 18)	50/50	72.1 ± 2.6	31.2 ± 4.7
PD (*n* = 18)	50/50	75.2 ± 1.8	44.6 ± 5.2
Prefrontal cortex			
Control (*n* = 24)	58/42	80.2 ± 1.5	38.8 ± 4.8
PD (*n* = 16)	56/44	74.8 ± 2.0	44.8 ± 5.8
Temporal cortex			
Control (*n* = 23)	61/39	80.3 ± 1.6	37.8 ± 4.9
PD (*n* = 18)	50/50	75.2 ± 1.8	44.6 ± 5.2
Cingulate gyrus			
Control (*n* = 22)	64/36	80.4 ± 1.7	38.5 ± 5.0
PD (*n* = 16)	50/50	75.5 ± 1.7	45.5 ± 5.8
Parietal cortex			
Control (*n* = 21)	62/38	79.7 ± 1.5	39.0 ± 5.1
PD (*n* = 18)	50/50	75.2 ± 1.8	44.6 ± 5.2

CSF				
Group	**Gender (M/F) (%)**	**Age**	**Disease duration (yrs)**	**H&Y score**
Control (*n* = 20)	50/50	66.6 ± 2.3	n/a	n/a
PD (*n* = 19)	47/53	65.1 ± 2.3	5.9 ± 1.3	3 ± 0.2

Plasma						
Group	**Gender (M/F) (%)**	**Age**	**Disease duration (years)**	**H&Y score**	**Total UPDRS**	**MoCA**
Control (*n* = 38)	50/50	63.2 ± 2.2	n/a	n/a	n/a	n/a
PD (*n* = 38)	53/47	64.7 ± 1.9	3.1 ± 0.8	2 ± 0.2	38.5 ± 3.6	22.7 ± 1.0

PBMCs		
Group	**Gender (M/F) (%)**	**Age**
Control (*n* = 5)	40/60	65.4 ± 3.3
PD (*n* = 5)	40/60	69.2 ± 3.9

PD, Parkinson's disease; F, female; M, male; n/a, not applicable; H&Y, Hoehn and Yahr; UPDRS, unified Parkinson's disease rating scale; MoCA, Montreal Cognitive Assessment; PBMCs, peripheral blood mononuclear cells. All measures are presented as mean + SEM, except H&Y score is presented as median ± SEM.

was collected and gently mixed in order to minimize the gradient influence. Cell counts were measured and samples were centrifuged in the original tube at 1800 × *g* for 10 min at 4°C. Blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 800 × *g* for 20 min. Plasma was collected from the top phase of the gradient. Both CSF and plasma were aliquoted in polypropylene tubes, frozen on dry ice and stored at −80°C until use. The maximum time interval from the sample collection until freezing was 30 min.

Preparation of Tissue Samples for Western Blotting

Western blot samples were prepared as previously described (Baek et al., 2016). Briefly, 100 mg of frozen tissue was taken from each brain region, which was then homogenized in 1 mL of ice cold buffer (pH 7.4) containing 50 mM Tris–HCL, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM EDTA, "complete protease inhibitor cocktail tablet" (Sigma), phosphatase inhibitor (PhosStop, Sigma), and 2 μg/mL pepstatin A dissolved in ethanol:dimethyl sulfoxide (DMSO) 2:1 (Sigma). Homogenization was performed using disposable pestles (Cat# BELAF199230001, VWR) until the liquid appeared homogenous. Protein concentration of each sample was measured by using BCA Protein Assay Kit (Thermo Fisher Scientific).

Western Blotting

Twenty micrograms of each sample was loaded on 8% SDS–polyacrylamide gel for protein separation then transferred to nitrocellulose membrane (Immobilon-F, Millipore). After blocking for nonspecific binding, the membranes were incubated with anti-GRP78 (rabbit polyclonal, 1:1000, Cat# ab21685, Abcam), anti-eIF2α (rabbit polyclonal, 1:1000, Cat# 9722, Cell signaling), anti-phosphorylated eIF2α (rabbit polyclonal, 1:1000, Cat# 9721, Cell signaling) primary antibodies followed by IRDye 800CW goat anti-rabbit secondary antibody (1:20,000, Cat#

926-32211, Li-Cor). Bands were detected using an Odyssey infrared fluorescent scanner, and the integral of intensity was quantified using Odyssey infrared imaging system application software version 2.1. β-Actin was chosen as a "house-keeping" protein in order to control for any inconsistency in loading samples. Each membrane was therefore probed for actin (mouse monoclonal, 1:10,000, Cat# A5441-100UL, Sigma–Aldrich) to normalize the level of immunolabeling of the protein-of-interest to actin, so that any potential variations in protein loading could be eliminated.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from 30 mg of frozen human brain tissues using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's protocol. The samples were then measured and evaluated for concentration and purity (260/280 nm ratio) using a Nanodrop (Marshall Scientific). RNA samples were stored at -80°C until use. cDNA was synthesized from 30 ng of total RNA using QuantiTect Reverse Transcription Kit (Qiagen). Levels of human GRP78 (Assay ID: Hs00946084_g1), eIF2α (Assay ID: Hs00187953_m1), and CHOP (Assay ID: Hs00358796_g1) transcripts were measured by qRT-PCR. Briefly, qRT-PCR reactions were prepared in duplicate for each sample with TaqMan assay (Thermo Fisher Scientific) and performed on a CFX96 Real-Time System (BioRad). All reactions were run at 55°C as an annealing temperature and for 40 s for elongation time. Transcript levels were determined by the comparative cycle threshold method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay ID Hs02758991_g1) was used as an internal control for normalization.

Quantification of Circulating GRP78 Protein by ELISA

The levels of GRP78 protein were measured in CSF (neat, i.e., no dilution) and in plasma (1:20 dilution) using the human GRP78 ELISA kit (Enzo Life Sciences) with a detection range of 1.4–4500 ng/mL. The assay was performed according to manufacturer's instruction.

Immunofluorescence Labeling of $CD4^+$ T Cells

Preparation of peripheral blood mononuclear cells (PBMCs) was performed as previously described (Green et al., 2017). $CD4^+$ T cells were isolated by using $CD4^+$ T Cell Isolation Kit (Cat# 130-096-533, Miltenyi Biotec) according to manufacturer's instructions. Purified $CD4^+$ T cells were resuspended in proliferation medium (RPMI 1640, glutamine, Pen/Strep, heat inactivated FCS, β-mercaptoethanol, non-essential amino acids, sodium pyruvate) containing Il-2 and Il-7 cytokines. Cells were then seeded in 96-well plates, pre-coated with 5 μg/mL anti-CD3 (clone 2C11), and 1 μg/mL anti-CD28 (clone 37.51) antibodies. The plate was then incubated in a humidified incubator with 5% CO_2 at 37°C for 7 days. After 7 days of incubation, cells were transferred to 10 μg/mL human ICAM-1-coated μ-slides (Ibidi), and incubated for 45 min at 37°C for adhesion and migration. Cells were then fixed and permeabilized for 20 min using Cytofix/Cytoperm solution (BD Biosciences). Cells were incubated with anti-GRP78 primary antibody (1:50, Cat# ab21685, Abcam), anti-α-syn primary antibody (1:50, Cat# 610787, BD Transduction), anti-calreticulin primary antibody (1:50, Cat# ab22683, Abcam) for overnight at 4°C, followed by goat anti-mouse Alexa Fluor™ 488 secondary antibody (1:500, Thermo Fisher Scientific), goat anti-rabbit Alexa Fluor™ 568 secondary antibody (1:500, Thermo Fisher Scientific) for 1 h at room temperature. All cells were counterstained with DAPI nuclear stain (300 nM, Sigma–Aldrich).

Confocal Microscopy and Image Analysis

Immunofluorescent images were acquired by ZEISS LSM 880 Airyscan confocal laser scanning microscopy equipped with ZEN2.1 software, using Plan-Apochromat 63×/1.4 Oil DIC M27 63× oil objective. The quantification of the GRP78 protein expression in $CD4^+$ T cells was pooled from five independent experiments, in which more than 20 cells were analyzed from each group (control or PD) for every experiment. The analyses were done using ImageJ. The outline of a cell was defined by image threshold, and the total immunofluorescence was measured by maximum projections of Z stack images after background subtraction. The mean fluorescent intensity of PD group was normalized to that of control group.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5. All descriptive statistics for the variables in the study were reported as means ± standard error of means (SEM), unless otherwise stated. Normality tests were run to assess data distribution. One-way ANOVA or parametric unpaired *t*-test was used for variables with normal distribution, whereas Mann–Whitney non-parametric analysis was used for the distorted distribution. Differences were considered statistically significant with $P < 0.05$.

RESULTS

Changes in the Level of GRP78 Protein and the Ratio Between p-eIF2α and Total eIF2α Proteins in Various Regions of the Brain of PD Patients

There was a significant decrease in the level of GRP78 protein in PD patients compared to control subjects in temporal cortex ($P = 0.0007$) and cingulate gyrus ($P = 0.001$, **Figure 1A**). Similar pattern was observed in the prefrontal cortex, in which the difference was very close to statistical significance ($P = 0.0663$, **Figure 1A**). In caudate and parietal cortex regions, there was an increasing trend in the level of GRP78 protein in PD patients compared to control subjects, though statistically insignificant (**Figure 1A**).

The ratio between phosphorylated-eIF2α (p-eIF2α) and total eIF2α proteins was significantly decreased in PD patients compared to control subjects in prefrontal cortex ($P = 0.0115$,

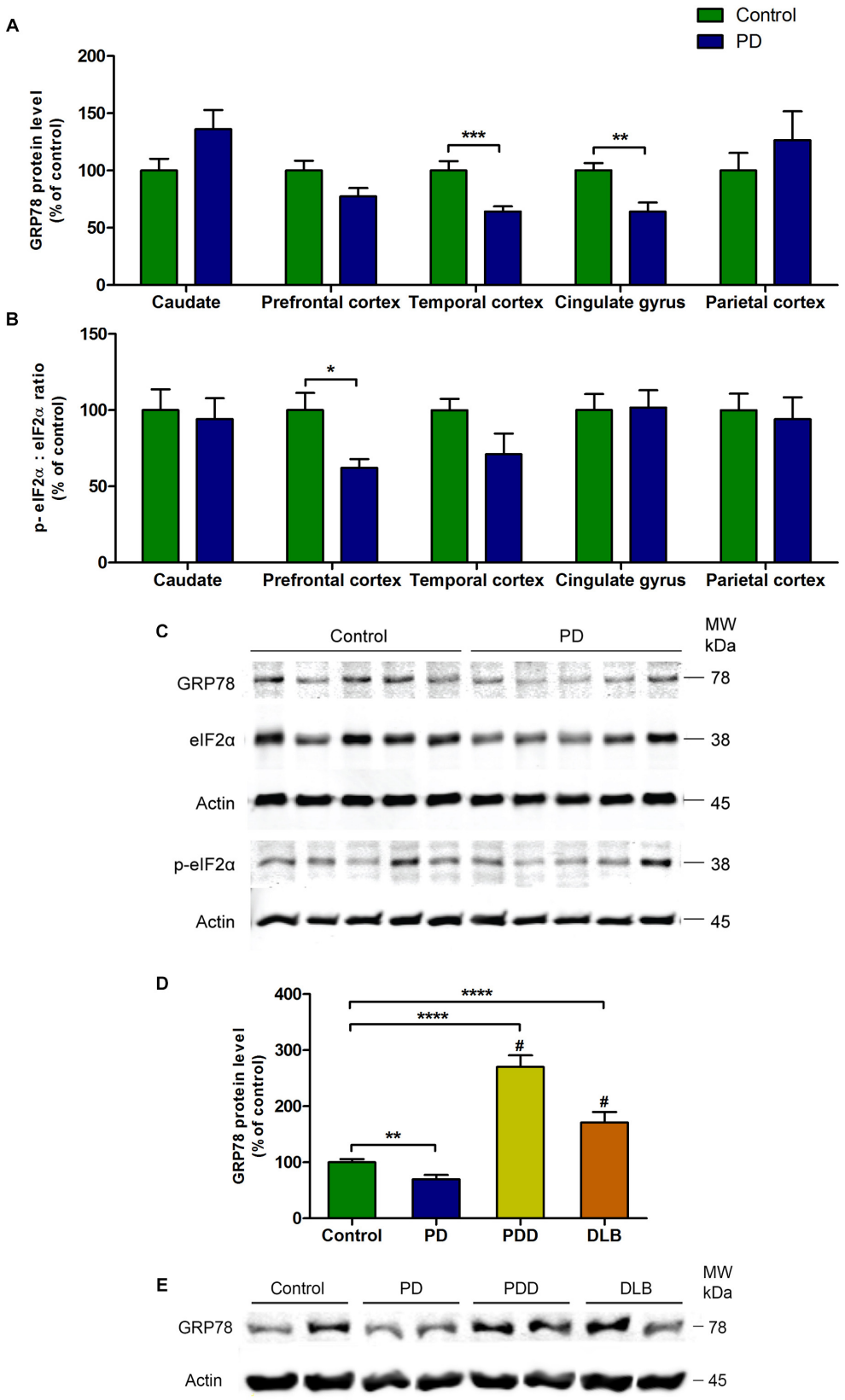

FIGURE 1 | Continued

FIGURE 1 | Changes in the level of GRP78 protein and the ratio between p-eIF2α and total eIF2α proteins in various regions of the brain from PD patients and control subjects. **(A)** The level of GRP78 protein was significantly decreased in temporal cortex and cingulate gyrus regions ($P = 0.0007$; $P = 0.001$, respectively). **(B)** The ratio between p-eIF2α and total eIF2α was significantly decreased in prefrontal cortex ($P = 0.0115$). There were no changes in the other regions. **(C)** A representative western blot image showing GRP78, total eIF2α, and p-eIF2α protein expressions in cingulate gyrus region in control and PD subjects. **(D)** The level of GRP78 protein in cingulate gyrus was significantly higher in PD with dementia (PDD) and dementia with Lewy bodies (DLB) patients compared to both control subjects and PD patients ($^{**}P = 0.0023$; $^{****}P < 0.0001$ compared to control; $^{\#}P < 0.0001$ compared to PD patients). **(E)** Representative western blot image illustrating the expression of GRP78 protein in cingulate gyrus in control, PD, PDD, and DLB subjects.

Figure 1B). This decrease was due to a significant increase in the level of total eIF2α protein ($P = 0.002$) in PD patients, while the p-eIF2α protein level remained unchanged (**Table 2**). In the temporal cortex, p-eIF2α and total eIF2α ratio was decreased almost to a significant level in PD patients compared to control subjects ($P = 0.0514$, **Figure 1B**). There were no changes in p-eIF2α and total eIF2α ratio in caudate, cingulate gyrus, or parietal cortex (**Figure 1B**). In order to validate this finding of a general decrease in the levels of GRP78 protein in PD patients, the western blot experiment from Baek et al. (2016) was repeated (**Figures 1D,E**), in which the authors showed that the levels of GRP78 protein in patients with Parkinson's disease with dementia (PDD) and dementia with Lewy bodies (DLB) were significantly higher compared to control subjects and AD patients in the cingulate gyrus. As expected, the results were consistent with the results of Baek et al. (2016), in which the level of GRP78 protein in cingulate gyrus was significantly higher in PDD and DLB patients compared to control subjects ($^{****}P < 0.0001$ for both PDD and DLB) and also to PD patients ($^{\#}P < 0.0001$ for both PDD and DLB) (**Figures 1D,E**).

Changes in the mRNA Level of *Grp78*, *eif2α*, and *Chop* in Various Regions of the Brain of PD Patients

There were significant increase in the levels of *Grp78* mRNA transcripts in all regions of the brain in PD patients compared to control subjects (caudate, $P = 0.0015$; prefrontal cortex, $P = 0.0025$; cingulate gyrus, $P = 0.0007$; parietal cortex, $P = 0.0047$; **Figure 2A** and **Table 3**). However, *eif2α* and *Chop* mRNA levels in PD patients were not significantly different to control subjects in any of the brain regions (**Figures 2B–D** and **Table 3**).

Circulating GRP78 Protein in Plasma and CSF of PD Patients

There was a high concentration of GRP78 protein in the plasma of PD patients as well as in control subjects. Although there was a slight decrease in GRP78 protein level in PD patient compared to control subjects, it was not statistically significant (**Figure 3A**). In contrast to plasma level, the concentrations of GRP78 protein in CSF of control and PD patients were negligible, and there were no differences between PD patients and control subjects (**Figure 3B**).

GRP78 Protein Expression in CD4$^+$ T Cells of PD Patients

Double immunofluorescence staining data with α-syn and calreticulin showed cytoplasmic localization of GRP78 in CD4$^+$ T cells (**Figures 4A,B**). The GRP78 protein was expressed in the cytoplasm of CD4$^+$ T cells in both control subjects and in PD patients (**Figure 4C**). However, the level of expression in PD patients was not significantly different from that of control subjects (**Figure 4D**).

DISCUSSION

Growing experimental evidence suggests that UPR is involved in PD; and this is not surprising since the accumulation of misfolded α-syn is a central pathogenic process in PD. Indeed, changes in the expression of GRP78 protein and other UPR activation markers (p-PERK and p-eIF2α) have been observed in the brain of PD patients (Hoozemans et al., 2007; Selvaraj et al., 2012; Baek et al., 2016; Mercado et al., 2018). Nevertheless, there are only a handful of studies examining UPR activation in PD using *post mortem* human brain tissues, and furthermore, almost all of these human studies employ semi-quantitative immunohistochemical method. In the present study, changes in the levels of UPR proteins in various regions of the brain from PD patients were measured by means of quantitative western blot. Surprisingly, among five regions analyzed, there were significant decreases in the level of GRP78 protein in temporal cortex and cingulate gyrus of PD patients compared to control subjects, while there were no changes in caudate, prefrontal, or parietal cortical regions (**Figure 1A**). Although the observed decrease in the level of GRP78 protein in PD patients is in contrast to previous *post mortem* human studies, this decrease has been observed in other neurodegenerative diseases and during normal aging. For example, in the brain of AD patients, the level of GRP78 protein has been shown to be reduced compared to control subjects

TABLE 2 | Summary of changes in the UPR proteins in PD patients compared to control subjects.

Brain regions	UPR proteins			
	GRP78	p-eIF2α	eIF2α	p-eIF2α:eIF2α
Caudate	ns	ns	ns	ns
Prefrontal cortex	ns	ns	↑ $P = 0.002$	↓ $P = 0.0115$
Temporal cortex	↓ $P = 0.0007$	↓ $P = 0.0014$	↓ $P = 0.0008$	ns
Cingulate gyrus	↓ $P = 0.001$	ns	ns	ns
Parietal cortex	ns	↓ $P = 0.0006$	↓ $P = 0.0145$	ns

Upward arrow: An increase in expression. Downward arrow: A decrease in expression. ns: Not significant.

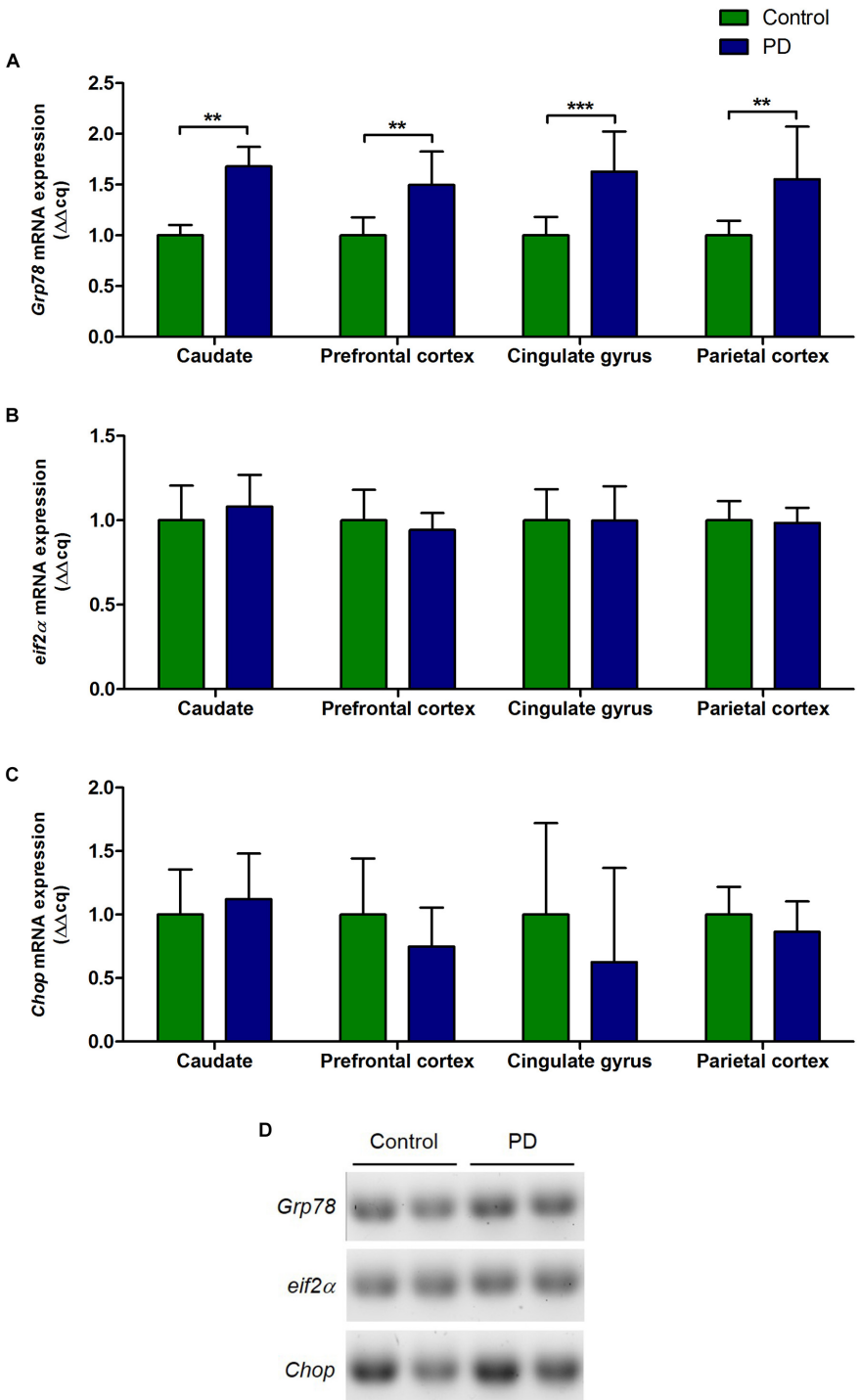

FIGURE 2 | Changes in the level of *Grp78*, *eif2α*, and *Chop* mRNA expression in various regions of the brain from PD patients and control subjects. **(A)** The level of *Grp78* mRNA in PD patients was significantly increased in all regions of the brain compared to control subjects (P = 0.0015, 0.0024, 0.0007, 0.0047 respect to brain regions). **(B,C)** There were no changes in the levels of *eif2α* and *Chop* mRNA in PD patients in any of the brain regions. **(D)** Representative gel image showing the expression of *Grp78*, *eif2α*, and *Chop* mRNA in prefrontal cortex in control subjects and PD patients.

(Katayama et al., 1999; Baek et al., 2016) or remained unchanged (Sato et al., 2000). In a mouse model of over-expressing mutated human presenilin-1 gene, the most prevalent mutation found in cases of familial AD, the expression of GRP78 was also found to be decreased (Katayama et al., 1999). Furthermore, Salganik et al. (2015) have shown the loss of GRP78 during normal aging, in

TABLE 3 | Summary of changes in the UPR mRNA in PD patients compared to control subjects.

Brain regions	UPR mRNA		
	GRP78	eIF2α	CHOP
Caudate	↑ $P = 0.0015$	ns	ns
Prefrontal cortex	↑ $P = 0.0025$	ns	ns
Cingulate gyrus	↑ $P = 0.0007$	ns	ns
Parietal cortex	↑ $P = 0.0047$	ns	ns

Upward arrow: An increase in expression. Downward arrow: A decrease in expression. ns: Not significant.

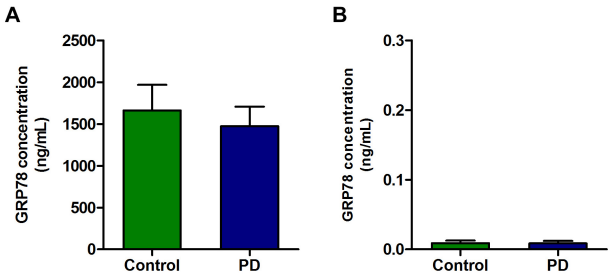

FIGURE 3 | The concentration of GRP78 protein in the plasma and CSF samples of PD patients and control subjects. **(A)** Although there was a modest level of GRP78 protein in plasma of PD patients and control subjects, there was no significant difference between the two groups. **(B)** The GRP78 protein level in CSF was negligible in both PD patients and control subjects.

which the old rats showed significantly lower levels of GRP78 protein in the nigrostriatal system compared to young animals. In the same study, it was shown that knockdown of GRP78 by specific small interfering RNAs in a rat model of over-expressing α-syn in the substantia nigra aggravated α-syn neurotoxicity in nigral dopamine neurons, which then lead to significantly greater neuronal loss and reduction of striatal dopamine. Moreover, the degree of GRP78 decline was correlated to the severity of neurodegeneration (Salganik et al., 2015).

In the present study, it was shown that changes in the level of GRP78 protein in a particular brain region did not directly correlate with the changes in the levels of downstream proteins such as eIF2α or p-eIF2α (**Figure 1B**). For instance, in the cingulate gyrus, there was a significant decrease in GRP78 protein level in PD patients compared to control subjects (**Figure 1A**), but there was no change in the ratio between p-eIF2α and eIF2α levels (**Figure 1B**). Similar results were found in a rodent study, in which neuronal GRP78 induction in α-syn over-expressing transgenic mice was not accompanied by an increase of p-eIF2α level, suggesting that α-synucleinopathy is linked to abnormal UPR which in turn could trigger cell death (Colla et al., 2012a). Taken together, absence of coherent changes in the level of proteins in the UPR pathway observed in the current study provide additional support for the above hypothesis that PD may be associated with impaired UPR.

Unlike GRP78 protein, the levels of GRP78 mRNA were significantly increased in PD in all areas of the brain analyzed (**Figure 2A**). However, mRNA levels of eIF2α or CHOP, a pro-apoptotic transcription factor, did not show any significant changes in PD patients compared to control subjects (**Figures 2B,C**). To explore the possibility whether the increased GRP78 mRNA expression is a compensatory response to the decreased protein level, individual GRP78 mRNA level was correlated to its matching protein level. Nevertheless, no correlation was found (data not shown). The mismatch between the levels of GRP78 mRNA and protein in PD further indicates that the UPR signaling may be dysfunctional, and that PD-related pathology that causes ER stress, likely the accumulation of misfolded α-syn, in some way impair the induction of GRP78 protein, which may also indicate an increase of the vulnerability of neurons to ER stress. Indeed, it was shown that α-syn inhibited processing of ATF6 directly via physical interactions and indirectly by inhibiting ER to Golgi transport of COPII vesicles (Credle et al., 2015). Moreover, the disease-causing mutant α-syn also reduced ER to Golgi trafficking and aggravated ER stress (Thayanidhi et al., 2010; Colla et al., 2012a,b). The phenomenon that decrease in UPR signaling leading to a possible increase in sensitivity to ER stress was demonstrated by Katayama et al. (1999). They showed that inhibition of endogenous IRE1 significantly increased vulnerability to ER stress, and increase in sensitivity to ER stress caused by treatment of an ER stress inducer was reversed by the expression of recombinant GRP78. The results from Katayama et al. (1999), together with the current study, suggest that activation of UPR signaling is important for protective effects against the ER stress, and that the reduction of GRP78 protein level may cause vulnerability to ER stress in PD, which may then potentiate disease progression.

It has been hypothesized that with aging and/or disease progression, soluble α-syn monomers that are present in the ER form insoluble α-syn oligomers/aggregates and attribute to chronic ER stress and neurodegeneration (Colla et al., 2012a). Recently, a new ER stress rat model using intranigral injection of a well-known ER stress inducer, tunicamycin, was employed to investigate whether ER stress is able to induce PD features (Coppola-Segovia et al., 2017). It was shown that ER stress not only induced locomotor impairment and dopaminergic neuronal loss, but also substantial α-syn oligomerization in substantia nigra pars compacta, astroglial activation, and increased expression of ER stress markers (Coppola-Segovia et al., 2017). These results reinforce the notion that ER stress, hence the UPR, could be an important contributor to the pathophysiology of PD. Nevertheless, there are still not enough evidence to decipher the fact whether the activation of the UPR is a cause or consequence of neurodegeneration observed in PD. Furthermore, how and what controls the changeover switch of the UPR between neuroprotection and neurotoxicity remains largely obscure.

While the present study confirms that there is a turbulence in the UPR in different areas of the brain of PD patients, due to technical limitations with reagents, it was difficult to efficiently

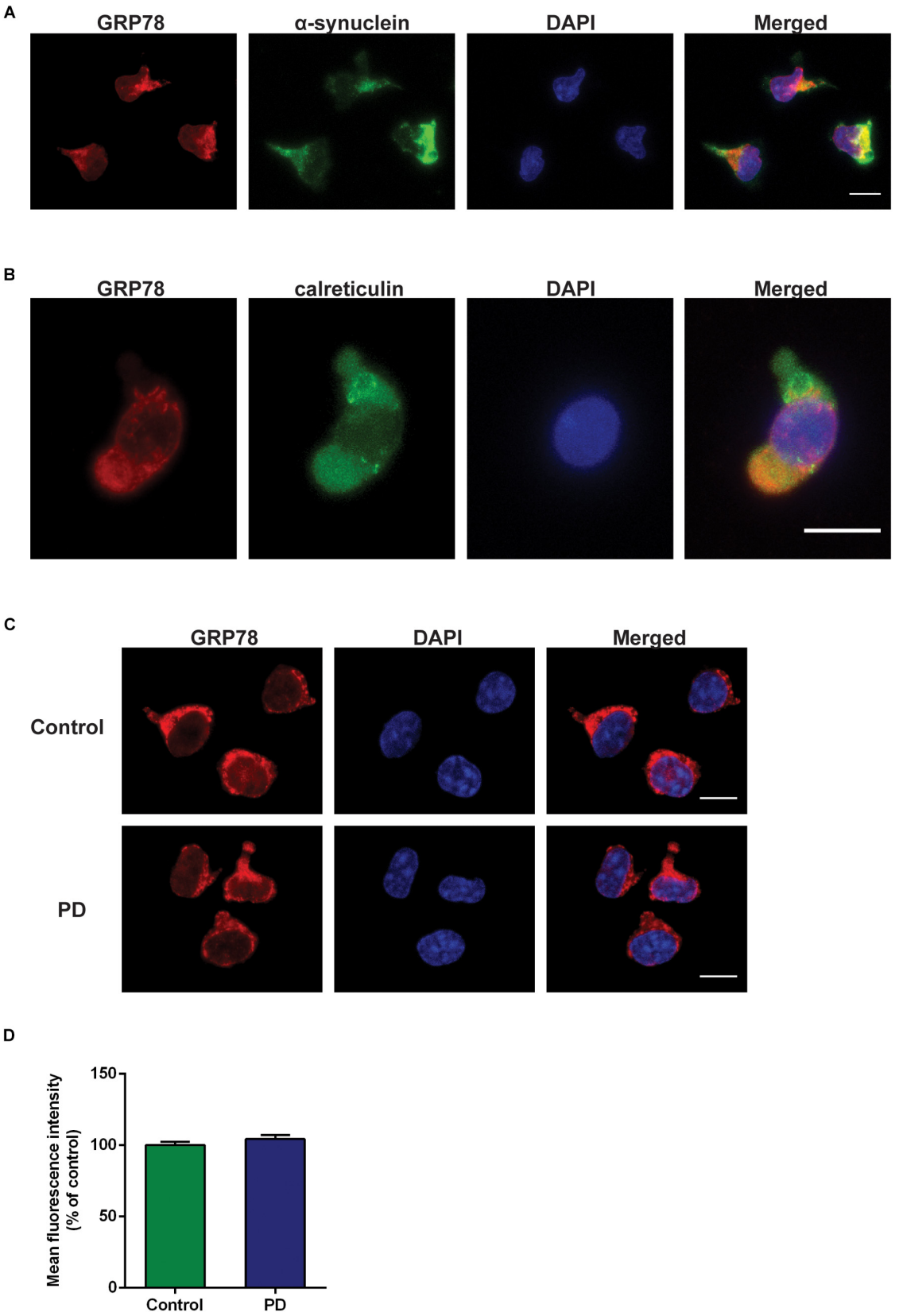

FIGURE 4 | Continued

FIGURE 4 | GRP78 protein cellular localization and expression in $CD4^+$ T cells derived from control subjects and PD patients. **(A)** Double immunofluorescence staining of GRP78 (red) and α-synuclein (green) in $CD4^+$ T cells derived from control subjects. **(B)** Double immunofluorescence staining of GRP78 (red) and calreticulin (green) in $CD4^+$ T cells derived from control subjects. **(C)** There was a moderate level of GRP78 protein expression (red) in both control subjects and PD patients. GRP78 was localized in the cytoplasm. Scale bar represents 10 mm. **(D)** The level of GRP78 protein expression in PD patients was not significantly different from control subjects.

evaluate the state of other UPR reporters such as XBP1 and PERK. Since induction of the ER chaperones and the XBP1 cleavage can occur independent of UPR mechanisms (Kim et al., 2008), lack of GRP78 and/or p-eIF2α inductions in PD patients observed in the present study may reflect activation of processes other than UPR. Moreover, to further validate the UPR changes observed in the present study, changes in the level of other ER resident proteins, such as GRP94, calnexin, and protein disulfide isomerase, should also be examined in the future.

Given that the UPR is a homeostatic stress response, it means that it is greatly controlled by positive and negative feedback loops. The interaction between the three signaling pathways of the UPR proposes that the alteration of one pathway will affect signaling of the other two pathways also. For example, inhibition of one pathway may in fact increase signaling through one of the other pathways. This phenomenon was demonstrated by Harding et al. (2001) where deletion of PERK resulted in an increased activity of IRE1α. Therefore, additional studies investigating the changes in the proteins of the other two arms of the UPR pathway, that is, IRE1α and ATF6, are needed to fully understand the relationship between different arms of the UPR pathway in PD, and how each arm of the UPR pathway is involved in the neurodegenerative process in PD.

Diagnosis of PD remains tricky and misdiagnosis rate with vascular or atypical parkinsonian disorders reaches up to 20–30% (Rajput and Rajput, 2014). At present, the assessment of clinical motor symptoms underlies the diagnosis of PD. The absence of a reliable biomarker with high sensitivity and specificity has significantly hindered the validation of potential therapies. One of the novel results of this study was the measurement of the GRP78 protein in plasma and CSF samples of PD patients as to discover a potential biomarker for PD. Although GRP78 protein has been detected in the plasma of endometrial cancer and obese patients (Ciortea et al., 2016; Khadir et al., 2016), it has never been measured in PD patients. In the plasma from control subjects and PD patients, high levels of GRP78 protein were detected, though its level in PD patients was not significantly different to control subjects (**Figure 3A**). To the best of authors' knowledge, the present study is first to evaluate the presence of GRP78 protein in CSF of PD patients. Nevertheless, unlike plasma, GRP78 protein in CSF was almost undetectable in both control subjects and PD patients (**Figure 3B**). A possible reason for the undetectable level of GRP78 in CSF could be that "whole" GRP78 protein is too large to be secreted in CSF. Therefore, it would be interesting to investigate whether fragments of GRP78 protein could be detected in CSF. Although the current study did not provide a clear evidence that GRP78 could serve as a possible biomarker for PD, further studies are required in order to investigate whether other UPR proteins and/or ER stress-related proteins have potential to be novel biomarkers for PD. A relatively high level of GRP78 protein in plasma from both control subjects and PD patients (**Figure 3A**) led us to further understand the implication(s) of this result in the disease state. Therefore, we investigated the level of GRP78 protein expression in PBMCs, specifically in $CD4^+$ T cells derived from control subjects and PD patients. However, when we compared the expression level of GRP78 in PD patients vs. control subjects, we found no difference (**Figures 4C,D**). Delpino and Castelli (2002) showed that extracellular GRP78 are mostly derived from an active release from living cells and are not solely due to the protein leakage from dead cells. Recent studies have also demonstrated that GRP78 release is increased in cancer, obesity, or upon ER stress (Khadir et al., 2016; Steiner et al., 2017). Taken together, it may be hypothesized that the high level of GRP78 protein observed in plasma of PD patients could be due to circulating PBMCs releasing GRP78 into the extracellular domain. However, since the level of GRP78 protein in plasma in PD patients was not different from control subjects, further investigation is inevitable to determine the difference in the state of GRP78 protein between control subjects and PD patients.

CONCLUSION

In conclusion, the present study showed that there are changes in the level of UPR proteins and mRNAs, particularly GRP78, in various regions of the brain of PD patients (**Figures 1**, **2**). However, while there were central changes, there were no peripheral changes, as observed in the levels of GRP78 protein in CSF, plasma, and in immune cells (**Figures 3**, **4**). Based on these results, one can cautiously postulate that UPR changes may be limited to the site of neurodegeneration, and not influenced elsewhere. In other words, it may be that the UPR in PD is quite a specific response in terms of location of action rather than a generic reaction to ER stress or progression of PD. This highlights an attractive opportunity to explore the UPR as a novel therapeutic target for PD with negligible peripheral side effects.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Alzheimer's Disease Neuroimaging Initiative recommended protocol with written informed consent from all subjects. All subjects gave written informed consent in

accordance with the Declaration of Helsinki. The protocol was approved by the UK National Research Ethics Service and by the Regional Ethics Review Board of Stockholm.

AUTHOR CONTRIBUTIONS

J-HB and PS conceived and designed the experiments. J-HB, BT, MP, DM, and YH performed the experiments. J-HB, BT, MP, and DM analyzed the data. J-HB wrote the manuscript. All authors have read and approved the final manuscript.

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REVIEW
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Bioenergetics and Autophagic Imbalance in Patients-Derived Cell Models of Parkinson Disease Supports Systemic Dysfunction in Neurodegeneration

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Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder worldwide affecting 2–3% of the population over 65 years. This prevalence is expected to rise as life expectancy increases and diagnostic and therapeutic protocols improve. PD encompasses a multitude of clinical, genetic, and molecular forms of the disease. Even though the mechanistic of the events leading to neurodegeneration remain largely unknown, some molecular hallmarks have been repeatedly reported in most patients and models of the disease. Neuroinflammation, protein misfolding, disrupted endoplasmic reticulum-mitochondria crosstalk, mitochondrial dysfunction and consequent bioenergetic failure, oxidative stress and autophagy deregulation, are amongst the most commonly described. Supporting these findings, numerous familial forms of PD are caused by mutations in genes that are crucial for mitochondrial and autophagy proper functioning. For instance, late and early onset PD associated to mutations in Leucine-rich repeat kinase 2 (*LRRK2*) and Parkin (*PRKN*) genes, responsible for the most frequent dominant and recessive inherited forms of PD, respectively, have emerged as promising examples of disease due to their established role in commanding bioenergetic and autophagic balance. Concomitantly, the development of animal and cell models to investigate the etiology of the disease, potential biomarkers and therapeutic approaches are being explored. One of the emerging approaches in this context is the use of patient's derived cells models, such as skin-derived fibroblasts that preserve the genetic background and some environmental cues of the patients. An increasing number of reports in these PD cell models postulate that deficient mitochondrial function and impaired autophagic flux may be determinant in PD accelerated nigral cell death in terms of limitation of cell energy supply and accumulation of obsolete and/or unfolded proteins or dysfunctional organelles. The reliance of neurons on mitochondrial oxidative metabolism and their post-mitotic nature, may explain their increased vulnerability to undergo degeneration upon mitochondrial challenges or autophagic insults. In this scenario, proper mitochondrial function and

turnover through mitophagy, are gaining in strength as protective targets to prevent neurodegeneration, together with the use of patient-derived fibroblasts to further explore these events. These findings point out the presence of molecular damage beyond the central nervous system (CNS) and proffer patient-derived cell platforms to the clinical and scientific community, which enable the study of disease etiopathogenesis and therapeutic approaches focused on modifying the natural history of PD through, among others, the enhancement of mitochondrial function and autophagy.

Keywords: neurodegeneration, mitochondria, autophagy, Parkin, LRRK2, fibroblasts

PARKINSON'S DISEASE

Parkinson's disease (PD) is the most common movement disorder and the second most frequent neurodegenerative disease affecting more than 6.5 million people worldwide (Teves et al., 2017), representing 2–3% of the population over 65 years (Connolly and Lang, 2014; Poewe et al., 2017). As the global life expectancy increases, the number of people with PD is expected to rise by more than 50% in 2030, constituting an important burden for public health (Kalia and Lang, 2015).

Although it was already known in ancient India under the name of "Kampavata," PD was first described in 1817 by James Parkinson in the *Essay on the Shaking Palsy* and later on refined and expanded by Jean-Martin Charcot who named the disorder "malaldie de Parkinson"(Kempster et al., 2007; Corti et al., 2011; Goetz, 2011). Despite PD was described more than two centuries ago, the conceptualization of the disease continues to evolve and it is now recognized as a systemic disease with multiple layers of complexity.

The cardinal symptoms of PD described by James Parkinson in 1817 and then refined by Jean-Martin Charcot, include bradykinesia, muscular rigidity, rest tremor and postural and gait impairment (Goetz, 2011) (**Figure 1**).

Pathologically, PD is a complex neurodegenerative disorder characterized by the prominent death of dopaminergic neurons (DAn) in the *substantia nigra* (SN) *pars compacta* (SNpc) located in the mesencephalon and the consequent striatal dopamine (DA) deficit that leads to the classical motor symptoms of the disease (Kalia and Lang, 2015). In addition to the loss of DAn, another hallmark of PD is the presence of intraneuronal inclusions in the soma of the remaining DAn. These inclusions, named Lewy bodies (LB) as well as Lewy neurites (LN), are collectively referred as Lewy pathology (LP). LB are round eosinophilic inclusions mainly formed by insoluble α-synuclein aggregates as well as ubiquitin and other proteins (Shults, 2006). The aggregation of these misfolded proteins has been shown to be common to PD, dementia with LB, and multiple system atrophy (Goedert et al., 2013).

Clinical Features

The dramatic loss of DAn in the SNpc, even in early stages of the disease, suggests that the degeneration in this region starts long before the motor symptoms appear (Poewe et al., 2017). In this context, PD is considered to occur in three stages: preclinical PD, when the neurodegeneration has started but no clinical signs or symptoms are present; premotor or prodromal PD, when clinical signs and/or symptoms are present but are insufficient to establish a diagnosis of PD; and clinical PD, when the diagnostic criteria are met (Kalia and Lang, 2015, 2016). Non-motor features are frequently present in the prodromal phase of the disease, which can last for 20 years or more, and involve a multitude of non-motor features including rapid eye movement (REM) sleep behavior disorder (RBD), constipation and hyposmia, as well as depression and anxiety (Poewe, 2008).

In this scenario, it is now widely accepted that PD is not a movement disorder simply induced by the loss of the DAn in the SNpc. The SN is not the only damaged region in PD, nor the first affected one. Brain sites other than the SN, such as the cerebral cortex and the limbic system, have also been reported to be impaired in patients during the presymptomatic phase (Dickson, 2018). In fact, several studies have shown that the degenerative process in PD is much more extensive and affects not only the central nervous system (CNS) but also the peripheral autonomic nervous system (PNS) and the organs outside the brain that the latter innervates (Braak et al., 2004). PNS dysfunction underlies the presence of some of the specific non-motor features that appear in the prodromal phase of PD and remain present over the course of the disease (Jain, 2011). In line with this, LP is not restricted to the brain but has also been encountered in the spinal cord and PNS including the vagus nerve, sympathetic ganglia, cardiac plexus, enteric nervous system (ENS), salivary glands, adrenal medulla, cutaneous and sciatic nerves (Tolosa and Vilas, 2015).

Risk Factors

Parkinson's disease was thought to be primarily caused by environmental factors, but research reveals that the disease develops from a complicated interplay of ageing, genetics and environment. In fact, the vast majority of cases occur sporadically and genetic forms of the disease account for about 10% of patients (Klein and Westenberger, 2012).

In general, the average age of onset of PD is the late fifties, with a broad range from <40 to >80 years of age depending, among others, on its pattern of inheritance. Young-onset PD is commonly defined by an age of onset <45 years and $>10\%$ of these cases have a genetic basis; the proportion of genetically defined cases rises to $>40\%$ of those with disease onset before 30 years of age (Alcalay et al., 2010; Marder et al., 2010).

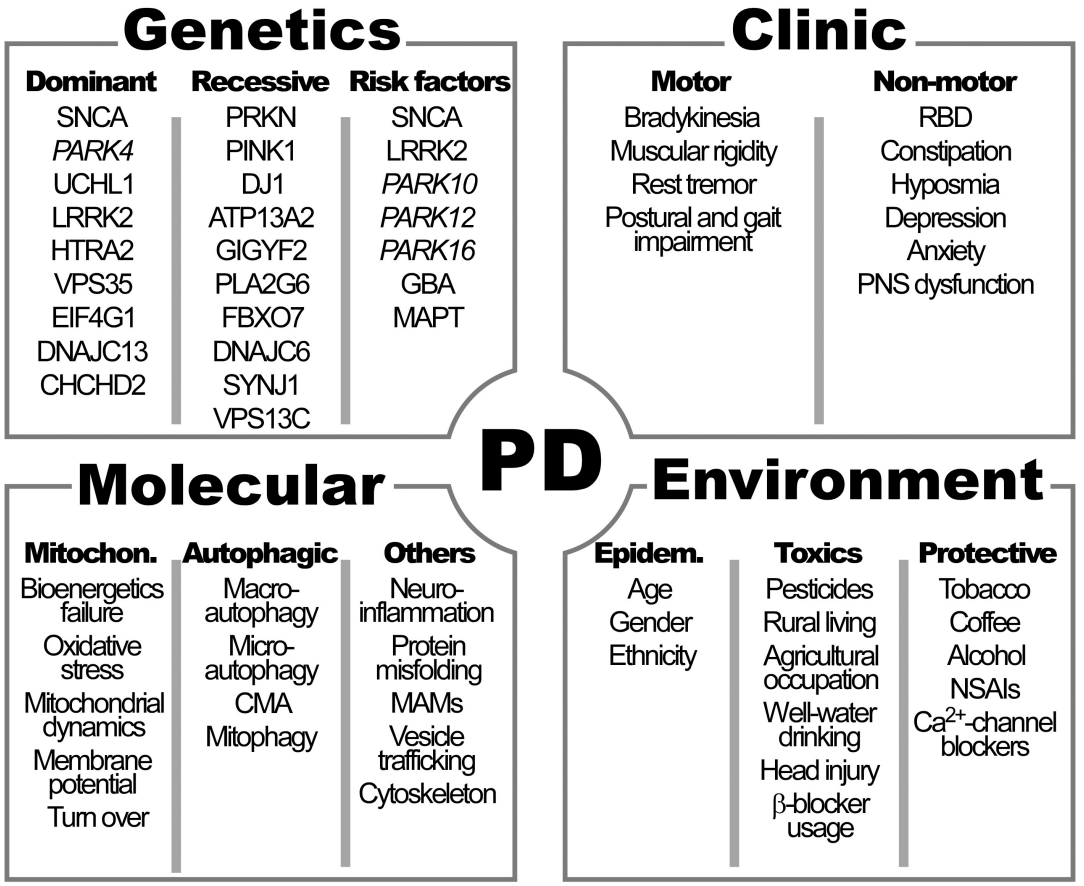

FIGURE 1 | Clinical manifestations and genetic, molecular and environmental factors characteristics of Parkinson's disease (PD). See **Table 1** for the names of genes. RBD, rapid eye movement sleep behavior disorder; PNS, peripheral autonomic nervous system; CMA, chaperon-mediated autophagy; MAMs, mitochondrial associated membranes; NSAIs: non-steroidal antinflammatory drugs; Ca^{2+}, calcium.

Non-genetic Risk Factors

The greatest risk factor for the development of neurodegenerative diseases, including PD, is ageing (Kalia and Lang, 2015). Incidence increases nearly exponentially from the sixth to the ninth decade of life by 5–10 fold (Kalia and Lang, 2015; Poewe et al., 2017). Total global prevalence is 0.3% and rises with age up to 3% in those >80 years of age (Poewe et al., 2017) (**Figure 1**). Many lines of evidence suggest that some molecular pathways including mitochondrial dysfunction, oxidative stress and abnormal cell wasting clearance (autophagy) have a central role in both, physiological aging and age-related neurodegenerative diseases, such as PD (Lin and Beal, 2006). This may be especially relevant in neurons, due to its post-mitotic nature and scarce replacement, that prone them to store defects as they age. Accelerated or healthy aging and all factors responsible of modulating brain fragility play a major role in PD development, together with genetic and environmental factors.

Gender has also been reported to be a risk factor for PD, with approximately a 3:2 male-to-female ratio. Sex hormones have been proposed to play a neuroprotective role in the disease. In case of female hormones, the antioxidant capacity of estradiol, for instance, has been proposed to prevent neurodegeneration (Aguirre-Vidal et al., 2017). Interestingly, estradiol has also been demonstrated to activate metabolic signaling by regulating mitochondrial function, emerging as protective hormone in case of bioenergetic deficits (Pozdniakova et al., 2018). The neuroprotecting role of progesterone is also being evaluated (Bourque et al., 2019). Alternatively, gender associated differences could also be associated to sex-associated genetic mechanisms, to specific differences in exposure to environmental cues or to the contribution of inequality in health care (Kalia and Lang, 2015). Interestingly, in a few populations, including one study from Japan, no differences in gender, and even increased prevalence in females, was observed (Kusumi et al., 1996). The explanation for equal gender PD prevalence in these populations remains elusive. Dietetic, socio-cultural, economic or even hormonal or molecular characteristics of Japanese population (as particular mitochondrial DNA haplogroups) may justify gender equivalence in PD development.

Other risk factors for PD are directly associated with environmental features are pesticide exposure, rural living, agricultural occupation, well-water drinking, prior head injury and β-blocker use. A special mention must be done for exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP), with

similar structure of some herbicides that increase the risk of PD, due to its historical importance. Since its incidental discovery in drug abusers after inadvertent self-administration, MPTP has been widely used to induce PD in animal models (Zhang et al., 2019). MPTP is lipid-soluble molecule that penetrates the blood–brain barrier and, once converted to its oxidized product (MPP+), interferes with mitochondrial respiration. MPP+ acts specifically in mitochondrial complex I, such as other toxic PD inducers (including rotenone and pesticides). Blockade of mitochondrial respiration has three main toxic consequences to cells: the inhibition of ATP generation and associated bioenergetic failure, the derived elevation of intracellular Ca^{2+} that promotes cell death and the promotion of oxidative stress responsible of cell damage, all hallmarks of PD pathogenesis.

In contrast, tobacco smoking, coffee drinking, non-steroidal anti-inflammatory drug use, calcium channel blocker intake, and alcohol consumption have been associated with a decreased risk of PD development (Kalia and Lang, 2015).

Additionally, the incidence of PD seems to vary within different ethnicities. The prevalence of PD is high in Ashkenazi Jews of Israel, Inuit, Alaska native and native American populations (de Lau and Breteler, 2006; Poewe et al., 2017) and only one study reports that PD is more common in Hispanic and non-Hispanic whites compared to African Americans and Asian in United States (Van Den Eeden et al., 2003). The higher incidence of PD in these populations has been classically attributed to specific genetic burden.

Genetic Risk Factors

There are two different classes of genetic contributions to PD: gene mutations directly associated to genetically inherited forms of PD and genetic variations, indirectly accounting for risk factors of disease.

With respect to the first, the existence of heritable forms of PD was originally established through the discovery in 1997 that mutations in *SNCA*, the gene encoding for the α-synuclein protein, caused PD and the demonstration that α-synuclein was the major component of LB (Corti et al., 2011). Since then, the list of mutations causing monogenic types of PD continues to grow associated either to dominant or recessive inherited forms of PD (**Table 1** and **Figure 1**). Currently 8–10% of cases are familial (result from a genetic alteration leading to PD). They are caused by a subset of *locus* usually encoded by the prefix PARK and a number referring to their order of discovery. Those mutations affecting genes with an autosomal recessive pattern of inheritance usually result in early onset cases of PD, while mutations affecting genes with dominant autosomal inheritance usually cause forms of PD that resemble late-onset idiopathic PD.

The discovery of the molecular pathways orchestrated by proteins encoded by these genes associated with monogenic forms PD, have reinforced the notion that impaired mitochondrial and autophagy homeostasis are key events in disease etiology (Park et al., 2018). In fact, impaired mitochondrial function and autophagy have been directly linked to mutations of at least eleven of the genes associated to familial PD (**Table 1**). Among them, Leucine-rich repeat kinase 2 (*LRRK2*) and Parkin (*PRKN*) genes emerge amongst the most frequent forms of autosomal dominant and recessive forms of PD, respectively.

With respect to the second, genetic risk factors of PD account for the rest of 90–92% of non-inherited cases of PD, so-called idiopathic. They are caused by the complex interplay of an array of unknown factors, a part from the numerous genetic risk factors of PD (see **Table 1**): modifying effects by susceptibility alleles, environmental exposures and gene-environmental interactions that may condition gene expression. Some of these genetic risk factors that may conditionate the development of PD are in common to lysosomal storage diseases, mitochondrial pathologies or genes governing autophagic processes.

The definition of genetic and environmental cues in the development of PD is one of the novel areas of study in which growing and coming efforts should be focused and, probably, thanks to the development of new generation sequencing tools, the discovery of novel gens responsible of PD will arise, together with new putative genetic risk factors, thus reducing the number of idiopathic PD cases.

Table 1 includes mutations directly associated to genetically inherited forms of PD (either of dominant or recessive inheritance) and genetic variations indirectly accounting for risk factors of disease. Gene name, *locus* and symbol have been described, together with the protein encoded by the gene and the function it exerts (the cell pathway governated), as well as the kind of PD associated to the genetic mutation or variation (responsible of early, late, sporadic or unknown onset). Among these entities, *LRRK2* and *PRKN* genes emerge amongst the most frequent forms of autosomal dominant and recessive inherited-forms of PD, respectively.

MODELS TO STUDY PARKINSON'S DISEASE

A major challenge to study PD is the inaccessibility of the target tissue of the disease (DAn), which is currently only available *post-mortem*. In addition, by the time that clinical symptoms manifest, most of the cells targeted by PD have already been lost (Grosch et al., 2016). Thus, finding models that faithfully recapitulate the events in PD is essential to understand the impaired molecular processes that underlie the disease etiopathogenesis and its progression. In this regard, different experimental *in vitro* and *in vivo* models of study have been consistently used (Blesa et al., 2012; Falkenburger et al., 2016).

Animal Models

Animal models have allowed the study of PD *in vivo*, partially reproducing the specific pathogenic events and behavioral outcomes of the disease. In fact, after the study of brain necropsies from PD patients, much of the current understanding of the etiology and the pathogenesis of PD has been obtained from the study of neurotoxin-based animal models (Bezard et al., 2013), only recently complemented by experimental approaches targeting genes responsible of the disease. Typical cases of toxic exposure to induce PD in animal models is the use rotenone and MPTP exposition, which reinforced mitochondrial implication

TABLE 1 | Classification of genes associated with familial forms of Parkinson's disease.

Gene name	Gene symbol	Gene locus	Protein	Protein function and cell pathway governated	Onset of disease
Autosomal dominant inheritance Parkinson's disease					
SNCA	PARK 1 or PARK4	4q22.1	Alpha-synuclein	Synaptic vesicles trafficking	Early
Unknown	PARK3	2p13	Unknown	Unknown	Late
UCHL1	PARK5	4p13	Ubiquitin C-terminal hydrolase L1	Proteasome system	Late
LRRK2	PARK8	12q12	Leucine-rich repeat kinase 2	Autophagy processing	Late
HTRA2	PARK13	2p13.1	HtrA serine peptidase 2	Mitophagy development	Unknown
VPS35	PARK17	16q12	Vacuolar protein sorting 35	Endosome regulation	Late
EIF4G1	PARK18	3q27.1	Eukaryotic translation initiation factor 4 gamma 1	Protein translation	Late
DNAJC13	PARK21	3q22.1	DnaJ heat shock protein family (Hsp40) member C13	Endosome regulation	Late
CHCHD2	PARK22	7p11.2	Coiled-coil-helix-coiled-coil-helix domain containing 2	Mitochondria-mediated apoptosis and metabolism	Late/Early
Autosomal recessive inheritance Parkinson's disease					
PRKN	PARK2	6q26	Parkin	Mitophagy development	Early
PINK1	PARK6	1p36.12	PTEN-induced putative kinase 1	Mitophagy development	Early
DJ-1	PARK7	1p36.23	DJ-1	Mitophagy development	Early
ATP13A2	PARK9	1p36.13	ATPase cation transporting 13A2	Lysosomal function	Early
GIGYF2	PARK11	2q36-7	GRB10 interacting GYF protein 2	Insulin-like growth factors (IGFs) signaling	Early
PLA2G6	PARK14	22q13.1	Phospholipase A2 group VI	Lipids metabolism	Early
FBXO7	PARK15	22q12.3	F-box protein 7	Mitophagy development	Early
DNAJC6	PARK19	1p31.3	DnaJ heat shock protein family (Hsp40) member C6	Endosome regulation	Early
SYNJ1	PARK20	21q22.11	Synaptojanin 1	Endosome regulation	Early
VPS13C	PARK23	15q22.2	Vacuolar protein sorting 13 homolog C	Mitophagy development	Early
Risk factors for developing PD					
SNCA	PARK 1 or PARK4	4q22.1	Alpha-synuclein	Synaptic vesicles trafficking	Early
LRRK2	PARK8	12q12	Leucine-rich repeat kinase 2	Cytoskeleton/vesicle transporting/autophagy regulation	Late
Unknown	PARK10	1p32	Unknown	Unknown	Unknown
Unknown	PARK12	Xq21-q22	Unknown	Unknown	Unknown
Unknown	PARK16	1q32	Unknown	Unknown	Unknown
GBA	–	1q22	Glucosylceramidase beta	Lysosomal function	Late
MAPT	–	17q21.31	Microtubule associated protein tau	Microtubule structure	Sporadic

Modified from Del Rey et al. (2018).

in PD. However, toxin-based models rely on acute insults to the nervous system and do not model the slow neural degeneration and development of clinical manifestations characteristic of PD (Westerlund et al., 2010) or the presence of LB, hallmark of the disease, thus raising concerns on the recapitulation of PD pathology.

Later, with the identification of PD-related genes, transgenic models including yeast, *Drosophila melanogaster*, *Caenorhabditis elegans* and murine models have been developed (Blesa et al., 2012) as an alternative to the classical toxin-based ones. These models enabled to gain insights in the molecular events underlying the disease, such as mitochondrial and autophagic deregulation, widely demonstrated in the target tissue of PD. These models have shed light into PD pathogenic processes, but have fallen short in replicating the phenotype and pathology of human disease (Dawson et al., 2010). One of the main drawbacks in the use of animal models to study PD is life span difference between species, which may not allow reproducing age-related events that are relevant to disease pathogenesis. On the other hand, there is an important risk when specifically using invertebrates to study PD as relevant pathogenic factors are vertebrate-specific and may be absent in these models. Finally, most of them do not recapitulate the key clinical and neuropathological features of the disease (as trembling and neuromelanin withdrawal in DAn). Maybe this explains why biomarkers of disease previously verified in murine models and treatments that have shown positive outcomes in these models, have not later been predictive of therapeutic success in humans (Vandamme, 2014). The biological differences between mice and humans may be accountable for this fact, and it is an issue that researchers must be aware of and carefully account for when using these animal models.

In this regard, and probably due to a closer similarity, non-human primates (NHP) have been used to generate the most robust and clinically useful models of PD. The current gold standard animal model of PD is a toxin-based NHP induced

model, which shows stable, bilateral clinical features that closely resemble idiopathic PD (Johnston and Fox, 2014) and may even exhibit some features of RBD (Verhave et al., 2011). However, this model does neither recapitulate the major pathophysiological hallmark of idiopathic PD, LB, and its utility for the study of prodromal PD has still not been validated (Barraud et al., 2009).

The development of experimental models to elucidate disease etiology, find novel diagnostic/prognostic biomarkers and assay new therapeutic strategies remains as one of the most challenging gaps in PD research.

The use of toxic or genetic animal models of disease has strengths as reproducing the complex interplay between different neural and non-neural brain cells directly in the target tissue of the disease, and the assay of different therapeutic approaches in physiologic context. Unfortunately, in parallel, animal models fail to recapitulate important hallmarks of PD as clinical and anatomopathological features. Other weaknesses, a part of ethical concerns, high economic and facility costs, are that most of them fail to reproduce the influence of aging, epigenetic, and genetic modifying factors characteristic of PD patients. Novel cell models overcome part of these limitations.

Cell Models

Immortalized cell lines of neural origin, either animal or human, that can also be subjected to toxin exposition or gene editing, have been widely used to model PD (Obinata, 2007). These cell models have provided consistent and reproducible results. Among their main strengths there is their identical genetic background that confers them a large homogeneity (Carter and Shieh, 2015). Moreover, they represent wide platforms for disease modeling due to low cost maintenance and editing easiness. One example is the human-derived neuroblastoma cell line SHSY5Y that can be used undifferentiated or differentiated to DAn to model PD (Lopes et al., 2010; Alvarez-Erviti et al., 2011).

However, immortalized cell lines are also associated to important weaknesses including the presence of genetic instability or the high rate of glycolytic metabolism which pushes toward the use of patient-derived cell models (Frattini et al., 2015).

The fact that the greatest proportion of PD is of unknown cause and the urgent need to find novel biomarkers at the prodromal phase of the disease has encouraged the development of specific patient-derived models. The advantages of these patient-derived cell models is that they recapitulate PD pathogenicity at an individual basis, thus partially circumventing the drawbacks of animal and established cell line models (Teves et al., 2017). In this context, the use of patient-derived cells that conserve patient-specific features has constituted a substantial progress in the study of PD, considering the great complexity and individual variability of the disease that encompasses unknown genetic and epigenetic factors, including aging, as well as environmental insults, has been recapitulated.

The use of patient-derived neural stem cell models has stirred up the field of PD research. Some works on PD have been done directly studying these stem cells (Sanberg, 2007) and some others by differentiating them into neural precursors or mature neurons (Le Grand et al., 2015; Yang et al., 2017). For instance, neural precursors as neurosphere models (free-floating clusters of neural stem cells) are widely used for the study of neuronal differentiation and neuronal disease (Matigian et al., 2010). For the study of mature-derived neurons, the development of induced pluripotent stem cells (iPSCs) has spawned a new approach to model PD allowing researchers to generate disease-specific DAn *in vitro* by reprograming somatic cells from patients with the disease (Fernandez-Santiago and Ezquerra, 2016). It is expected that the access to iPSCs-derived neurons from PD patients will shed light into mechanistic insights of PD pathogenesis and serves as a platform for drug screening and early diagnosis.

One of the latest applications of iPSC is the generation of different brain cell lineages to create brain organoids that resemble neuronal architecture, self-organization and cell to cell interaction from the physiologic brain. They are three-dimensional (3D) *in vitro* culture systems that recapitulate the developmental processes and organization of the developing human brain. These "mini-brains" provide a physiologically relevant model for the study of neurological development and disease processes that are unique to the human nervous system, together with other 2D and 3D models including neurospheres, neural aggregates, neural rosettes, and cortical spheroids. They all are emerging and promising models for the study of brain fragility and neurodegenerative diseases that will bring light into PD field in the next coming years but that are currently handicapped because of their novelty and setting up troubleshooting (Schwamborn, 2018).

Additionally, and despite being an exciting prospect for PD research, stem cell-derived neural lines and iPSC technologies have some other important inconveniences, including a considerable phenotypic variability unrelated to their genotype and their high maintenance costs and time-inefficiency (Jacobs, 2014). Additionally, stem cell or iPSC differentiation into neurons generally leads to low yields of DAn generation (Jacobs, 2014) thus obtaining heterogeneous cell pools where undifferentiated and DAn-derived cell types coexist (Fernandez-Santiago et al., 2015). Of note, whilst stem cells and iPSCs reprogramed from somatic cells highly rely on glycolytic metabolism, neurons are mainly energetically sustained by mitochondrial oxidative metabolism. In this scenario, one of the limitations of these cell models is the analysis of certain cell processes such as bioenergetics, oxidative stress or autophagy may become biased due to this confounding factor. Additionally, the genetic manipulation required to generate iPSC-derived DAn is frequently associated to genetic aberrations (e.g., copy number variations, somatic coding mutations, and chromosomal defects). Thus, the development of alternative patient-derived cell models is gaining in strength.

As previously mentioned, accumulating evidences suggest that PD is a multisystem disorder rather than a solely dopaminergic motor syndrome that encompasses central and peripheral clinical features (Djaldetti et al., 2009; Cersosimo and Benarroch, 2012). In line with this, PD pathological and molecular changes are also not confined in the CNS but are also present in the PNS and the organs that the latter innervates (Djaldetti et al., 2009). For instance, a great number of studies have reported α-synuclein

deposits in many different peripheral tissues derived from PD patients (Tolosa and Vilas, 2015). On the other hand, many other alterations at molecular level including transcriptional changes, mitochondrial dysfunction and associated increased oxidative stress as well as autophagy deregulation have been described in PD-derived peripheral tissues such as muscle (Cardellach et al., 1993), blood cells including platelets and leukocytes (Haas et al., 1995; Muftuoglu et al., 2004; Mutez et al., 2014) and fibroblasts (Mortiboys et al., 2010; González-Casacuberta et al., 2018; Juarez-Flores et al., 2018), supporting the use of novel peripheral approaches.

The use of patient skin-derived fibroblasts has been widely utilized to model numerous diseases of metabolic, neurodegenerative, and lysosomal origin (Solini et al., 2004; McNeill et al., 2014; Alvarez-Mora et al., 2017; Konrad et al., 2017). For mitochondrial diseases, fibroblasts constitute the model of choice to diagnose and often to support research of these entities (Cameron et al., 2004; Soiferman and Saada, 2015; Ferrer-Cortès et al., 2016). Several studies in PD have used human skin-derived fibroblasts to investigate the molecular mechanisms underlying disease etiopathogenesis (Auburger et al., 2012). Skin-derived fibroblasts offer considerable advantages (**Table 2**): they constitute a patient-specific cellular system that retains the genetic (mutations, polymorphisms, polygenic risk factors, etc.) and epigenetic background of the patients while potentially preserving the specific environmental, toxic and cumulative age history. They show relevant expression of most PD genes which make them also suitable for the study of monogenic forms of PD (Auburger et al., 2012; Ivanov et al., 2016). Since they are accessible peripheral cells, they can be obtained from PD patients and healthy controls through an easy and minimally invasive procedure. Furthermore, fibroblasts can be propagated in culture, frozen, and stored for long periods of time, and transformed in cell types that exhibit molecular characteristics of the target tissue of the disease. It should be noted that fibroblasts make dynamic cell-to-cell contacts when cultured, which is similar to neuronal cells (Konrad et al., 2017).

Studies in skin-derived fibroblasts from patients with sPD have thrown relevant and consistent information regarding molecular pathways altered in this type of neurodegeneration. A recent study showed that fibroblasts from sPD patients have higher growth rates, altered morphology, increased mitochondrial susceptibility to UV-exerted stress and autophagic alterations (Teves et al., 2017).

Other works focused on the study of molecular alterations in monogenic-PD patient fibroblasts have reported disease-relevant changes further supporting the adequacy of this model to study these forms of the disease. These changes include altered transcript (González-Casacuberta et al., 2018) and protein expression (Lippolis et al., 2015; Azkona et al., 2016), *SNCA* gene expression up-regulation (Hoepken et al., 2008), altered GCase enzyme activity (McNeill et al., 2014), microtubule destabilization (Cartelli et al., 2012), impaired autophagy (Dehay et al., 2012; Rakovic et al., 2013; Juarez-Flores et al., 2018), increased sensitivity to neurotoxins (Yakhine-Diop et al., 2014), bioenergetic deficits (Papkovskaia et al., 2012; Ambrosi et al., 2014; Juarez-Flores et al., 2018), mitochondrial alterations (Mortiboys et al., 2008, 2010), and enhanced apoptosis (Klinkenberg et al., 2010; Romani-Aumedes et al., 2014).

In summary, skin-derived fibroblasts show certain disadvantages (see **Table 2**) but constitute, at the same time, a patient-specific cellular system that, without genetic manipulation, can potentially recapitulate the main features of the disease (Auburger et al., 2012). In fact, many of the molecular hallmarks occurring in nigral DAn have been reported in fibroblasts from patients with sporadic and monogenic forms of the disease (Hoepken et al., 2008; Mortiboys et al., 2010; Ambrosi et al., 2014; Haylett et al., 2016).

TABLE 2 | Advantages and disadvantages of using skin-derived fibroblasts as a cell model to study PD.

Advantages	Disadvantages
• Low facility and maintenance costs • Easy to obtain, propagate, maintain and store • Maintain genetic and epigenetic background of the patients • Reflect environmental cues and age-related events of patients • Relative poor invasiveness of the procedure • Presents relevant expression of most PARK genes • As primary cells, do not exhibit maximal glycolisis • Can be genetically manipulated to be reprogrammed to iPSCs and redifferentiated to dopaminergic neurons or to perform gene silencing or protein rescuing experiments	• As mitotic cells exhibit high renewal rates in contrast to the postmitotic nature of neural tissue • Not part of the central nervous system • Mixed metabolism (glycolitic and oxidative) to obtain cell energy • Mycoplasma contaminations are frequent and may lead to artificial phenotypes • Pure fibroblast culture is not achieved until passage 3 • Growing fibroblasts can be at different cell cycle phases representing a confounding factor

Modified from Auburger et al. (2012).

MOLECULAR MECHANISMS UNDERLYING PD PATHOGENESIS: MITOCHONDRIAL DYSFUNCTION, OXIDATIVE STRESS AND AUTOPHAGY IMPAIRMENT

Most of accumulated evidences derived from studying brains of PD patients, animal or cell models of disease stand for common molecular mechanisms underlying PD pathogenesis. Among them, neuroinflammation, apoptosis, proteasomal dysfunction, and especially mitochondrial impairment, reactive oxygen species production (ROS) and autophagic failure, emerge as key players in PD development. However, there are different schools of thought regarding the triggers of the disease. Two main hypotheses raised: according to the "protein depot cascade hypothesis," alpha-synuclein and other misfolded proteins stored as protein depots in neurons are the cause of PD. Other stand for the "mitochondrial cascade hypothesis," that foresees the origin of PD in a defect in the oxidative phosphorylation (OXPHOS) system. Interestingly, protein deposition and bioenergetics

appear to be closely related. Thus, alpha-synuclein can reduce OXPHOS function and OXPHOS deficiency can increase alpha-synuclein production (Cardoso, 2011; Haelterman et al., 2014).

In line with the "mitochondrial cascade hypothesis," some primary mitochondrial diseases caused by monogenic mutations in mitochondrial-related genes that usually translate to OXPHOS deficiencies, clinically manifests as brain disease such as neurodegeneration and parkinsonism (Diez et al., 2017; Suomalainen and Battersby, 2017). In fact, mitochondria contribute to neurodegeneration through deficiencies in the mitochondrial respiratory chain (MRC) or OXPHOS and associated overproduction of ROS, as well as the accumulation of mitochondrial DNA (mtDNA) mutations or defects in its quality (deletions) or quantity (depletion) (Suomalainen and Battersby, 2017). Many evidences point out that mitochondrial dysfunction-derived oxidative stress is mainly centralized at the level of the MRC complexes I and III and play a central role in brain damage of PD patients (Poewe et al., 2017) (**Figure 1**).

The study of mitochondrial contribution to PD and other neurodegenerative diseases has been widely validated by the use of cybrids (Ghosh et al., 1999; Llobet et al., 2013). They allow the exploitation of mutation-independent, mitochondrial-derived impairments (age-associated, mostly). Arduíno et al. described the generation of cytoplasmic hybrid cells (or cybrids) as a promising cellular model for the study of sPD. This approach consists on the fusion of platelets harboring mtDNA from sPD patients with cells in which the endogenous mtDNA has been depleted (Rho0 cells). This allows the comparison of different mtDNAs in the same nuclear context. The sPD cybrid model has been successful in recapitulating most of the hallmarks of sPD (including CI dysfunction, ROS generation, loss of calcium homeostasis, changes in mitochondrial morphology, increased proton leak and decreased maximal respiratory capacity, as well as protein aggregation in the form of Lewy body-like inclusions) (Swerdlow et al., 1996; Cassarino et al., 1997; Sheehan et al., 1997; Ghosh et al., 1999; Trimmer et al., 2004; Esteves et al., 2009, 2010a,b; Arduíno et al., 2012, 2013; Llobet et al., 2013), thus constituting a validated model for addressing the link between mitochondrial dysfunction and sPD pathology.

Mitochondrial function is essential in almost all cells of the organism, but specially in neurons. There are different reasons to explain their dependence on proper mitochondrial function, controlled oxidative stress production and adequate mitochondrial replacement through autophagy.

Neural metabolism is highly oxidative. Glucose is the obligatory energy substrate of the adult brain. However, under certain circumstances the brain has the capacity to use alternative blood-derived energy substrates, such as ketone bodies (during starvation or development) (Magistretti, 2006) and lactate (during periods of intense physical activity) (van Hall et al., 2009). Once inside the cell, glucose can be metabolized through glycolysis (leading to lactate production or mitochondrial metabolism) or through the pentose phosphate pathway (as glycogenesis can only be performed in astrocytes). Both mitochondrial metabolism and pentose phosphate pathway are the proper bioenergetic pathways enhanced in physiologic conditions to provide of ATP and antioxidant power to the cell, respectively. Contrarily, the metabolic activation of lactate production through the anaerobic glycolysis may be detrimental for long-term neuronal function and should be only sustained in certain punctual circumstances (Falkowska et al., 2015).

Metabolic oxidative activity of neurons explains they dependence on mitochondria to obtain energy. Noticeably, brain is one of the highest energy-demanding organs of the body due to the its intrinsic physiological activity. It is constituted by postmitotic cells (neurons) with less capacity for cellular regeneration compared to other organs. Thus, it is believed to be particularly vulnerable to mitochondrial dysfunction, ROS damaging effects and detrimental autophagic renewal of cell components, that may explain why is prone to manifest clinical evidences of mitochondrial, oxidative or autophagic alterations (Picard and McEwen, 2014). This is especially relevant in the case of DAn that synthesize the most pro-oxidant neurotransmitter of the CNS (DA), thus becoming especially vulnerable to oxidative environments, mitochondrial failure or autophagic imbalance.

In this scenario, mitochondrial dysfunction, associated oxidative stress and autophagic development becomes critical for neuronal survival. Several lines of evidence have implicated mitochondrial dysfunction as a key element in PD pathogenesis. Reduction of mitochondrial CI activity has been reported in several tissues isolated from PD patients including the CNS (Schapira and Gegg, 2011). In addition, the target genes of the mitochondrial master transcriptional regulator PGC1α have been reported to be under expressed in PD, together with strong evidences of increased oxidative stress and reduced autophagic function that may be finally responsible for defective mitochondrial and protein depot (Schapira and Gegg, 2011; Nixon, 2013; Siddiqui et al., 2015).

Unfortunately, when postmortem brain tissue of PD patients is studied, most of DAn have disappeared (Teves et al., 2017), thus difficulting the establishment of any potential etiologic causal link. In consequence, the study of experimental models of PD conveys the opportunity to foresee the triggers of neuronal degeneration.

As previously mentioned, initial models of PD were developed by using MPTP, a mitochondrial neurotoxin that specifically targeted DAn in primates and mice which was discovered to produce parkinsonism in humans (Langston, 2017). MPTP-based models and many other models using mitochondrial neurotoxins such as 6-hydroxydopamine (6-OHDA), rotenone or paraquat, have been used over the past years in the PD research field to replicate features of disease neuropathology (Jagmag et al., 2015). On the other hand, the depletion of mitochondrial proteins in mice that are essential for mtDNA maintenance (TFAM and En1) leads to neuronal degeneration of DAn in the SN which accounts for the development of several important features of PD neuropathology (Pickrell et al., 2013).

In PD patients, reduction of mitochondrial CI activity has been reported in several isolated tissues including the SN and peripheral tissues (Schapira and Gegg, 2011). In addition, the downregulation of the target genes of the master transcriptional regulator involved in mitochondrial biogenesis (peroxisome proliferator-activated receptor gamma coactivator 1-alpha or PGC-1α) have been reported in PD (Siddiqui et al., 2015). Low

levels of α-synuclein have been encountered in mitochondria in physiologic conditions and *in vitro* abnormal accumulation of this protein has proven to lead to mitochondrial CI dysfunction and associated oxidative stress, importantly linking these two events that have been repeatedly reported in PD (Poewe et al., 2017).

Different levels of ROS damage have been reported within the target brain region that undergoes selective neurodegeneration in PD. Specifically, lipid peroxidation markers such as 4-hydroxynonenal and malondialdehyde, have been identified in the SN of PD patients (Dias et al., 2013). However, it remains elusive whether oxidative stress occurs early in the disease or later during the decease of neurons and thus, as a consequence of cell degeneration.

Mitochondria are not isolated or static entities but instead are highly dynamic organelles that are transported on cytoskeletal proteins responsible for mitochondrial trafficking and are continuously subjected to fusion and fission processes in order to maintain their homeostasis (Johri and Beal, 2012). Mitochondrial dynamics have also been reported to be altered in PD (Van Laar and Berman, 2009). Similarly, mitochondrial turnover and quality control through selective autophagy (mitophagy), or mitochondrial relationship with other organelles as endoplasmic reticulum and its associated membranes (MAMs) have also been associated to PD (Hattori et al., 2017). Mitochondrial dysfunction and oxidative stress are associated with the impairment of the autophagy process through the accumulation of damaged mitochondria due to their defective turnover and through depletion of lysosomes, evidencing that different molecular pathways involved in PD pathogenesis are intimately related (Poewe et al., 2017).

Although our understanding of the regulatory pathways that control autophagy is still limited, recent advances have shed light on the importance of autophagy in a panoply of physiological processes and human diseases including neurodegeneration (Jing and Lim, 2012). The post-mitotic status of neurons prone them to be strictly dependent on optimal regulation of autophagy for the removal of dysfunctional or obsolete cell constituents, especially as the brain ages. In fact, the most prevalent pathological feature of many neurodegenerative diseases is the aggregation of misfolded proteins and the loss of certain neuronal populations (Guo et al., 2018), closely related to lysosomal function. This becomes more evident with the observation that the brain is often the most severely affected organ in primary lysosomal disorders and that mutations in genes involved in autophagy are causatively linked mechanisms to neurodegenerative disorders with exceptional frequency. This link between lysosomal performance and neurodegenerative diseases explains the high prevalence of genetic lysosomal variants that are found in PD by genome wide association studies (Levine and Kroemer, 2019).

On the other hand, it has been reported that in neurodegenerative disorders, such as Alzheimer's disease, amyotrophic lateral sclerosis and familial PD, defects arise at different stages of the autophagy pathway and have different implications for pathogenesis and therapy (Nixon, 2013). Recent research in the field of PD points out to alterations in specific steps of autophagic processes that could be of high relevance in the etiopathogenesis of the disease (Jing and Lim, 2012; Nixon, 2013; Guo et al., 2018) (**Figure 1**). For instance, an upregulation of macroautophagy following an overwhelmed chaperone-mediated autophagy (CMA) system as a result of overexpression of misfolded aggregates of α-synuclein has been shown in mice and *in vitro* PD model systems (Spencer et al., 2009; Yu et al., 2009; Ebrahimi-Fakhari et al., 2011). Moreover, an accumulation of autophagosomes (AP) and an early decrease in lysosome content as a result of lysosomal membrane destabilization and cytosolic release of cathepsins has been reported in DAn of a neurotoxin-based mouse model (Dehay et al., 2010). In addition, a decrease in lysosomal acidification and consequent decline of lysosomal protein turnover has been reported in mice overexpressing α-synuclein (Stefanis et al., 2001; Cuervo et al., 2004).

In *post mortem* brain samples of PD patients dysfunctional lysosomes and accumulation of AP were observed in neurons, indicating a pathogenic role of autophagy in PD (Guo et al., 2018).

Mitochondrial Dysfunction, Oxidative Stress, and Autophagy Disruption in Familial PD

Amongst all familial forms of PD, late and early onset PD associated to mutations in *LRRK2* and *PRKN* genes, respectively, are responsible for the most frequent dominant and recessive inherited forms of PD. These genes have emerged as promising examples of disease due to their established role in commanding bioenergetic and autophagic balance (**Figure 2**).

Numerous genes responsible of inherited PD cause the impairment of essential functions for mitochondrial homeostasis: OXPHOS function, mitochondrial trafficking, oxidative stress, calcium imbalance, mitochondrial biogenesis, mitochondria dynamics and mitochondrial autophagy.

LRRK2-Associated PD (LRRK2-PD)

The *LRRK2* gene (at the PARK8 *locus*) is located in chromosome 12, contains 51 exons and spans a genomic distance of 144 kb that includes 7500 nucleotides of coding sequence. Up to date, about 80 "probably pathogenic" and seven pathogenic *LRRK2* mutations have been described, being the G2019S (G2019S-*LRRK2*) the most frequent pathogenic mutation (Corti et al., 2011). Most of *LRRK2* mutations correspond to missense variants, which, along with the dominant inheritance, are consistent with a gain-of-function pathogenic mechanism (Corti et al., 2011). A number of non-pathologic variants are also known and some others variants that may act as PD risk factors have been reported using genome wide association studies (Islam and Moore, 2017).

LRRK2 encodes a 2527 amino-acid multi-domain protein (LRRK2), which is also known as dardarin, from the Basque word "dardara" which means trembling. *LRRK2* has the particular feature of encoding a leucine-rich repeat (LRR), a ROC-COR GTPase, a mitogen-activated protein kinase, and WD40 domains in the same protein (Singh et al., 2019) (**Figure 3**). Of note,

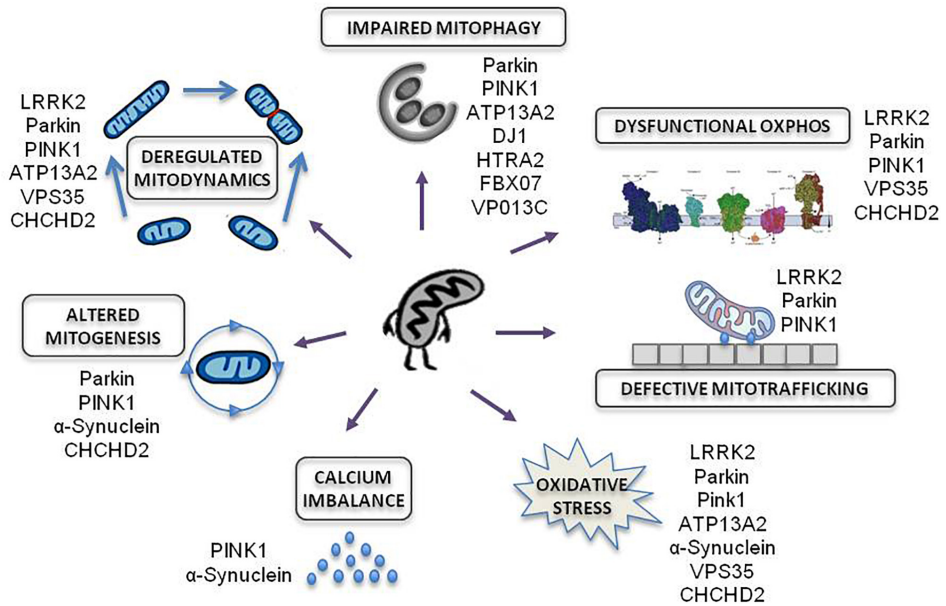

FIGURE 2 | Mitochondrial effects of genetic mutations associated to familial PD. Modified from Park et al. (2018).

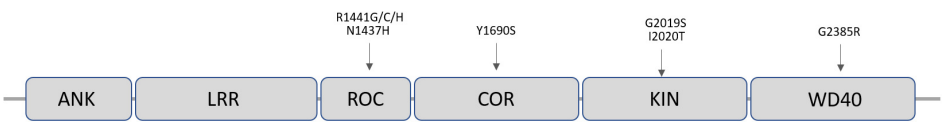

FIGURE 3 | Schematic representation of the LRRK2 protein showing its functional domains and the most frequent pathogenic missense point mutations responsible for PD.

pathogenic mutations appear to be located in functionally relevant regions of the protein such as in the specific case of the G2019S-*LRRK2* mutations that affect the kinase domain making it more active (Corti et al., 2011) (**Figure 3**). LRRK2 is expressed in most organs including brain, heart, liver, and circulating immune cells.

Mutations in *LRRK2* account for ∼10% of familial PD and for a significant fraction of sPD cases (Kalinderi et al., 2007). *LRRK2* mutation frequencies vary between ethnic groups, being North African Arabs and Ashkenazi Jews the most affected populations (Correia Guedes et al., 2010). The G2019S-*LRRK2* mutation is responsible for 1% of apparent sPD and 4% of familial PD worldwide (Healy et al., 2008). Importantly, even when proven pathogenic mutations are present, penetrance is age dependent and estimated between 30 and 74% (Ozelius et al., 2006). This fact makes the *LRRK2*-mutation carriers a very interesting target of study, as they present a subclinical stage with molecular alterations potentially determinant for disease progression.

Clinically, *LRRK2*-associated PD (*LRRK2*-PD) presents with a PD-typical phenotype with no sex association. A large systematic review of *LRRK2*-PD case reports that age of onset is around 57 years, with a mean disease duration of 10 years. The cardinal PD symptoms were reported with the following frequency: bradykinesia in 99%, rigidity in 99%, tremor in 88% and postural instability in 65%, while atypical signs of PD have been reported only anecdotally. In addition, autopsies of such patients showed prominent loss of melanized DAn in the SNpc (Trinh et al., 2018). Disease progression is slow and response to treatment is as good as in sPD (Corti et al., 2011). *LRRK2*-PD has demonstrated an unprecedentedly significant role of LRRK2 in PD pathogenesis as most of the clinical and pathological features are indistinguishable from those of sPD (Gosal et al., 2005). Thus, since its discovery, great efforts have been focused on the study of this form of the disease.

Within cells, *LRRK2* associates with various intracellular membranes and vesicular structures including the endosomes, the lysosomes, the multivesicular bodies, the outer mitochondrial membrane (OMM), lipid rafts, microtubule associates vesicles, Golgi complex, and the endoplasmic reticulum (Cookson, 2012), thus it is highly associated to MAMs. Accordingly to its multi-domain nature, LRRK2 has been implied in many cellular functions such as cytoskeleton remodeling, vesicle trafficking and movement, protein translation, autophagy and mitochondrial function homeostasis (Cookson, 2010; Liu et al., 2012; Taymans et al., 2015; Roosen and Cookson, 2016; Juarez-Flores et al., 2018; Price et al., 2018). Specifically, studies consisting of modifications in the expression of *LRRK2* in different neural-cell based models have reported an altered synaptic vesicle

trafficking and endocytosis (Shin et al., 2008; Piccoli et al., 2011). LRRK2 dysfunction has also been demonstrated to play a role in different pathologic scenarios, such as ∝-synuclein phosphorylation, microtubule dynamics, alterations in the uncoupling protein system (UPS) as well as in neurite growth and branching regulation that may trigger neurodegeneration (Dächsel et al., 2010). Silencing of LRRK2 reduced the inflammatory response in different human cell-derived and animal models (Lopez de Maturana et al., 2014).

A growing body of evidence supports a role for LRRK2 in mitochondrial dynamics and function. In fact, it interacts with a number of crucial proteins that regulate mitochondrial dynamics such as dynamin-related protein1 (DRP1), Mitofusin (MFN1) and 2 and optic atrophy1 (OPA1) (Wang et al., 2012; Stafa et al., 2014; Park et al., 2018). Thus, *LRRK2* might directly affect mitochondrial homeostasis while indirectly regulating it through autophagy and cytoskeletal dynamics (Singh et al., 2019). This hypothesis is also supported by many studies reporting mitochondrial dysfunction in various animal models of G2019S-*LRRK2* PD, in *postmortem* human tissues from LRRK2-PD patients (Mortiboys et al., 2010; Cooper et al., 2012; Sanders et al., 2014; Yue et al., 2015) and in different patient-derived cell models (Niu et al., 2012; Wang et al., 2012; Cherra et al., 2013; Su and Qi, 2013). Such studies reported mtDNA damage, decreased mitochondrial membrane potential (MMP) and ATP production, as well as altered mitochondrial dynamics and mitophagy (Singh et al., 2019). In addition, a protective role against oxidative stress has been reported for wild-type LRRK2, which seems to be lost in mutant forms of the protein (Liou et al., 2008).

Studies in Fibroblasts From *LRRK2*-Associated PD

Mitochondrial phenotypes have been characterized in *LRRK2*-fibroblasts at baseline and under conditions of pharmacological stress. The most common pharmacological approaches used to date include mitochondrial toxins such as MPTP, valinomycin, oligomycin, CCCP, and rotenone. As these approaches may mimic mitochondrial toxicity, they are far from dissecting the mitochondrial pathways affected under physiological conditions (Trentadue et al., 2012). Smith et al. (2015) demonstrated an increased sensitivity to valinomycin in a subset, but not all, of fibroblasts derived from PD patients, pinpointing again the great interindividual variability of the disease and, outstandingly, that the molecular characteristics of patient-derived cell models do not always correlate with the clinical presentation of the disease.

Mortiboys et al. (2010) reported for the first time mitochondrial alterations in fibroblasts of human G2019S-*LRRK2* mutation carriers, consisting of reduced MMP, reduced intracellular ATP levels, mitochondrial elongation and increased mitochondrial interconnectivity. These findings were further confirmed by Papkovskaia et al. (2012), who described decreased MMP and ATP levels as well as increased proton leakage and ROS levels with the associated increase in uncoupling protein 2 (UCP2) in fibroblasts from G2019S-*LRRK2* PD patients. Several studies have repeatedly observed alterations in mitochondrial dynamics such as increased mitochondrial fragmentation in fibroblasts from *LRRK2*-PD patients (Su and Qi, 2013; Grünewald et al., 2014; Smith et al., 2015; Falkenburger et al., 2016).

A recent study compared mitochondrial function and autophagy in fibroblasts of G2019S-LRRK2-mutation carriers without clinical symptoms (so called non-manifesting carriers or NMC), with patients harboring G2019S-LRRK2-mutation and clinical manifested PD. Interestingly, fibroblasts of NMC showed an enhanced mitochondrial performance upon forcing mitochondrial oxidative metabolism with galactose and upregulation of autophagy (Juarez-Flores et al., 2018). These findings suggested that the exhaustion of the bioenergetic and autophagy reserve might contribute to the onset of clinical PD symptoms. Other authors have reported heightened autophagic flux and higher expression of autophagy markers as well as an increased mitophagy in G2019S-LRRK2-mutation carriers with clinical diagnosed PD (Smith et al., 2015; Su et al., 2015). The reduction in mitophagy and increased ROS production has been associated to defective histone acetyltransferase and deacetylase activities contributing to cell death also in LRRK2-fibroblasts (Yakhine-Diop et al., 2019). Additionally, the novel role of key regulators of autophagy (as TMEM230) interacting with Rab proteins as Raba or Rab32been described in LRRK2-patients fibroblasts is emerging as a promising new target in disease (Waschbüsch et al., 2014; Kim et al., 2017).

PRKN-Associated PD (*PRKN*-PD)

Other forms of PD have also been genetically associated to mitochondrial and autophagic imbalance, in this case through a recessive inheritance. This is the case of PRKN.

The locus of *PRKN* is mapped to the telomeric region of the long-arm of chromosome 6. More than 170 different mutations have been identified throughout the sequence of this particularly large gene (1.35 Mb) ranging from point and missense mutations to large deletions or multiplications and small deletions/insertions (Bruggemann and Klein, 1993; Klein and Westenberger, 2012) (**Figure 4**). Rare deletions extending in the neighboring *PRKN* coregulated gene (*PACRG*) result in the same early onset parkinsonism phenotype (Corti et al., 2011).

PRKN is a 465 amino acid protein that contains an NH2-terminal homologous to a ubiquitin-like domain (UBL) followed by three really interesting new gene (RING) finger domains (RING 0–2) separated by an In-Between-RING (IBR) domain in the COOH-terminal part, each of which bind two Zn^{2+} (Zhang et al., 2015) (**Figure 4**). Functionally, PRKN is a member of a family of E3 ubiquitin protein-ligases responsible for the labeling of selected cargos, such as obsolete proteins and organelles, which need to be degraded through the ubiquitination process. This process comprises the transfer of activated ubiquitin molecules to the lysine residues of specific substrate proteins. Depending on the site and type of ubiquitination (mono, poly or multi-ubiquitination), certain cell signaling processes are activated, including proteosomal degradation but also non-degradative signaling roles (Dawson and Dawson, 2010).

Along with the original discovery of the PRKN function as an E3 ubiquitin ligase in PD-associated *PRKN* mutations, the hypothesis that loss of PRKN function would lead to the toxic

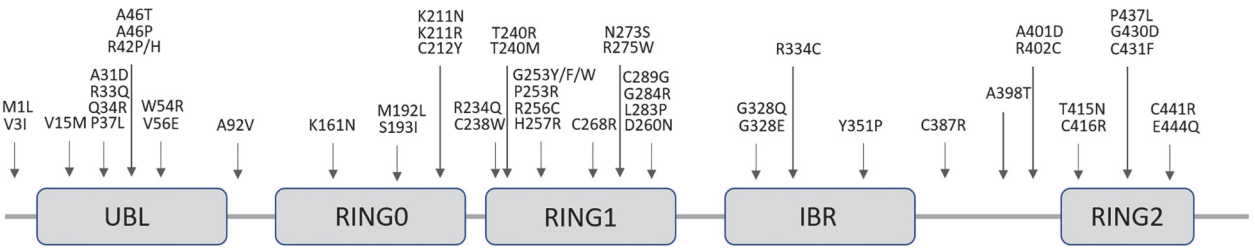

FIGURE 4 | Schematic representation of the Parkin protein showing its functional domains and the most frequent pathogenic missense point mutations responsible for PD. Dozens of alternative frameshift and nonsense mutations, insertions, deletions, duplications or triplications have also been associated to disease.

accumulation of one or several of its substrates raised. To date, no less than 25 PRKN putative substrates have been reported and new substrates continue to emerge periodically, especially those related to mitochondria (Zhang et al., 2015). In addition, many dynamically regulated ubiquitination sites in dozens of proteins have been identified, with strong enrichment for OMM proteins, indicating that PRKN dramatically alters the ubiquitination status of the mitochondrial proteome (Sarraf et al., 2013).

Nigral cell loss in *PRKN*-PD patients appears to be caused by a loss of function of the protein due to biallelic homozygous or compound heterozygous mutations in the *PRKN* gene. However, there is an ongoing debate with regard to whether heterozygous *PRKN* mutations may confer increased susceptibility to PD as heterozygous *PRKN* pathogenic variants have been detected in a large number of individuals with PD (Bruggemann and Klein, 1993; Mortiboys et al., 2008).

Although the population-based prevalence of *PRKN*-PD is largely unknown (Bruggemann and Klein, 1993), it is thought that *PRKN* mutations account for up to 50% of recessive familial forms and 80% in those patients with a PD onset before the age of 20 years (Corti et al., 2011). Women and men are equally affected, with an age at onset usually <40–50 years (Mizuno et al., 2001) although some individuals may not develop PD until age 60 or 70 years (Klein et al., 2000; Lohmann et al., 2003). In addition to an earlier age at onset, *PRKN*-PD patients show a clinical phenotype similar to that of sPD being bradykinesia and tremor amongst the most common signs, but also a number of specific clinical features. *PRKN*-PD is also characterized by a relatively benign course with slow progression, remarkable and maintained response to low levodopa doses but with frequent severe treatment-related motor complications such as early motor fluctuations and the development of dyskinesias (Cheon et al., 2012). Pyramidal signs, cerebellar features, and psychiatric disorders have been reported, but dementia or dysautonomia seem to be rare (Corti et al., 2011; Johansen et al., 2018).

In the limited neurophathologic studies, *PRKN* mutations are associated with selective DAn loss in the SN and some cases reported a moderate decrease of noradrenergic neurons in the *Locus coeruleus* with gliosis and without LB. However, a few cases of LP have been reported in PRKN-PD, especially those associated to a later onset of the disease (Bruggemann and Klein, 1993; Johansen et al., 2018).

One of the best characterized functions of PRKN is its role in the process of mitophagy, which is the selective targeting of a damaged mitochondrion for autophagy. Compelling evidence suggests that PRKN acts together with and downstream of PINK1 in a common mitochondrial quality control pathway responsible for the detection and clearance of damaged mitochondria through mitophagy (Eiyama and Okamoto, 2015). In healthy mitochondria, PINK1 is constitutively imported into the OMM and inner mitochondrial membranes (IMM), cleaved by several proteases and subsequently degraded. Loss of MMP impedes the import of PINK1 in the IMM, thereby stabilizing PINK1 on the OMM and consequently recruiting PRKN from the cytosol. In its native state, PRKN is auto-inhibited by its N-terminal UBL domain, which blocks the binding site for any incoming E2 ubiquitin conjugate, required for PRKN ubiquitination activity. Upon mitochondrial depolarization, PINK1 phosphorylates cytoplasmic PRKN in its UBL domain, relieving PRKN autoinhibition (Eiyama and Okamoto, 2015). Activated PRKN ubiquitinates many OMM proteins including VDAC1, mitofusins and the translocase of the OMM 20 (TOMM20) (Kondapalli et al., 2012; Ivankovic et al., 2016). Together, PINK1 and phosphorylated PRKN extensively modify the OMM with phosphorylated ubiquitin (pUb) chains. pUb chains serve as a mitochondrial receptor for further allosteric activation and recruitment of PRKN to the OMM, resulting in a self-amplifying feed-forward loop. Ubiquitination of these substrates primes mitochondria for recruitment to phagophores that then mature to AP and fuse with lysosomes resulting in the degradation of dysfunctional mitochondria (Ichimura et al., 2013). In addition, recent evidence suggests that PRKN is also involved in the aggresome-autophagy pathway in which PRKN promotes the sequestration of misfolded proteins into aggresomes and its subsequent clearance by autophagy (Olzmann and Chin, 2008; Yung et al., 2016).

On the other hand, PRKN has been implicated in mitochondrial biogenesis specifically, through the regulation by ubiquitination of the protein levels of one of its substrates named PARIS (ZNF746) (Shin et al., 2011). PARIS represses the expression of the transcriptional coactivator PGC-1α, which is considered a master regulator of mitochondrial biogenesis. In this line, PARIS has been reported to accumulate in models of PRKN inactivation and in human PD brain (Shin et al., 2011). Thus, PRKN potentially acts as an intermediary between

mitochondrial biogenesis and autophagy, by both blocking mitochondrial biogenesis and mitochondrial turn-over, thus resulting in mitochondrial aging.

Studies in Fibroblasts From *PRKN*-PD Patients

Amongst all the studies using skin-derived fibroblasts as a cell model for PRKN-PD, it is worth stressing that many of them have focused on studying mitochondrial function leading to controversial outcomes.

Alterations in the enzymatic activities of the MRC have been previously reported in PRKN-PD fibroblasts (Mortiboys et al., 2008; Grunewald et al., 2010; Pacelli et al., 2011). Mortiboys et al. (2008) and Pacelli et al. (2011) described CI enzymatic deficiency in *PRKN*-PD fibroblasts, while Grunewald et al. (2010) observed preserved enzymatic activities in isolated mitochondria from a larger cohort (Mortiboys et al., 2008; Grunewald et al., 2010; Pacelli et al., 2011). Mitochondrial complex IV deficiency has only been described by Pacelli et al. (2011) in two PRKN-PD fibroblasts lines while others have reported unaltered enzymatic activity of this complex (Mortiboys et al., 2008; Grunewald et al., 2010). Mitochondrial respiration is frequently measured to assess how MRC enzymatic activities translate to global mitochondrial function. Haylett et al. (2016) and Zanellati et al. (2015) consistently observed increased basal mitochondrial respiration in PRKN-PD fibroblasts but reported opposite outcomes in ATP-linked respiration. In contrast to these findings, a previous study described an overall decrease in all respiratory parameters of PRKN-PD fibroblasts (Pacelli et al., 2011).

Mitochondrial membrane potential has also been widely explored as a general marker of mitochondrial integrity in PRKN-PD fibroblasts. While two authors were not able to demonstrate alterations in this parameter (Grunewald et al., 2010; Haylett et al., 2016), others reported decreased MMP (Zanellati et al., 2015; Koentjoro et al., 2017), especially when exposing cells to mitochondrial-challenging conditions (Mortiboys et al., 2008; Grunewald et al., 2010).

Many evidences point to the involvement of PRKN in the entire process of mitochondrial dynamics, including organelle biogenesis, fusion/fission, and mitochondrial clearance via mitophagy (Lim et al., 2012). In this context, mitochondrial network morphology has been of interest in *PRKN*-PD fibroblasts studies but with controversial results (Mortiboys et al., 2008; Grunewald et al., 2010; Pacelli et al., 2011; van der Merwe et al., 2014; Zanellati et al., 2015; Haylett et al., 2016). Although some studies seem to agree that mitochondrial length is conserved in these cells (Mortiboys et al., 2008; van der Merwe et al., 2014; Haylett et al., 2016), others have observed a fragmented mitochondrial network (Pacelli et al., 2011; Zanellati et al., 2015). While most of studies did not show significant alterations in mitochondrial branching (Grunewald et al., 2010; van der Merwe et al., 2014; Zanellati et al., 2015), Haylett et al. (2016) observed decreased levels and Mortiboys et al. (2008) reported increased rates. In line with this, mitochondrial content has been assessed in several works but only Grunewald et al. (2010) reported a significant increase in this feature in *PRKN*-PD fibroblasts whereas others have observed conserved (Mortiboys et al., 2008; van der Merwe et al., 2014) or decreased levels (Pacelli et al., 2011).

As previously discussed, oxidative stress is a hallmark of mitochondrial dysfunction that has often been related with neurodegeneration, specifically in PD (Guzman et al., 2010; Surmeier et al., 2011; Poewe et al., 2017). In accordance, previous authors demonstrated increased protein and lipid oxidation in small cohorts (Grunewald et al., 2010; Pacelli et al., 2011).

Surprisingly, to our knowledge, studies assessing mitophagy or autophagy in *PRKN*-PD fibroblasts are scarce. Only recently, Koentjoro et al. (2017) elegantly demonstrated that a *PRKN*-PD patient fibroblast cell line failed in initiating mitophagy upon induction of mitochondrial depolarization. Interestingly, they also examined an unusual homozygous *PRKN* mutation carrier who did not develop clinical PD by her eight decade and found preserved mitochondrial function due to the induction of a PINK1/Parkin-independent mitophagy mediated by Nix, which is a selective autophagic receptor located on the OMM (Koentjoro et al., 2017).

Other studies in *PRKN*-PD fibroblasts have reported alterations in alternative important cell processes which represent promising targets of disease pathogenesis to be further explored. For instance the regulation of endoplasmic reticulum-to-mitochondrial contacts by Parkin via Mfn-2 (Basso et al., 2018). Also, Pacelli et al. (2019) reported altered severe damping of the bioenergetic oscillatory patterns associated to circadian rhythms and molecular clockworks in fibroblasts from PRKN-PD patients that may conditioning mitochondrial quality control and mitophagy. One study performing a whole-genome expression analysis by RNA-sequencing found that different *PRKN* mutations were associated with a large number of gene expression changes at the transcriptome level (González-Casacuberta et al., 2018). Specifically, authors reported the upregulation of 1C-dependent anabolic biosynthetic pathways, which has been related with the activation of the mitochondrial integrated stress response (ISRmt) in front of mitochondrial dysfunction (Bao et al., 2016; Celardo et al., 2017; Suomalainen and Battersby, 2017). Additional studies in PRKN-PD fibroblasts have reported alterations in the protein expression and lipidome profiles (Lippolis et al., 2015; Lobasso et al., 2017) as well as cytoskeleton alterations such as microtubule destabilization (Cartelli et al., 2012; Vergara et al., 2015).

The characterization of fibroblasts of PD patients point out disrupted pathways to be targeted and therapeutic platforms, but some concerns and controversies arise. The low reproducibility of mitochondrial function analysis presented in most studies performed up to date could be attributed to the small sample sizes tested. Also differences in methodological approaches, protocols and experimental conditions (e.g., site of skin biopsy, passage number of cells, etc.) may partially account for the large variation obtained. For instance, the use of different high-resolution respirometry approaches in which oxygen consumption is measured from seeding fibroblasts or from cells in suspension. Similarly, assessing MRC enzymatic activities in intact cells or in mitochondrial enriched fractions may contribute to outcome disparities. Moreover, all the studies were performed in glycolytic conditions that may partially unveil mitochondrial deficits and contribute to controversy (Mortiboys et al., 2008; Grunewald et al., 2010; Pacelli et al., 2011; van der Merwe et al., 2014; Zanellati et al., 2015; Haylett et al., 2016). In this sense, the

use of alternative sources of energy, as galactose, serves for two purposes: to force and challenge oxidative metabolism (usage widely extended for the diagnosis of mitochondrial disorders) and mimic neuronal metabolism (mainly based in OXPHOS function). Novel studies focused in exploring mitochondrial or autophagic function in galactose may be useful to unveil pathogenic mechanisms of disease.

In summary, the particular area of research focused on the study of mitochondrial function in *PRKN*-PD fibroblasts has proved to be contentious, with several groups either describing similar defects or no apparent abnormalities. It would be of great importance that researchers join efforts on homogenization of protocols and analyzing a more significant number of *PRKN*-PD patient-derived cells in order to unveil if mitochondrial and autophagic dysfunction is a crucial event in PRKN-PD pathogenesis.

DISEASE MODIFYING THERAPIES

Since the disseverment of PD, different therapeutic options have been developed to ameliorate the symptoms of PD. The first one to be developed was levodopa, in 1960, a precursor that is transformed to DA in the brain, supplying the amount of DA that degenerated neurons are not able to produce. Other medications include DA agonists and monoamine oxidase-B (MAO-B) or Catechol-O-methyltransferase inhibitors (COMT) inhibitors, selegiline, and rasagiline, that decrease the activity of MAO-B, enzyme responsible of degrading DA. There are also other options to severe cases who do not respond to DA based on the use of apomorphine and duodopa administered by pumps, surgical interventions or deep brain stimulation (Rizek et al., 2016).

However, these treatments are supportive, only control the symptoms of the disease, the neurodegeneration is not stopped or reversed, consequently, there are no curative treatments for PD.

To unveil the pathophysiology of the diseases and develop new therapeutic strategies to reduce the impact of PD and find a cure is essential to develop novel models of disease.

The experimental models herein discussed hold potential for the development of PD modifying therapies. The complementary assay of any potential candidate in different experimental models confers strength to the potential therapeutic efficacy before translation into the clinical settings. Patient-derived cell models offer usefulness either as platforms for testing novel therapeutic approaches or for prompting the discovery of novel targets from disrupted pathways reported in these models.

The use of these experimental models in PD has permitted the discovery of different therapeutic candidates, with different degree of evidence on their potential therapeutic activity and security concerns. This is the case of FGF20, echinacoside, rosmarinic acid or autophagic modulators, as Threalose or Torin 1, among many others, targeting different disrupted cell pathways in disease (Liang et al., 2019; Lv et al., 2019; Wang et al., 2019). Depending on the subtype of PD, specific treatments have been proposed. This is the case of *LRRK2*-PD carriers, where the use of LRRK2 inhibitors has been proposed. They are currently being tested in clinical I trials. Unfortunately, their systemic action may unveil secondary effects, somehow bypassed by the targeting of specific neural effectors (as PAK6 or Rab GTPases) to modulate neural disrupted protein and organelle trafficking in PD (Kiral et al., 2018). Similarly, for *PRKN*-patients, selective mitochondrial drugs have been proposed. Experimental data supports the use of fusion or fission inhibitors (as MDIVI-1), that still rank in experimental settings (Manczak et al., 2019). Antioxidant and mitochondrial principles (as coenzyme Q, that failed in a phase III assay) and peroxisome proliferator-activated receptor-γ agonists that reduces proinflammatory cytokines and modulate mitochondrial biogenesis (as Pioglitazone) were also tested in clinical trials, but failed to demonstrate further efficacy.

Apart from symptomatic treatments (such as levodopa or surgical interventions), disease modification through neuroprotection remains as the main milestone in PD research. Neuroprotection tested by pramipexole (CALM-PD), ropinirole (REAL-PD), and pramipexol (PROUD-PD) failed to establish disease modification (Bartus et al., 2013; Obeso et al., 2017). In this sense, calcium channel blockers aimed to prevent calcium influx on nigral neurons are being tested (isradipine is being evaluated in phase I and II clinical trials), together with compounds able to increase urate antioxidant protection (inosine is undergoing phase II studies) (Obeso et al., 2017).

Additionally, aiming to support neuroprotection through the enhancement of neuronal viability, trophic factors are also being evaluated in PD, showing moderate or null therapeutic success. Of them, glial family ligands as glial derived neurotrophic factor (GDNF) and neurturin in preclinical studies demonstrated strong neuroprotection in multiple animal models. However, multiple clinical trials, including 2 phase II trials, failed to demonstrate their efficacy or showed significant side effects (Kordower et al., 2000; Obeso et al., 2017).

Alpha-synuclein has become lately the major target for PD therapeutics. Initial preclinical efforts concentrated on synuclein-lowering treatments such as siRNAs directed against alpha-synuclein, that resulted toxic in animals. Novel attempts focused to disaggregate aggregated synuclein, facilitate its clearance by augmenting autophagy pathways, or using antibodies to prevent its propagation from the periphery to the brain and once in the brain across the neural axis. Vaccines against alpha-synuclein as both active and passive immunization approaches have been attempted. Active immunotherapy attempts to stimulate the immune system against specific antigens (Bergström et al., 2016). Passive immunotherapy uses monoclonal antibodies against alpha-synuclein molecule. Initial phase 1 safety trials are currently underway and show promising results in terms of safety and tolerability profiles. The enhancement of glucocerebrosidase (GBA) lysosomal activity to reduce alpha-synuclein levels is also being tested through small molecule chaperones in clinical trials (McNeill et al., 2014).

Probably the next coming years will open future perspectives for the development of new supportive and curative therapies in PD, where personalized medicine, mainly based on genetic and molecular counseling, will help to direct specific PD patients to a wide panoply of therapies.

The development of novel therapeutic options will depend of the efficacy of candidate compounds previously tested in preclinical settings and experimental models of disease, as the herein described, and the target of disrupted pathways, as the herein explained.

CONCLUSION

Parkinson's disease encompasses a wide panoply of genetic and molecular etiologies leading to common clinical manifestations. Different schools of thought differ in considering either mitochondria or protein deposition-cascade as the triggers of PD, but all they convey that PD pathogenesis is associated to the deregulation of both mitochondrial and autophagic clearance pathways, supporting its role in the disease. Mitochondrial dysfunction and their turnover through autophagy directly targets some types of PD (as those carrying mitochondrial or autophagic mutations) but also stand at the base of the rest of PD by providing the overdose of energy needed to support alternative deregulated pathways while maintaining oxidative stress levels within control ranges. Thus, proper mitochondrial and autophagic function protects against PD and exhaustion of mitochondrial and autophagy contributes to PD development, independently of the genetic base. These molecular alterations have been consistently reported in skin-derived fibroblasts from PD patients carrying mutations in *LRRK2* and *PRKN* genes. These findings demonstrate the presence of molecular damage characteristic of the PD target tissue beyond the CNS and the usefulness of these patient-derived cells to model PD, models that can be metabolically upgraded to resemble neuron behavior and challenge mitochondrial and autophagic function by the use of galactose.

Current research gaps in PD research stand for the development of novel therapeutic candidates aimed to promote healthy brain aging and avoid or even cure PD, probably based in personalized-medicine guided by genetic and molecular counseling. New generation sequencing will increase the number of genes responsible of familial PD and the number of genetic risk factors accounting for sporadic PD, thus unveiling molecular imbalances underlying PD. Novel compounds against these targets will be discovered in experimental settings and disease models to set the path for further clinical trial testing. Complementary models of disease will be needed to dissect the disrupted pathways in PD and design specific therapeutic targets, but the use of patient-derived cells such as fibroblasts is gaining in strength because they constitute platforms to model disease etiopathogenesis and try new therapeutic approaches in the genetic and epigenetic background of the patient.

New challenges and potential developments in the field of PD entail the validation of these novel therapeutic candidates focused on modifying the course of PD through, among others, promoting mitochondrial and autophagic performance.

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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MINI REVIEW
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Intracellular and Intercellular Mitochondrial Dynamics in Parkinson's Disease

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The appearance of alpha-synuclein-positive inclusion bodies (Lewy bodies) and the loss of catecholaminergic neurons are the primary pathological hallmarks of Parkinson's disease (PD). However, the dysfunction of mitochondria has long been recognized as a key component in the progression of the disease. Dysfunctional mitochondria can in turn lead to dysregulation of calcium homeostasis and, especially in dopaminergic neurons, raised mean intracellular calcium concentration. As calcium binding to alpha-synuclein is one of the important triggers of alpha-synuclein aggregation, mitochondrial dysfunction will promote inclusion body formation and disease progression. Increased reactive oxygen species (ROS) resulting from inefficiencies in the electron transport chain also contribute to the formation of alpha-synuclein aggregates and neuronal loss. Recent studies have also highlighted defects in mitochondrial clearance that lead to the accumulation of depolarized mitochondria. Transaxonal and intracytoplasmic translocation of mitochondria along the microtubule cytoskeleton may also be affected in diseased neurons. Furthermore, nanotube-mediated intercellular transfer of mitochondria has recently been reported between different cell types and may have relevance to the spread of PD pathology between adjacent brain regions. In the current review, the contributions of both intracellular and intercellular mitochondrial dynamics to the etiology of PD will be discussed.

Keywords: alpha-synuclein, tunneling nanotube, Parkinson's, mitophagy, mitochondria

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INTRODUCTION: PD, α-SYNUCLEIN AND MITOCHONDRIA

The principal histopathological marker of Parkinson's disease (PD) is the presence in neurons of α-synuclein (α-syn) protein aggregates that occur in inclusion bodies known as Lewy bodies (Shults, 2006; McCann et al., 2014). α-Syn is primarily expressed pre-synaptically and evidence exists of α-syn transfer from neurons to neuronal and non-neuronal cells *in vitro*, indicating that α-syn pathology spreads between anatomically adjacent brain regions by a cell-to-cell transfer mechanism (Valdinocci et al., 2017). α-Syn is a small (14 kDa), acidic protein expressed in the brain, peripheral nervous system and circulating erythrocytes (Thakur et al., 2019). Its pre-synaptic localization and high abundance implicate an important role in synaptic transmission (Burre et al., 2010) with specific functions implicated in synaptic vesicle recycling and regulating soluble NSF

attachment protein receptor (SNARE) interactions and dopamine biosynthesis (Theillet et al., 2016; Sulzer and Edwards, 2019). *In vitro* α-syn is a dynamically unfolded protein, although *in vivo* the membrane-associated tetrameric form is proposed to be α-helical (Bartels et al., 2011). Various factors, such as raised copper or calcium concentration, oxidative stress and post-translational modifications can trigger intracellular α-syn aggregation (Fujiwara et al., 2002; El-Agnaf et al., 2006; Reynolds et al., 2008; Rcom-H'cheo-Gauthier et al., 2016). Whilst a definitive link between mitochondrial dysfunction and initiation of PD still does not exist, it is clear that dysfunctional mitochondria are omnipresent in PD (Chen et al., 2019). Moreover, α-Syn can be located at mitochondrial membranes, especially under stress conditions (Cole et al., 2008; Devi et al., 2008), and its aggregation can be linked to mitochondrial dysfunction in PD (Perfeito et al., 2013; Celardo et al., 2014). α-Syn aggregates may in turn cause deleterious alterations in mitochondrial function, including intracellular dynamics. This review focusses on α-syn interactions with mitochondria in PD.

INTRACELLULAR MITOCHONDRIAL DYNAMICS IN PD

Mitochondria are highly dynamic and interconnected entities, with the ability to change their morphology in addition to mobilizing within the cell to help power critical functions (Burte et al., 2015). Mitochondrial dynamic processes include fusion/fission, transport and clearance, and are so interconnected and interdependent that they have been proposed to form an interactome that ultimately controls mitochondrial quality, quantity and metabolism (Dorn and Kitsis, 2015; Shirihai et al., 2015). Under normal conditions, mitochondria constantly undergo cycles of fusion and fission that affect their morphology and shape, processes that may be perturbed in PD. α-Syn has been shown to influence mitochondrial size both independently and dependent on fusion/fission proteins, with recent reports detailing these interactions as an attribute of the pathological variants such as oligomers and fibrils, ruling out any negative effects on mitochondrial dynamics as a normal function of the monomer (Wang et al., 2019). Factors such as the GTPases Mitofusin1 and 2 (Mfn1/2), and optic atrophy protein 1 (OPA1) are involved in fusing the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM), respectively, forming elongated structures that are more efficient in ATP generation (Chan, 2012; Tilokani et al., 2018). As illustrated in **Figure 1**, oligomeric α-syn can bind to lipids in the OMM and distress the membrane curvature, leading to a decrease in mitochondrial fusion rate (Pozo Devoto and Falzone, 2017). In addition, overexpression of α-Syn in transgenic mice led to reductions in Mfn1/2 protein levels, correlating with a decrease in mitochondrial fusion and smaller mitochondria (Xie and Chung, 2012). α-Syn knockdown was shown to trigger mitochondrial elongation (Kamp et al., 2010).

Conversely, mitochondrial fission is dependent on dynamin-related protein 1 (Drp1), mitochondrial fission factor (Mff), mitochondrial fission protein 1 (Fis1) and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49/51) for shrinkage of structures typically promoted during cellular replication (Korobova et al., 2013; Pagliuso et al., 2018). Depending on the isoform, post-translational modification of Drp1 by the small ubiquitin-like modifier (SUMO) drastically alters the effect on mitochondrial fission. SUMO-2/3 for instance has been shown to inhibit Drp1-Mff interaction preventing mitochondrial fragmentation, however, SUMO-1 stabilizes Drp1 leading to enhanced fragmentation; with evidence establishing a link between SUMO and PD, this further implicates mitochondrial dynamics in the pathogenesis of the disease (Guo et al., 2013, 2017; Fu et al., 2014; Vijayakumaran et al., 2015; Henley et al., 2018; Vijayakumaran and Pountney, 2018). Mitochondrial complex I inhibitors, Rotenone and MPP^+, inducers of parkinsonian phenotypes, were shown to promote mitochondrial fission (Barsoum et al., 2006; Thomas et al., 2011) and Drp1 inactivation prevented the fission phenotype (Wang et al., 2011a). In the substantia nigra of sporadic PD patients, the short form of OPA1 (OPA1-S) was decreased in the absence of changes in Mfn1, further suggesting mitochondrial fusion deficiency (Zilocchi et al., 2018). Thus, a net increase in mitochondrial fission over fusion may ultimately lead to a fragmented mitochondrial network negatively impacting the efficiency of neuronal signaling in PD. Worth noting are reductions in Drp1 in the later stages of degeneration in transgenic mice (Xie and Chung, 2012). Mitochondria are also a major source of intracellular calcium which is released upon electron transport chain dysfunction, such as that caused by rotenone-mediated inhibition, and interacts with α-syn to promote aggregation. Indeed, induction of the endogenous neuronal calcium buffering protein, calbindin-D28k (CB), was able to block rotenone-induced α-syn aggregation and neurons expressing high levels of CB excluded α-syn inclusion bodies in both human and mouse model tissues (Rcom-H'cheo-Gauthier et al., 2016, 2017; McLeary et al., 2019). Moreover, the Miro1 protein acts as a calcium sensor at the mitochondrial outer membrane, reacting to high calcium by augmenting mitophagy (Nemani et al., 2018).

Intracellular mitochondrial dynamics involves the transport of mitochondrial units from one area of the cell to another, and is of key importance in neuronal cells that are polarized with long axons and dendrites (Lin and Sheng, 2015). Mitochondria need to be transported to synaptic terminals, active growth cones and axonal branches, where they maintain energy and Ca^{2+} homeostasis (Sheng, 2017). Trafficking of mitochondria is microtubule (MT)-based, relying on ATP and motor proteins. MTs are polar α/β-tubulin polymers with the minus end within the cell body and the plus end to the cell extremity (Tas and Kapitein, 2018), and neurons can move mitochondria in both directions using independent motor proteins. Kinesins drive transport toward the cell extremities (anterograde transport) whereas dynein motors are responsible for retrograde transport (Hirokawa et al., 2010). Hydrolysis of ATP is essential to fuel mitochondrial movement in both directions (Zala et al., 2013). Bridging between mitochondria and MT-bound motor proteins are motor adaptor proteins such as Trafficking Kinesin Proteins 1 and 2 (TRAK1 and 2), which connect the OMM protein Mitochondrial Rho GTPase 1 (Miro1) to

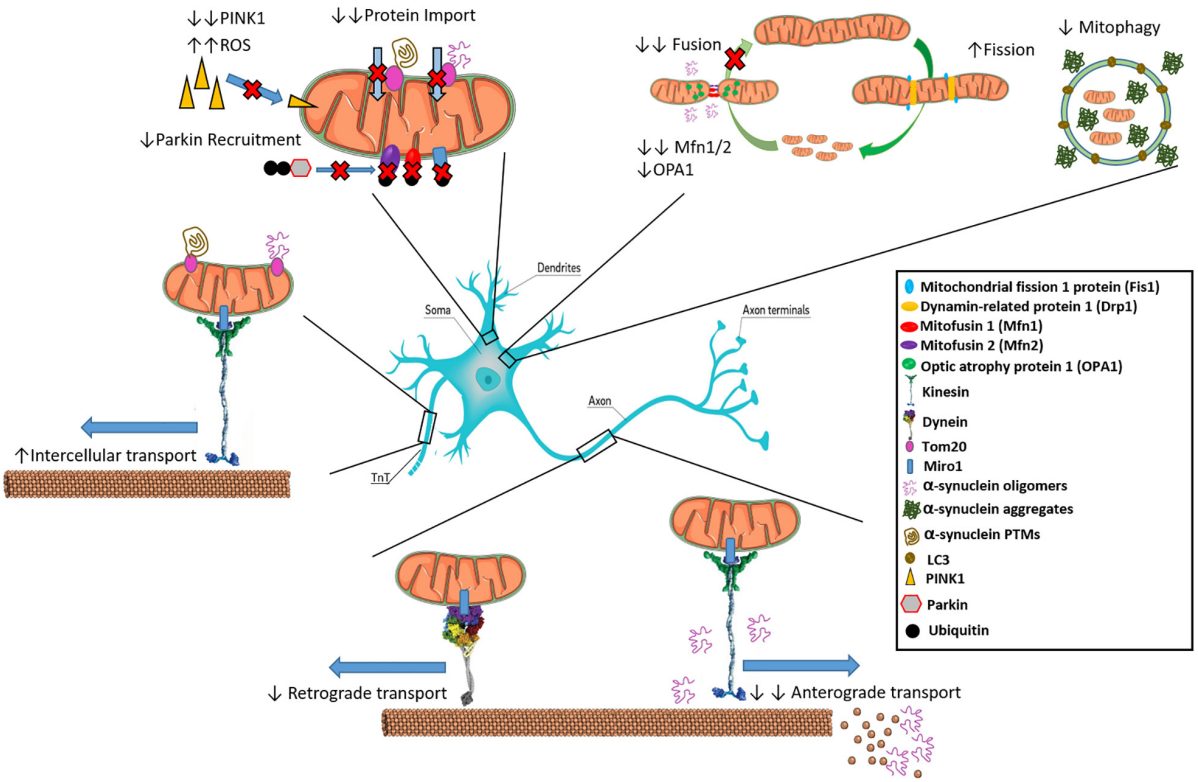

FIGURE 1 | Interactions of α-synuclein with inter- and intra-cellular mitochondrial dynamics. α-syn inhibits mitochondrial fusion as over expression leads to reduced Mfn1/2 and OPA-1. Oligomeric α-syn can interact with kinesin to dislodge it from microtubules preventing anterograde transport along axons. Oligomeric α-Syn also promotes the depolymerization of microtubules, preventing transport of mitochondria. Increased aggregated α-syn is associated with reduced mitophagy. Certain α-Syn types such as oligomers and mutated variants like S129E bind to Tom20 resulting in inhibition of mitochondrial protein import. Reduction in PINK1 leads to inability to signal for mitophagy of α-syn bound mitochondria through Parkin recruitment, leading to ROS generation. Movement of pathological aggregates between cells via TnTs may occur if bound to Tom20 on actively transferring mitochondria.

kinesin (Melkov and Abdu, 2018). Indeed, Miro1 also mediates Drp1/Fis1-independent mitochondrial shape transition (Mist), needed for mitophagy (Nemani et al., 2018). Syntabulin can also link mitochondria and kinesins whilst syntaphilin acts as an anchor stopping mitochondrial movement (Cai et al., 2005; Chen and Sheng, 2013).

An increase in α-syn concentration was shown to result in mitochondrial traffic arrest even before axonal degeneration, affecting both anterograde and retrograde transport (O'Donnell et al., 2014; Pozo Devoto and Falzone, 2017). Nigral dopaminergic neurons especially display a "dying back" pattern, wherein anterograde trafficking of mitochondria becomes disrupted in early stage sporadic PD followed by retrograde transport at late stage (Chu et al., 2012). Loss of this trafficking severely reduces nigral neuron ability to regulate the conditions necessary for axonal signaling and neurotransmitter release at terminals. Regarding anterograde trafficking, α-syn oligomers disrupt through direct binding interactions between kinesin and the MT in addition to increasing expression of tau, a MT structure disruptor (Prots et al., 2013, 2018). α-Syn appears to induce MT fragmentation directly as well, hindering mitochondrial movement from distal cell areas (Melo et al., 2017). Interestingly, this finding produced the opposite outcome to Chu et al. (2012) whereby retrograde transport was disrupted first. This may be due to differences in α-syn species interaction with trafficking complexes disrupting mitochondrial transport as Chu et al. (2012) brought about "dying back" through viral overexpression of α-Syn whilst Melo et al. (2017) utilized an A53T transgenic model. Moreover, the PD-linked protein leucine rich repeat kinase-2 (LRRK2) seems to alter MT polymerization/depolymerization cycles, affecting mitochondrial trafficking (Gillardon, 2009; Godena et al., 2014). Impairment of mitochondrial transport is also induced by the parkinsonian toxin MPP^+, which inhibits kinesin-1-mediated anterograde transport leading to an increase in dynein-dependent retrograde transport (Morfini et al., 2007). LRRK2 and PTEN-induced kinase 1 (PINK1) mutant drosophila also exhibit disturbed mitochondrial calcium homeostasis with functional involvement of Miro1 (Lee et al., 2018).

ROLE OF MITOPHAGY IN PD

Mitochondrial dynamics also includes mitochondrial clearance by mitophagy, a mitochondrion-specific autophagic process that drives dysfunctional mitochondria to degradation in

autophagosomes (Rodolfo et al., 2018). Several mitophagy mechanisms have been described, which can be dependent on or independent of mitochondrial receptors (Martinez-Vicente, 2017), including B-cell lymphoma 2 nineteen kilodalton interacting protein 3 (BNIP3), Nix, Bcl-2-like protein 13 (Bcl2-L-13) and Fun14 domain-containing protein 1 (FUNDC1), which interact with microtubule-associated proteins 1A/1B light chain 3B (LC3), recruiting the autophagosomal machinery (Chu, 2018). Cardiolipin may also mediate mitophagy, driving mitochondrial degradation when this phospholipid (that has a LC3-binding motif) moves from the IMM to the OMM (Chu et al., 2013). Receptor independent mitophagy involves the priming of mitochondria after PINK1 translocation from the cytosol to the mitochondria. The loss of mitochondrial membrane potential, and inhibition of PINK1-degrading proteases, leads to PINK1 accumulation in mitochondria, where it recruits the E3 ubiquitin ligase Parkin that initiates mitophagy by ubiquitinating OMM proteins, such as Mfn1 and Mfn2, Miro1, translocase of outer mitochondrial membrane 20 (TOM20), and voltage-dependent anion channel (VDAC) (Meissner et al., 2015). Poly-ubiquitinated OMM proteins act as mediators that ultimately cause mitochondrial engulfment by autophagosomes (Yoshii and Mizushima, 2015).

Evidence for a role of mitophagy in PD includes the observation that α-syn overexpression decreases the level of LC3-positive vesicles in human neuroblastoma cells (Winslow et al., 2010). Interestingly, mutations in PINK1 and Parkin genes cause autosomal recessive forms of familial early onset PD (Valente et al., 2001; Mata et al., 2004; Kumar et al., 2017), implicating a role of mitophagy in the aetiopathogenesis of PD (Dawson and Dawson, 2010). Mitochondrial morphology aberrations were found in a PINK1-mutant Drosophila model, and overexpression of Parkin was shown to rescue the phenotype (Clark et al., 2006). The role of Parkin mutations in mitophagy impairment was confirmed using iPSC-derived dopaminergic neurons with mutations in the Parkin gene (Suzuki et al., 2017). Additionally, Parkin has been demonstrated to be highly insoluble in PD, compromising autophagic systems (Lonskaya et al., 2013). Increased pathological α-syn leads to increased cytosolic Ca^{2+} and Miro1 upregulation, however, the adaptor function of Miro1 between mitochondria and motor transport complexes is abrogated at high Ca^{2+} concentration (Saotome et al., 2008; MacAskill et al., 2009; Wang and Schwarz, 2009). In addition to transport, increased Miro1 protects mitochondria from mitophagy. Thus, in PD it is likely that Miro1 upregulation in combination with PINK1 reduction serves to delay degradation allowing for unregulated ROS generation (Shaltouki et al., 2018). Interestingly, an increase in Miro1-dependent anterograde transport of mitochondria was found in a PINK1-knockout model (Liu et al., 2012). It is perhaps possible that in the early stages of PD, whilst cytosolic Ca^{2+} is low, that the transport function of Miro1 is unaffected, then changing to act as a protector especially in later stages. Phosphorylation of Miro1 by PINK1 is required for Miro1 degradation, however, mutations in PD likely affect this function (Wang et al., 2011b; Shlevkov et al., 2016). In combination with the protective capabilities of Miro1, this may explain why there is a frequent accumulation of dysfunctional mitochondria in distal axonal areas in PD models whilst still ubiquitinated by Parkin. Recent work by Grassi et al. (2018) suggests the possibility of differing α-syn variants affecting different cellular systems, with a non-fibrillar phosphorylated α-syn species described to induce mitophagy. However, understanding of mitophagy in PD is relatively limited due to the lack of amenable *in vivo* experimental approaches.

INTERCELLULAR TRANSFER OF MITOCHONDRIA AND α-SYNUCLEIN

Genes move from progenitors to progeny, i.e., in a vertical manner, while horizontal gene transfer (HGT) is rare among eukaryotes (Keeling and Palmer, 2008; Davis et al., 2014; Davis and Xi, 2015). Recently, HGT has been reported for mitochondrial genes, via horizontal transfer of mitochondria between cells *in vitro* (Rustom et al., 2004; Spees et al., 2006; Rogers and Bhatacharya, 2013; Ahmad et al., 2014; Wang and Gerdes, 2015; Rustom, 2016; Sinha et al., 2016). Wang and Gerdes (2015) showed that organelles, including mitochondria, move between cells via so-called tunnelling nanotubes (TNTs), narrow inter-cellular bridges with actin as a structural protein and with tubulin fibers as "tracks" for movement of subcellular structures between cells (Rustom et al., 2004). Co-culture studies showed that transfer of mitochondria from mesenchymal stem cells (MSCs) into cancer cells with defects in mitochondrial DNA (mtDNA) resulted in recovery of mitochondrial respiration in cancer cells (Spees et al., 2006) and mitochondria, moving from healthy cells via TNTs, rescued cancer cells exposed to mitochondrial insults during early stages of apoptosis (Wang and Gerdes, 2015). Mice with experimental lung disease were grafted with allogenic MSCs with labeled mitochondria resulting in movement of the mitochondria to the diseased cells, recovering their respiration and alleviating the pathology (Islam et al., 2012). Inter-cellular transfer of mitochondria maintains balanced heteroplasmy of mtDNA in outbred individuals (Jayaprakash et al., 2015), pointing to mitochondrial HGT as a more frequent event than previously considered (Berridge et al., 2015, 2016).

In relation to disease, transfer of damaged mtDNA has been observed in various mouse models of engrafted tumor cells with other works such as that by Dong et al. (2018) revealing that damaged mtDNA is transported within the mitochondria (Tan et al., 2015). Such a process allows for the damaged mtDNA to affect the acceptor cell through the generation and leakage of ROS, especially so if mitophagy or systems regulating the prevention of damaged mitochondrial spread are faulty. Regarding PD, knowledge on mtDNA and mitochondrial spread in general is still unclear. mtDNA mutations are observed in neuronal cells in early stages of PD (Braak Stage 3 onward) due to damage attained via oxidative stress (Lin et al., 2012). This is not observed in late stage PD. However, this could potentially be due to the increased chances of neurons that previously hosted the damaged mtDNA of being destroyed, in addition to a lower population of neurons with damaged mtDNA prior to death. Various interactions

of α-syn with mitochondria lead to the generation of ROS and thus increase risk of damaged mtDNA, including certain pathological α-syn species shown to bind with high affinity to the Tom20 mitochondrial outer membrane protein, thereby inhibiting mitochondrial protein uptake and promoting ROS generation (Di Maio et al., 2016; Grassi et al., 2018). Recent work by Wang et al. (2019) further reveals α-syn bound mitochondria only occurs with pathological α-syn aggregate species and not physiological monomers. This finding did not pertain only to PD but other synucleinopathies such as Dementia with Lewy Bodies and Multiple System Atrophy. In addition α-syn is also shown to inhibit Complex I directly, further compromising the energy production of the mitochondrion and increasing the generation of ROS (Reeve et al., 2015). No evidence has emerged to suggest that α-syn-affected mtDNA or mitochondria can spread to healthy cells, however, further investigation is required.

Mitochondrial transport is driven by motor complexes via binding through specific adaptor proteins, such as Miro1, suggesting that a potential mechanism of mitochondrial transfer between cells is mediated via the motor systems using TNTs (Ahmad et al., 2014; Sinha et al., 2016). This is based on experimental data (Ahmad et al., 2014) as well as analogy with the movement of mitochondria along axons in neuronal cells, where the kinesin and dynein motor systems include the motor protein plus two adaptor proteins Milton and Miro1, the latter with high affinity for mitochondria (Hase et al., 2009; Wang and Schwarz, 2009; MacAskill and Kittler, 2010; Kimura et al., 2012; Schiller et al., 2013). Indeed, mutations in the Miro1 gene, RHOT1, have recently been linked to PD cases, wherein the mutations led to fewer ER-mitochondria contact sites, calcium dyshomeostasis and exacerbation of calcium-dependent mitochondrial fragmentation and increased mitochondrial clearance (Grossmann et al., 2019). How the movement of mitochondria between cells is triggered and regulated is still not fully understood. Recent work has shown α-syn utilizing TNTs for spread. Thus, Dieriks et al. (2017) demonstrated the establishment of TNTs and transport of α-syn from neurons and pericytes in culture. Interestingly, Rostami et al. (2017) revealed TNT formation and α-syn transfer between astrocytic cells, transport of healthy mitochondria to rescue stressed mitochondria damaged by α-syn and transport of α-syn from α-syn affected astrocytes to healthy astrocytes. There were no reports of α-syn utilizing mitochondria for TNT-mediated migration, however, mitochondria may also represent an efficient carrier of α-syn between certain cell types. Furthermore, prion proteins, which have some similarities in mode of propagation to α-syn aggregates, have been established to hijack TNTs to mediate cell-to-cell transfer of the infectious protein (Gousset et al., 2009; Dorban et al., 2010; Prusiner et al., 2015; Steiner et al., 2018). Although there are many potential mechanisms of α-syn spread (Valdinocci et al., 2017), in view of findings that α-syn can migrate within TNTs bound to organelles such as lysosomal vesicles (Abounit et al., 2016) it is tempting to speculate that α-syn may transfer between neighboring cells bound to mitochondria that are translocated actively along TNTs (**Figure 1**).

POTENTIAL FOR MITOCHONDRIALLY TARGETED PD THERAPEUTICS

The influences of pathological α-syn on mitochondrial dynamics in PD are potentially wide-ranging. Mitochondria-targeted drugs, such as Mito-Q and Mito-Apocynin showed therapeutic potential in experimental PD models whilst others such as Metformin are variable likely attributed to model utilized (Patil et al., 2014; Bayliss et al., 2016; Ismaiel et al., 2016; Lu et al., 2016; Langley et al., 2017; Xi et al., 2018). Although therapies that target calcium mobilization or oxidative stress tackle the effects of mitochondrial dysfunction, future innovative approaches could ameliorate mis-location of mitochondria or intercellular mitochondrial transfer. Further work is clearly needed to articulate the full significance of changed mitochondrial dynamics in PD etiology.

AUTHOR CONTRIBUTIONS

All authors have contributed to the preparation of the manuscript, with DP taking responsibility for editing the final version of the manuscript.

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The Impairments of α-Synuclein and Mechanistic Target of Rapamycin in Rotenone-Induced SH-SY5Y Cells and Mice Model of Parkinson's Disease

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Parkinson's disease (PD) is characterized by selective degeneration of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc). α-synuclein (α-syn) is known to regulate mitochondrial function and both PINK1 and Parkin have been shown to eliminate damaged mitochondria in PD. Mechanistic target of rapamycin (mTOR) is expressed in several distinct subcellular compartments and mediates the effects of nutrients, growth factors, and stress on cell growth. However, the contributions of these various regulators to DAergic cell death have been demonstrated mainly in culture with serum, which is known to dramatically influence endogenous growth rate and toxin susceptibility through nutrient and growth factor signaling. Therefore, we compared neurotoxicity induced by the mitochondrial inhibitor rotenone (ROT, 5 or 10 μM for 24 h) in SH-SY5Y cells cultured with 10% fetal bovine serum (FBS), 1% FBS, or 1% bovine serum albumin (BSA, serum-free). In addition, C57BL/6J mice were injected with 12 μg ROT into the right striatum, and brains examined by histology and Western blotting 2 weeks later for evidence of DAergic cell death and the underlying signaling mechanisms. ROT dose-dependently reduced SH-SY5Y cell viability in all serum groups without a significant effect of serum concentration. ROT injection also significantly reduced immunoreactivity for the DAergic cell marker tyrosine hydroxylase (TH) in both the mouse striatum and SNpc. Western blotting revealed that ROT inhibited TH and Parkin expression while increasing α-syn and PINK1 expression in both SH-SY5Y cells and injected mice, consistent with disruption of mitochondrial function. Moreover, expression levels of the mTOR signaling pathway components mTORC, AMP-activated protein kinase (AMPK), ULK1, and ATG13 were altered in ROT-induced PD. Further, serum level influenced mTOR signaling in the absence of ROT and the changes in response to ROT. Signs of endoplasmic reticulum (ER) stress and altered expression of tethering proteins mediating mitochondria-associated ER contacts (MAMs) were also altered concomitant with ROT-induced neurodegeneration. Taken together, this study demonstrates that complex mechanism involving mitochondrial dysfunction, altered mTOR nutrient-sensing pathways, ER stress, and disrupted MAM protein dynamics are involved in DAergic neurodegeneration in response to ROT.

Keywords: rotenone, SH-SY5Y, α-synuclein, mitofusin, stereotaxic, mTOR

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INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disorder (NDD) characterized by progressive loss of dopaminergic (DAergic) neurons in the substantia nigra (SN) pars compacta (SNpc) along with intracellular aggregation of α-synuclein (α-syn) in structures known as Lewy bodies (LBs) (Lee and Trojanowski, 2006). Neurons use multiple feedback controls to regulate metabolism in response to nutrients and other signals. Neurons depend on oxidative phosphorylation (OXPHOS) for most energy needs, a process that consumes oxygen and glucose to generate energy-storing ATP (Hsu and Sabatini, 2008). This process relies on electron flow via electron transport chain (ETC) components in the inner mitochondrial membrane, culminating in the reduction of oxygen in the matrix, and the generation of a membrane potential across the inner membrane that is exploited to convert ADP to ATP (Whitworth and Pallanck, 2017).

Rotenone (ROT) is a naturally occurring insecticide, pesticide, and piscicide extracted from the roots of plants of the genera *Lonchocarpus* and *Derris*. It is highly lipophilic and therefore easily crosses all biological membranes including blood-brain barrier (Martinez and Greenamyre, 2012). The mitochondrial toxin ROT is widely used to induced a PD-like pathology in culture cells and experimental animals. ROT impairs OXPHOS by inhibiting mitochondrial ETC complex I (reduced nicotinamide adenine dinucleotide-ubiquinone reductase), leading to reduced ATP production and the formation of reactive oxygen species (ROS) that can induce oxidative stress (Duty and Jenner, 2011; Martinez and Greenamyre, 2012). The major advantages of ROT treatment for PD modeling are its ability to induce α-syn-positive cytoplasmic inclusions in nigral neurons resembling LBs (Betarbet et al., 2000) and progressive neurodegeneration accompanied by PD-like motor and non-motor symptoms (Johnson and Bobrovskaya, 2015).

In addition to external stressors, nutrient signals, growth factors, and cell energy balance (a product of mitochondrial function) are major regulators of cellular growth, proliferation, and survival in health and disease (Yang et al., 2019). However, the signaling pathways underlying nutrient effects in PD are largely unknown. Mechanistic/mammalian target of rapamycin (mTOR) is a serine-threonine kinase that controls several important aspects of mammalian cellular function through nutrient signal transduction (Saxton and Sabatini, 2017). It exists in two distinct multiprotein complexes, mTORC1 and mTORC2 (Switon et al., 2017), each with its own unique subunit composition and functions. The mTORC1 complex comprised of mTOR, mLST-8, FKBP38, Deptor, PRAS40, and rapamycin-sensitive adaptor protein of mTOR (Raptor) regulates cell growth, proliferation, and metabolism (Bai and Jiang, 2010), whereas mTORC2 comprised of mTOR, mSIN1, mLST8, and the rapamycin-insensitive subunit Rictor (Laplante and Sabatini, 2009) controls cell survival and cell-cycle dependent cytoskeleton assembly. Each complex also utilizes distinct cofactors and substrates for regulation of these processes according to nutrient and energy status. Collectively, these two complexes regulate multiple physiologic processes (Morrison Joly et al., 2016), such as axonal growth, neuronal development and survival, and synaptic plasticity, thereby contributing to learning and memory (Jaworski and Sheng, 2006; Swiech et al., 2008). The AMP-activated protein kinase (AMPK) is another widely recognized energy-sensing serine/threonine kinase (Mihaylova and Shaw, 2011). AMPK responds to oxidative stress and critically involved in NDDs (Jiang et al., 2013). In addition, AMPK-driven mTOR downregulation serves as a turn-off switch of the cellular anabolic program (Swiech et al., 2008). AMPK directly regulates the autophagy-associated kinase ULK1 through phosphorylation under nutrient signaling. Moreover, mTORC1 also phosphorylates ULK1 at Ser757 and affect the interaction between ULK1 and AMPK (Kim et al., 2011; Shang et al., 2011). Therefore, these mutually interacting protein signaling pathways may sense and integrate countless stimuli from nutrients and growth factors to direct normal cellular processes and pathogenic processes in NDDs like PD (Linke et al., 2017).

Endoplasmic reticulum (ER) stress also contributes to multiple pathophysiological processes in NDDs (Lavoie et al., 2011). Autophagy, Ca^{2+} homeostasis, lipid metabolism, mitochondrial ATP production, mitochondrial transport and biogenesis, ER stress, and the unfolded protein response (UPR) are fundamental cellular processes dependent on direct communication between the ER and mitochondria (Paillusson et al., 2017). Approximately 5–20% of the mitochondrial surface is closely apposed (within ∼10–30 nm) to ER membranes, forming specialized regions termed mitochondria-associated ER membranes (MAMs) (Paillusson et al., 2017). The MAM contains chaperones, oxidoreductases, calcium channels, calcium buffering proteins, and regulators of lipid metabolism. Thus, this subcellular compartment is likely involved in metabolic regulation by orchestrating protein folding, lipid synthesis, calcium buffering (Patergnani et al., 2011), and oxidation/reduction (Vance, 2014). MAM formation is dynamically regulated by tethering proteins between these organelles, such as glucose-regulated protein 75 (GRP75), mitofusin 1 (Mfn1), and Mfn2 (Ma et al., 2017). α-syn disrupts the MAM, which affects cellular exchange between the two organelles. Thus, MAM dysfunction may be a potential molecular mechanism linking α-syn to PD (Paillusson et al., 2017).

Despite extensive research efforts, it is still largely unclear how nutrients regulate the protein signaling pathways relevant to NDDs such as PD. Fetal bovine serum (FBS) has been used for mammalian cell culture to promote growth, differentiation, and survival (Piletz et al., 2018). However, serum is not a physiological fluid *in vivo*, and has been shown to induce aberrant cell growth characteristics, alter phenotype, and suppress neurotoxicity *in vitro* (Pirkmajer and Chibalin, 2011; Tekkatte et al., 2011). As many of these investigations have employed cells cultured in serum, the pathogenic pathways underlying the ROT-induced PD phenotype may differ substantially from PD pathogenesis *in vivo*. Therefore, it is critical to examine the effects of serum-containing cell culture media on cellular models of PD. Here we examined ROT toxicity in SH-SY5Y neuroblastoma cells under three different cell culture conditions: (1) 10% FBS, (2) low (1%) FBS, and (3) serum-free medium (0% FBS) containing 1% bovine

serum albumin (BSA) [used as synthetic serum as previously reported (Ramalingam and Kim, 2016)]. We also compared results to stereotaxic ROT injection in C57BL/6J mice. This study aimed to elucidate the molecular mechanism underlying ROT-induced toxicity, specifically the distinct contributions of mTOR/AMPK, and ER-mitochondrial tethering pathways.

MATERIALS AND METHODS

Chemicals, Reagents and Antibodies

Dulbecco's modified Eagle's medium (DMEM), penicillin streptomycin (Pen Strep), trypsin-EDTA, and FBS were purchased from Welgene (South Korea). Rotenone (R8875), dimethyl sulfoxide (DMSO; D2650), Avertin (2,2,2-Tribromoethanol; T48402) were purchased from Sigma-Aldrich (St. Louis, MO, United States). All other chemicals and reagents were from commercial suppliers and of the highest purity available. Plastic materials were purchased from SPL Life Science (SPL, Seoul, South Korea). The primary and secondary antibodies used in this study were tabled in **Supplementary Table 1**.

Cell Culture and Treatment

The human neuroblastoma cell line SH-SY5Y (CRL-2266) was obtained from ATCC (Manassas, VA, United States) and maintained in DMEM supplemented with 10% FBS, Pen Strep (100 U/ml; 100 mg/ml), and 2mM L-glutamine, at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Confluent cultures were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin-EDTA solution, reseeded as 1×10^5 cells/ml of DMEM containing 10% FBS or 1% FBS or 1% BSA and used for experiments after overnight incubation. SH-SY5Y cells were incubated with the absence or presence of ROT for 24 h. Combining floating cells in the medium and adherent cells detached by trypsinization and subjected to cell counting and Western blotting.

Cell Counting and Cell Morphology

After treated with ROT or solvent control (DMSO) at the indicated concentrations for 24 h, phase contrast images were taken using microscope Olympus CKX41 equipped with a camera. Damaged and deplated floating cells in the medium and adherent cells detached by trypsinization were combined and subjected to trypan blue cell counting method. Surviving cells, which cannot be stained with trypan blue dye, were counted under microscope. The cell count assay was performed in triplicates and expressed as a percentage (%) of control.

Preparation of Total Cell Lysates and Immunoblotting

After treated with indicated concentrations of ROT or DMSO for 24 h, cells were harvested by scraping with media, pelleted and washed twice with PBS. Then, exposed to RIPA buffer (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.251% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS): Thermo Fisher Scientific, United States) supplemented with protease and phosphatase inhibitors cocktail (Thermo Fisher Scientific, United States) and incubated for 30 min in ice. Lysates were centrifuged at 13,000 rpm for 20 min at 4°C and the supernatants were collected as total cell lysate. Protein concentrations were determined by BCA method (Kit). Proteins (30 μg) were separated on 6–12% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Bellerica, MA, United States). The membranes were washed with Tris buffered saline (TBS; 10 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.5% (v/v) Tween 20 (TBST) followed by blocking with 5% (v/v) non-fat dried milk solution prepared in TBST and then incubated overnight with primary antibodies at 4°C. The antibodies used are listed in **Supplementary Table 1**. After this, membranes were exposed to secondary antibodies conjugated to horseradish peroxidase for 2∼3 h at room temperature and further washed thrice with TBST. The immunoreactivity was detected by the luminol-based chemiluminescence (ECL) system. Equal protein loading was assessed by the expression level of β-actin. Densitometric analysis was performed using ImageJ (National Institute of Health, Bethesda, MD, United States) software.

Triton-X-100-Soluble and -Insoluble Fractionation

Following ROT toxicity for 24 h, SH-SY5Y cells were lysed on ice in RIPA buffer containing protease and phosphatase inhibitors with 1% Triton-X-100 for 30 min. Lysates were centrifuged at 12,000 rpm for 20 min at 4°C and the supernatants were collected as Triton-X-100-souble fraction. The cell pellets were washed with PBS then dissolved in the RIPA buffer containing protease and phosphatase inhibitors with 1% Triton-X-100 and 2% SDS and sonicated for 10 s and used as Triton-X-100-insoluble fraction. Protein samples were immunoblotted as described above.

Animals and Stereotaxic Surgery

Five-week-old C57BL/6J male mice were purchased from DBL (South Korea) were housed at room temperature under 12 h ligh/dark cycle. Food and water were provided *ad libitum* for 1 week before intrastriatal surgery. All animal experiments were approved by the Ethical Committee of Animal Research of DGIST, Daegu, South Korea accordance with international guidelines (DGIST-IACUC-18010204-01). Mice underwent unilateral stereotaxic surgery under injectable 2.5% Avertin anesthesia. A hole was drilled in the skull and a cannula inserted at following stereotaxic coordinates at AP + 1.0, ML −2.5 from bregma and DV −3.0 below dura in the right striatum, and 12 μg of freshly prepared ROT (dissolved in 2 μl DMSO) was infused (0.2 μl/min for 10 min for infusion with 5 min for diffusion). Control animals were injected with vehicle DMSO. Fourteen days after surgery, mice were anesthetized with avertin and perfused.

Immunohistochemistry

Mice were sacrificed by terminal anesthesia and transcardially perfused with 50 ml PBS followed by 50 ml 4% paraformaldehyde (PFA). Brains were rapidly removed, post-fixed in 4% PFA for 24 h and stored in a 30% sucrose solution for 48 h or

more. Serial coronal sections (35 μm) were cut using a freezing sledge microtome and a 1:4 series of sections was used for all quantitative immunohistochemistry. For immunohistochemical analyses, blocking of non-specific secondary antibody binding (using 3% normal horse serum in PBS with 0.2% Triton X-100 at room temperature for 1 h), sections were incubated overnight at room temperature with the primary antibody of TH (1:1000) diluted in PBS with 0.2% Triton X-100. Sections were then incubated in a biotinylated secondary antibody for 1 h [horse anti-mouse (1:2000, Vector, United Kingdom)] followed by 1 h incubation in streptavidin-biotin-horseradish peroxidase solution (Vector, United Kingdom). Sections were developed in 0.5% solution of diaminobenzidine (DAB) tetrahydrochloride (Sigma, Ireland) and mounted on microscope slides coverslipped using DPX mountant (BDH chemicals, United Kingdom). Immunostained sections were photographed and the total number of TH positive neurons in the SNpc was determined using the Optical fractionator probe in Stereo Investigator software (MicroBrightfield, Williston, VT, United States). All stereological counting was performed in a blinded manner to mice treatments.

Cell Lysates Preparation and Immunoblotting

After fourteen days of surgery, mice were anesthetized with avertin and perfused with PBS. Mice brain subregions of midbrain (MB) and striatum (ST) were located following procedures described previously (Jackson-Lewis and Przedborski, 2007). The lysates were prepared and Western blotting done as mentioned above.

Statistical Analysis

Data are expressed as mean ± standard error mean (SEM). The significance level of treatment effects was determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (*in vitro*; three or more groups) or an paired/unpaired two-tailed Student's *t*-test (*in vivo*; two-group comparisons). A probability of <5% ($p < 0.05$) was considered to be statistically significant. GraphPad Prism 5.0 software (La Jolla, CA) was used for data analyses and preparation of all graphs.

RESULTS

Rotenone-Induced Death in SH-SY5Y Cells

SH-SY5Y cells were cultured in the same medium containing different serum concentrations and then treated with ROT (0, 0.5, 1, 2.5, 5, and 10 μM) for 24 h. Microscopic examination revealed that the majority of cells were damaged and deplated following 5 and 10 μM ROT treatment in 1% FBS and 1% BSA culture medium (**Supplementary Figure 1**). Trypan blue cell viability assays combining both floating and adherent cells detached by trypsinization revealed little proliferation in 1% FBS or 1% BSA ($p < 0.001$) compared to control 10% FBS culture media. ROT dose-dependently increased cell death in all groups after 24 h (all $p < 0.001$; **Figure 1A**). However, cell death was substantially greater in the low-serum and no-serum groups.

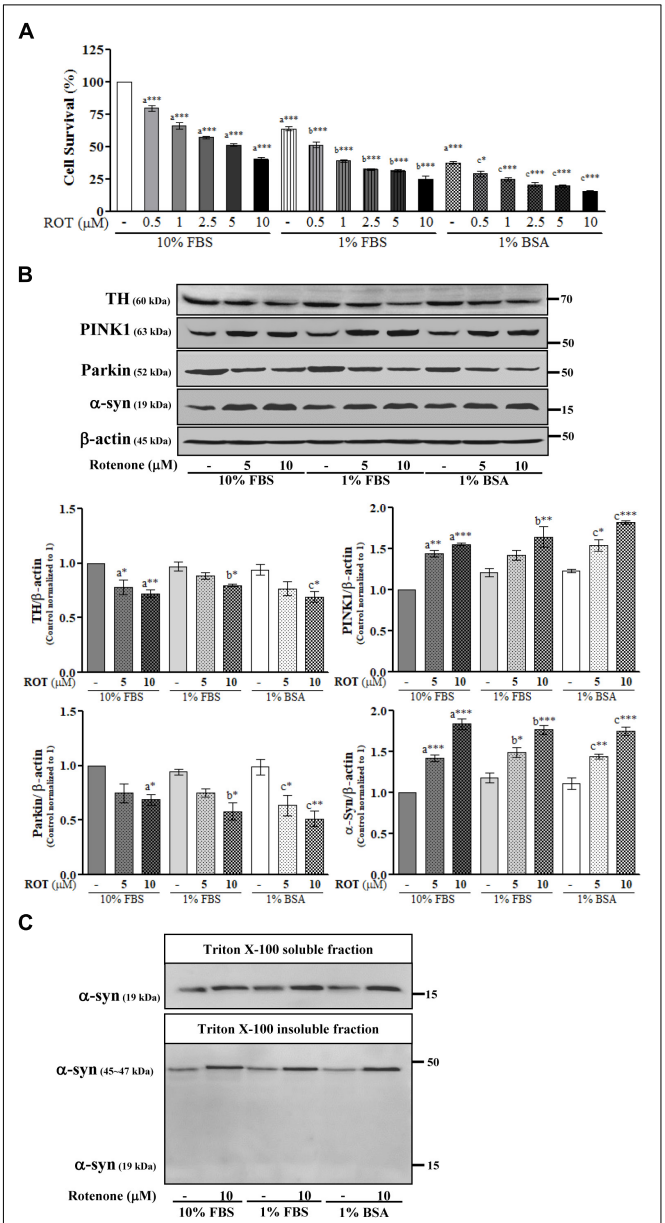

FIGURE 1 | SH-SY5Y cells were seeded as 1 × 10^5 cells/ml of DMEM containing 10% FBS or 1% FBS or 1% BSA and used for experiments after overnight incubation. Cells were incubated with the absence or presence of different concentrations of ROT (0, 0.5, 1, 2.5, 5, and 10 μM) for 24 h and assessed for trypan blue assay **(A)** or TH, PINK1, Parkin, α-syn and β-actin by Western blotting **(B)**. In addition, cells were fractionated into 1% Triton X-100 soluble and insoluble fractions and analyzed for α-syn by Western blotting **(C)**. Each picture is a representative of three independent experiments. Data are mean ± SEM of three independent experiments and analyzed by one-way of variance (ANOVA) followed by Tukey's *post hoc* test. Statistical significance: $_a$compared with 10% FBS control; $_b$compared with 1% FBS control; $_c$compared with 1% BSA control; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Rotenone Alters TH, Parkin, PINK1, and α-Syn Expression in SH-SY5Y Cells

To further evaluate ROT toxicity in SH-SY5Y cells, we measured expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine (DA) synthesis. Indeed, ROT significantly reduced TH protein expression in all serum groups ($p < 0.05$; **Figure 1B**). Moreover, ROT treatment (10 μM for 24 h) significantly increased expression of PINK1 ($p < 0.001$ in 10% FBS, $p < 0.01$ in 1% FBS, $p < 0.001$ in 1% BSA) and decreased expression of Parkin ($p < 0.05$ in 10% and 1% FBS, $p < 0.01$ in 1% BSA) as evidenced by Western blotting (**Figure 1B**), suggesting effects on mitochondrial function, quality control, and mitophagy.

Parkinson's disease is characterized by the presence of abnormal intracellular α-syn inclusions. ROT treatment increased α-syn in total cell lysates from all three serum concentrations groups ($p < 0.001$; **Figure 1B**), suggesting that ROT reduces SH-SY5Y DAergic neuron viability by promoting α-syn accumulation. Separate Western blot analyzes of the Triton-X100-soluble and insoluble lysate fractions, which are thought to include bioavailable and aggregated α-syn proteins, respectively revealed an increase of the oligomeric form in the Triton-X100-insoluble fraction and an increase of the monomeric form in the Triton-X100-soluble fraction. Thus, ROT may induce SH-SY5Y cell death through enhanced α-syn production and ensuing aggregation (**Figure 1C**).

Rotenone Alters mTORC and AMPK Expression Levels in SH-SY5Y Cells

The activities of mTORC1 and mTORC2 were assessed by measuring expression levels of phosphorylated (p)-mTORC1 (Ser2448) and p-mTORC2 (Ser2481), respectively. Control cells cultured in 1% FBS or 1% BSA exhibited slightly increased p-mTORC1 and significantly decreased p-mTORC2 ($p < 0.001$ and $p < 0.01$, respectively) (**Figure 2A**) compared to control cells cultured in 10% FBS. ROT (10 μM) dramatically increased p-mTORC1 ($p < 0.05$) and decreased p-mTORC2 ($p < 0.001$) expression in cells cultured with 10% FBS. Conversely, ROT decreased p-mTORC1 ($p < 0.05$ and $p < 0.001$, respectively) and increased p-mTORC2 (both $p < 0.001$) in cells cultured with 1% FBS or 1% BSA. These data suggest that FBS levels and ROT both alter mTOR signaling pathways in SH-SY5Y cells and that the effects of ROT differ depending on serum levels (nutrient availability). The overall effect of mTOR signaling depends on the specific complex activated, mTORC1 or mTORC2, which are distinguished by Raptor in mTORC1 and Rictor in mTORC2. Thus, we measured expression levels of p-Raptor (Ser792) and p-Rictor (Thr1135). Control cells cultured in 1% FBS or 1% BSA demonstrated no difference in p-Raptor expression but increased p-Rictor expression ($p < 0.01$ and $p < 0.001$, respectively) compared to control SH-SY5Y cells cultured in 10% FBS (**Figure 2B**), suggesting mTORC2 signaling predominance under low nutrient conditions. ROT treatment (10 μM for 24 h) decreased the expression levels of both p-Raptor (both $p < 0.001$) and p-Rictor ($p < 0.05$ in 10% and 1% FBS; $p < 0.01$ in BSA) in all three culture conditions, while total mTOR, Raptor and Rictor expression levels were unaffected (**Figures 2A,B**).

AMP-activated protein kinase is a major metabolic energy sensor that contributes to mTOR signaling through interactions with ULK1 and ATG13. Control cells cultured in 1% FBS or 1% BSA showed increased expression levels of p-AMPK (Thr172) (both $p < 0.001$), p-ULK1 (Ser757) ($p < 0.001$ and $p < 0.05$), and ATG13 (both $p < 0.001$) compared to control cells cultured in 10% FBS (**Figure 2C**). Treatment with ROT (10 μM for 24 h) significantly enhanced p-AMPK (Thr172), p-ULK1 (Ser757), and ATG13 expression by SH-SY5Y cells cultured in 10% FBS (all $p < 0.001$) but decreased expression levels of all three phosphorylated proteins in SH-SY5Y cells cultured with 1% FBS and 1% BSA, again indicating that serum influences mTOR signaling independently of ROT, and alters the mTOR signaling change in response to ROT.

Rotenone Induces ER Stress and Disrupts MAM in SH-SY5Y Cells

The endoplasmic reticulum is the central organelle responsible for protein folding, and there is compelling evidence that ER stress and protein misfolding are involved in ROT-induced PD-like toxicity. To examine ER stress in SH-SY5Y cells, we measured changes in the expression levels of protein kinase RNA (PKR)-like ER kinase (PERK) and inositol-requiring enzyme 1 α (IRE-1α). ROT (10 μM) increased expression of p-PERK at Thr981 ($p < 0.001$) and IRE-1α ($p < 0.01$) (**Figure 3**), suggesting that ER stress is involved in ROT-induced neuronal dysfunction. We further investigated the protein expression levels of MAM tethering proteins GRP75, Mfn1, and Mfn2. Control cells cultured with 1% FBS or 1% BSA showed significantly increased expression levels of GRP75 (both $p < 0.001$), Mfn1 ($p < 0.01$ in 1% FBS and $p < 0.001$ in 1% BSA), and Mfn2 (both $p < 0.05$) compared to control cells in 10% FBS (**Figure 3**). In addition, ROT (10 μM for 24 h) further increased the protein expression levels of GRP75 ($p < 0.001$) in all culture conditions. However, Mfn1 and Mfn2 were significantly increased by ROT (10 μM for 24 h) in cells cultured with 10% FBS ($p < 0.01$ and $p < 0.05$, respectively) but decreased in cells cultured in 1% FBS (both $p < 0.05$) or 1% BSA ($p < 0.001$ for Mfn1 and $p < 0.01$ for Mfn2).

Intrastriatal Rotenone Injection Alters TH, PINK1, Parkin, and α-Syn Expression Levels in C57BL/6J Mice

To examine the effects of ROT on mTOR signaling, ER stress, mitochondrial function, MAM function, and cell viability *in vivo*, we conducted single unilateral intrastriatal ROT infusions (12 μg) in C57BL/6J mice. Instrastriatal injection induced, significant depletion of TH immunoreactivity in the striatum and SN (**Figure 4A**), and significantly reduced TH-positive cell numbers in the ipsilateral (injection-side) SNpc after 14 days ($p < 0.01$). The expression levels of TH and Parkin were decreased while PINK1 and α-syn expression levels were increased in midbrain (**Figure 4B**) and striatum (**Figure 4C**) of ROT-injected mice compared to vehicle (DMSO)-injected mice

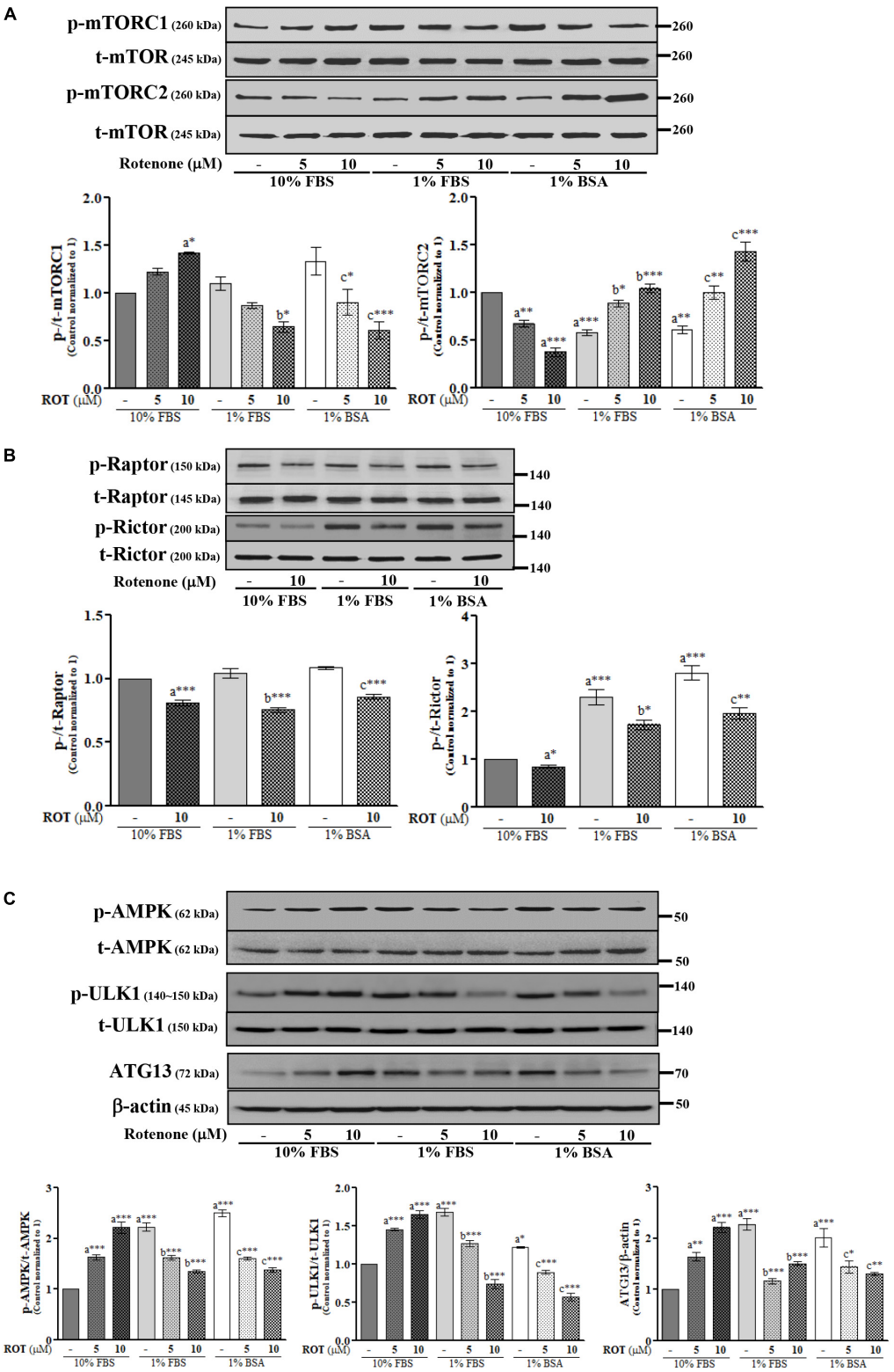

FIGURE 2 | SH-SY5Y cells were seeded as 1×10^5 cells/ml of DMEM containing 10% FBS or 1% FBS or 1% BSA and used for experiments after overnight incubation. Cells were incubated with the absence or presence of ROT (0, 5, and/or 10 μM) for 24 h and assessed for mTOR **(A,B)** and AMPK **(C)** signaling proteins and β-actin by Western blotting. Each picture is a representative of three independent experiments. Data are mean ± SEM of three independent experiments and analyzed by one-way of variance (ANOVA) followed by Tukey's *post hoc* test. Statistical significance: $_a$compared with 10% FBS control; $_b$compared with 1% FBS control; $_c$compared with 1% BSA control; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

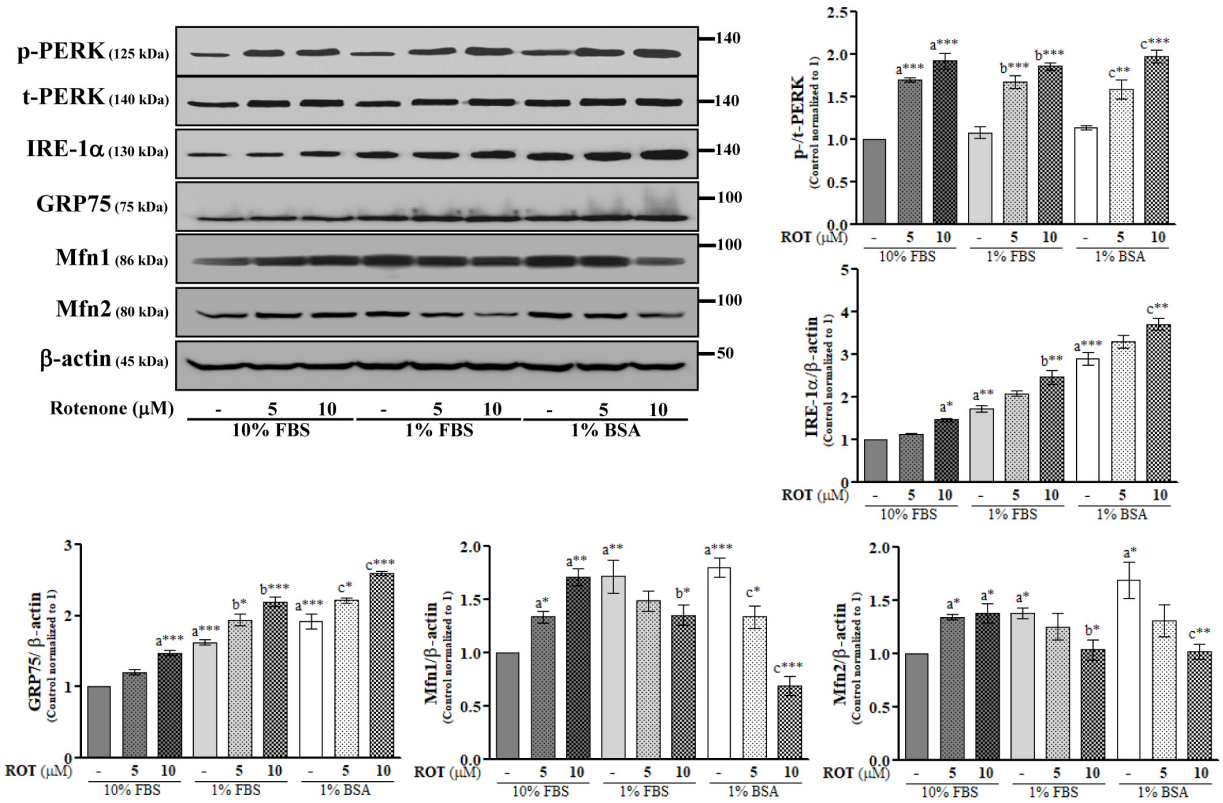

FIGURE 3 | SH-SY5Y cells were seeded as 1 × 10^5 cells/ml of DMEM containing 10% FBS or 1% FBS or 1% BSA and used for experiments after overnight incubation. Cells were incubated with the absence or presence of ROT (0, 5, and 10 μM) for 24 h and assessed for ER and MAM tethering proteins and β-actin by Western blotting. Each picture is a representative of three independent experiments. Data are mean ± SEM of three independent experiments and analyzed by one-way of variance (ANOVA) followed by Tukey's *post hoc* test. Statistical significance: $_a$compared with 10% FBS control; $_b$compared with 1% FBS control; $_c$compared with 1% BSA control; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

as measured by Western blotting, consistent with the effects of SH-SY5Y cells.

Intrastriatal Rotenone Alters mTOR and AMPK Signaling in C57BL/6J Mice

We then investigated the involvement of mTOR pathways in ROT toxicity. ROT injection increased p-mTORC1 (Ser2448) expression ($p < 0.01$) but reduced p-mTORC2 (Ser2481) expression ($p < 0.05$) in mouse midbrain (**Figure 5A**). Alternatively, ROT dramatically decreased both p-mTORC1 and p-mTORC2 in mouse striatum ($p < 0.001$; **Figure 5C**). In addition, p-Raptor and p-Rictor expression levels were reduced in the midbrain of ROT-injected mice ($p < 0.01$ and $p < 0.001$, respectively) (**Figure 5A**). Collectively, these findings suggest that ROT has region-specific effects on mTOR signaling pathways. Moreover, the protein expression levels of p-AMPK, p-ULK1, and ATG13 were decreased in ROT-injected mice (all $p < 0.01$), while t-AMPK and t-ULK1 expression levels remained unchanged (**Figure 5B**). In the striatum of ROT-injected mice, p-AMPK was increased while p-ULK1 and ATG13 expression levels were reduced (**Figure 5D**).

Intrastriatal Rotenone Alters ER Stress and Disrupts MAM in C57BL/6J Mice

To examine possible ROT-induced ER stress in the brain, we measured expression of the ER stress-associated proteins PERK and IRE-1α in midbrain (**Figure 6A**) and striatum (**Figure 6B**) lysates. Expression levels of p-PERK (Thr981) and IRE-1α were markedly increased by ROT in both midbrain and striatum (p-PERK: $p < 0.001$ in both regions; IRE-1α: $p < 0.001$ in midbrain, $p < 0.01$ in striatum) implicating ER stress in ROT induced neurodegeneration. Moreover, ER malfunction involved MAM dysfunction as the MAM tethering proteins GRP75, Mfn1, and Mfn2 were downregulated in midbrain ($p < 0.05$ for GRP75 and Mfn1; $p < 0.01$ for Mfn2) and striatum ($p < 0.05$ for GRP75; $p < 0.001$ for Mfn1 and Mfn2) of ROT-injected mice.

DISCUSSION

In recent years, many studies have been conducted to elucidate the molecular pathophysiology of PD progression. ROT, a fish poison that inhibits mitochondrial complex I, induces PD-like changes in cultured neurons, and rodent

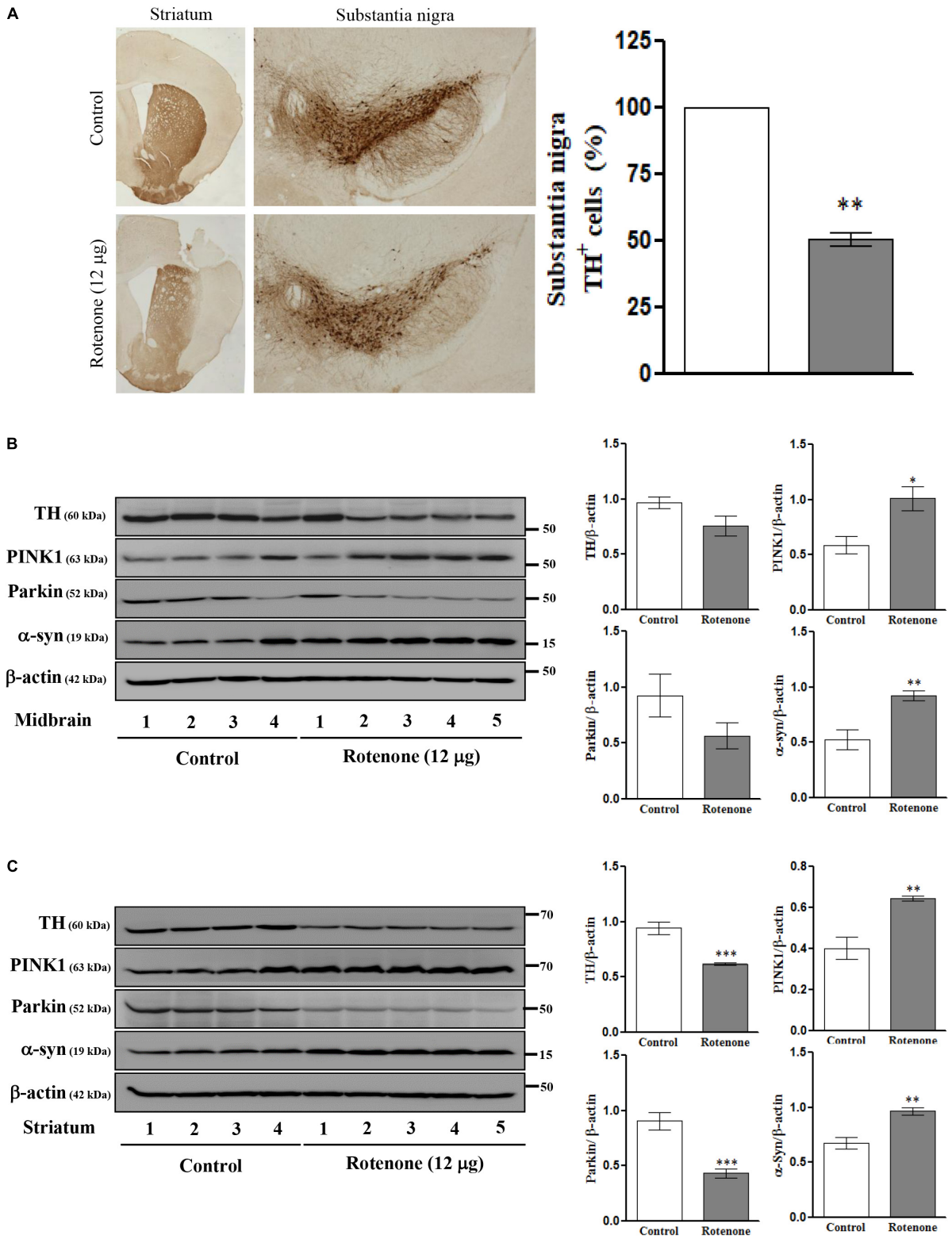

FIGURE 4 | Photomicrograph of ROT lesioned C57BL/6J mice striatum and substantia nigra pars compacta (SNpc) immunostained for TH (25×). The quantitative image analysis of TH positive cells in SNpc expressed as percentage (%). Each picture is a representative of three independent experiments. Data are mean ± SEM ($n = 3$) and analyzed by paired two-tailed Student's t-test. $^{**}p < 0.01$ **(A)**. Effects of ROT on TH, PINK1, Parkin, α-syn, and β-actin in the midbrain **(B)** and striatum **(C)** of stereotaxic C57BL/6J mice were analyzed by Western blotting. Data are mean ± SEM ($n = 4$ for control; $n = 5$ for ROT) and analyzed by unpaired two-tailed Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

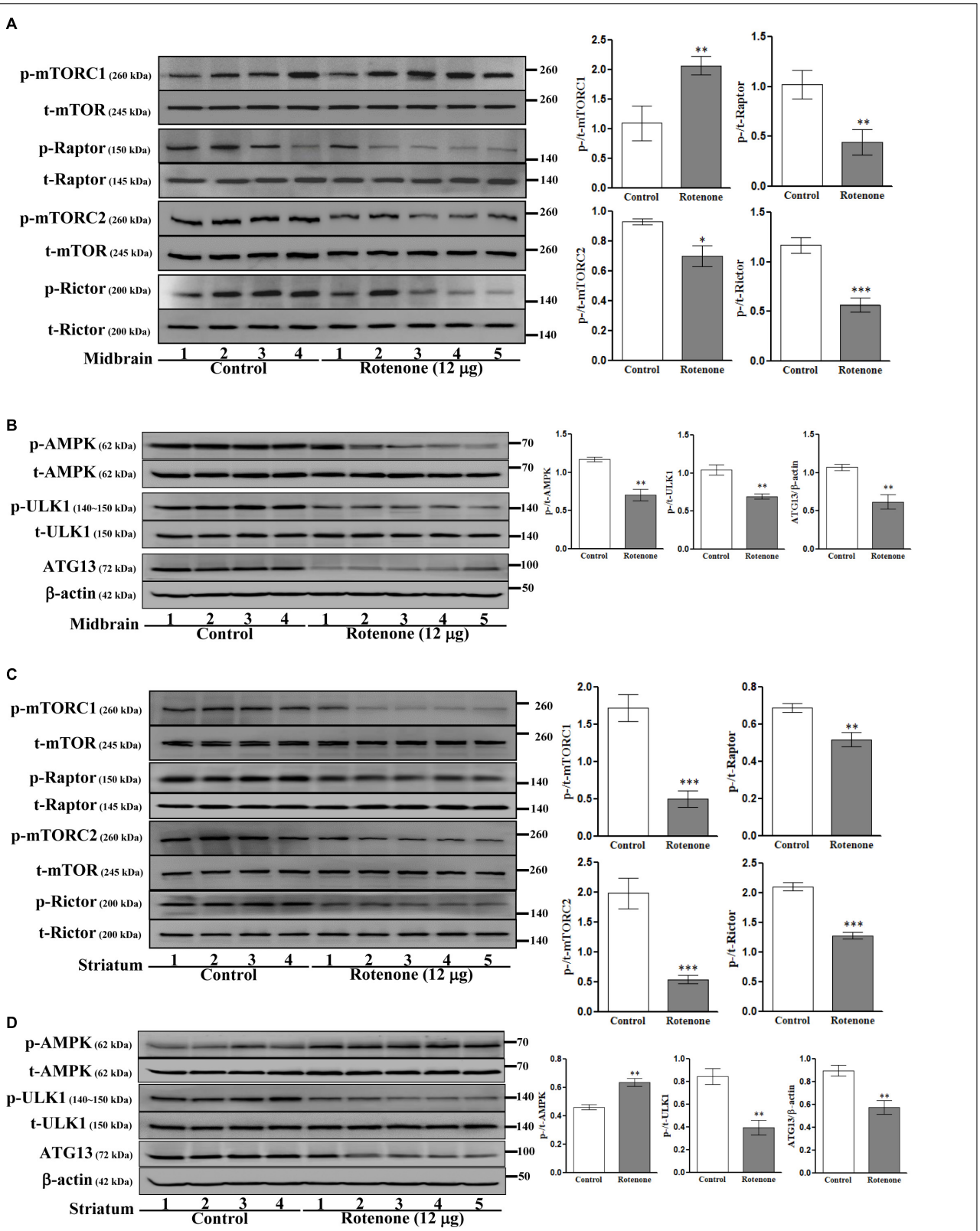

FIGURE 5 | Effects of ROT on mTOR **(A,C)** and AMPK **(B,D)** pathways in the midbrain **(A,B)** and striatum **(C,D)** of stereotaxic C57BL/6J mice were analyzed by Western blotting. Data are mean ± SEM ($n = 4$ for control; $n = 5$ for ROT) and analyzed by unpaired two-tailed Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

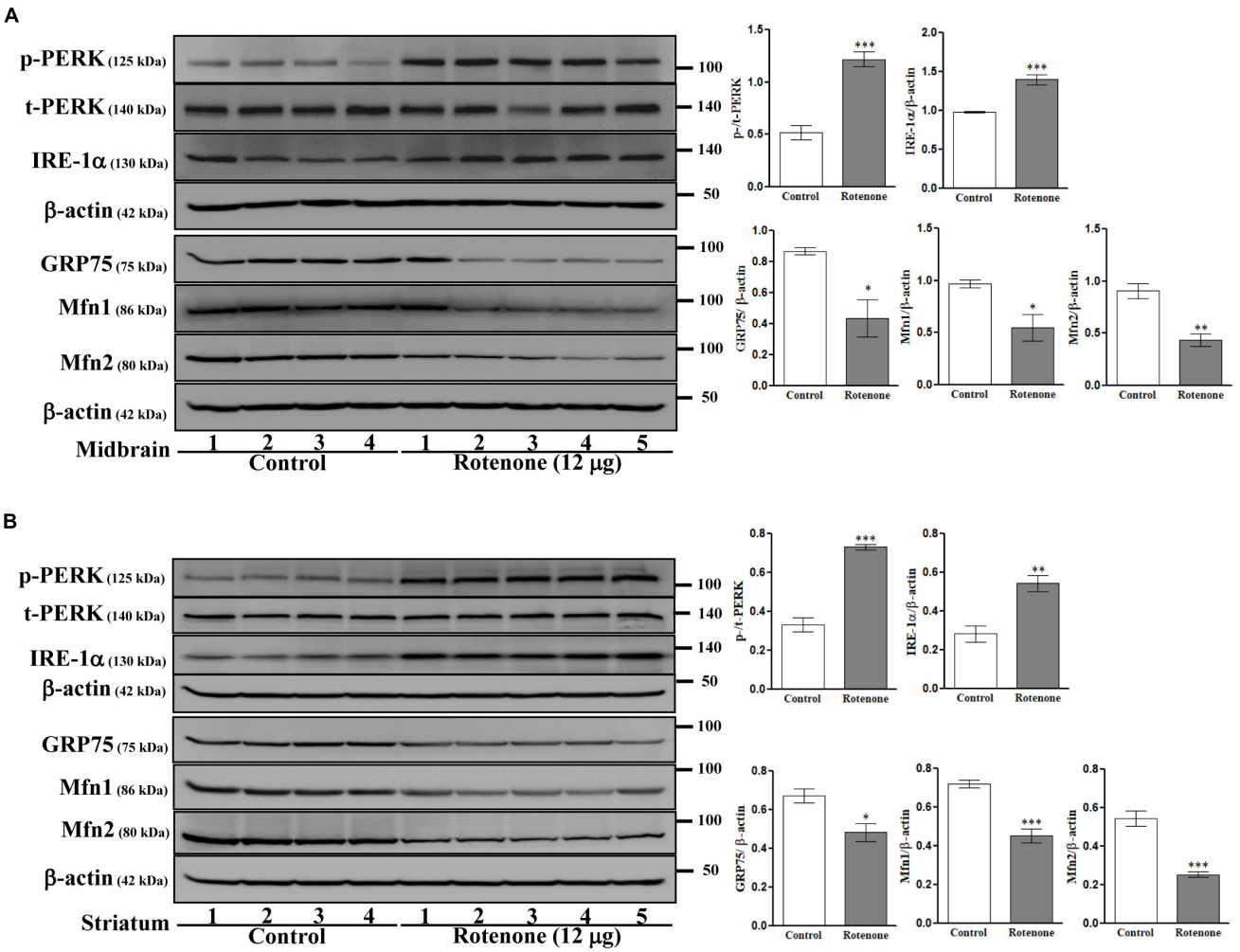

FIGURE 6 | Effects of ROT on ER and MAM pathways in the midbrain **(A)** and striatum **(B)** of stereotaxic C57BL/6J mice were analyzed by Western blotting. Data are mean ± SEM (n = 4 for control; n = 5 for ROT) and analyzed by unpaired two-tailed Student's t-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

brain (Sherer et al., 2003). SH-SY5Y is a DA-producing human catecholaminergic neuroblastoma cell line widely used as an *in vitro* dopaminergic cell model (Martins et al., 2013). ROT treatment for 24 h dose-dependently reduced SH-SY5Y cell number. Moreover, serum starvation by culture in 1% FBS or 1% BSA medium inhibited cell growth and proliferation in the absence of ROT. Cells under serum starvation alone or with ROT detached from the culture surface and lost typical neuronal morphology within 24 h consistent with previous studies showing that serum starvation induces cell death (Chou and Yung, 1997) and that ROT-induced impairment of mitochondrial complex I activity leads to apoptosis, likely via excess ROS formation (Imamura et al., 2006).

Tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, is obviously critical for phenotypic expression (Nagatsu et al., 1964). ROT treatment for 24 h dramatically reduced TH expression in SH-SY5Y cells. Moreover, ROT infusion in mouse striatum reduced TH immunoreactivity and reduced TH-positive cell count in the SNpc after 14 days, consistent with the dopaminergic neuronal degeneration along the nigrostriatal pathway that parallels the symptoms of PD (Carriere et al., 2016). In addition, surviving cells accumulate damaged mitochondria, leading to metabolic deficits, oxidative stress, mitophagy, and greater susceptibility to other pathogenic processes such as protein aggregation (Shaltouki et al., 2015).

Parkin (PARK2), a cytosolic ubiquitin ligase, and PTEN-induced kinase 1 (PINK1; PARK6), a mitochondria-targeted kinase, act important mediators of mitochondrial quality control (Whitworth and Pallanck, 2017) by removing damaged or dysfunctional mitochondria and preserving a healthy mitochondrial population (Oh et al., 2017). Dysfunctional mitochondria can trigger cell degeneration via mitophagy in PD (Li et al., 2015). In the present study, ROT inhibited Parkin expression and enhanced PINK1 expression in SH-SY5Y cells as well as in mouse midbrain and striatum, suggesting that loss of coordinated Parkin/PINK1 function contributes to ROT-induced mitochondrial impairment, oxidative stress, and cell death. Loss of Parkin results in an

initial accumulation of damaged mitochondria while PINK1 accumulation may cause proteosomal dysfunction (Wang et al., 2005; Shaltouki et al., 2015), which reduces Parkin solubility in toxin-induced PD models. Moreover, reduced Parkin in turn leads to the formation of protein aggregates resembling LBs in PD (Um et al., 2009, 2010).

Protein aggregation and filament formation are histopathological hallmarks of NDDs. For instance, PD is characterized by abnormal intracellular protein inclusions (LB and Lewy neurites or LN) mainly composed of aggregated α-syn fibrils (Faustini et al., 2017). The aggregation of α-syn causes proteasomal dysfunction, which may lead directly to neurodegeneration (Tanaka et al., 2001). We observed α-syn accumulation in SH-SY5Y cells and mouse midbrain and striatum following ROT treatment, consistent with previous studies demonstrating that ROT can trigger α-syn accumulation both *in vitro* and *in vivo* (Yuan et al., 2015). Aggregation of α-syn can be spread via a prion-like mechanism to neighboring neurons (Bae et al., 2012), resulting in the functional decline and death of dopaminergic neurons throughout the SNpc (Li et al., 2008; Lee et al., 2010). Moreover, α-syn can bind to TH, so accumulation of α-syn can reduce DAergic transmission by surviving cells (Perez and Hastings, 2004).

Under normal conditions, α-syn is cleared by proteolytic degradation in the extracellular space or lysosomal degradation in neighboring cells (Sung et al., 2005; Lee et al., 2008). However, impairment of these degradative mechanisms or an incapacity to clear proteins that have already aggregated is proposed the as primary defect leading to accumulation of insoluble α-syn (Cantuti-Castelvetri et al., 2005) within the LBs characteristic of PD (Myohanen et al., 2012), dementia with LB, and multiple system atrophy (Iwata et al., 2003). Previous *in vitro* studies have reported that the Triton-X100-soluble fraction represents bioavailable α-syn, whereas the Triton-X100-insoluble component may represent α-syn aggregated or sequestered in oligomeric forms (Klucken et al., 2003; Alves da Costa et al., 2006). In our present study, the oligomeric form of α-syn was detected only in the Triton-X100-insoluble fraction, while the monomeric form of α-syn was detected only in the Triton-X100-soluble fraction. ROT increased both Triton-X100-insoluble α-syn oligomers and Triton-X100-soluble monomer, consistent with previous studies reporting that ROT interacts with α-syn to drive accumulation of insoluble forms in SH-SY5Y cells (Sherer et al., 2002; Lee et al., 2004).

Growth factors and nutrients enhance protein synthesis and suppress protein degradation (Zhao et al., 2015). mTORs integrate signals from nutrients and growth factors with current energy status to regulate many neuronal processes, including autophagy, ribosome biogenesis, and growth (Sarbassov et al., 2005), as well as synaptic plasticity, learning and memory, and food uptake in adult brain (Zhou et al., 2015). Our present study suggests that both mTORC1 and mTORC2 regulate the responses to FBS and ROT in SH-SY5Y cells. ROT increased p-mTORC1 but decreased p-mTORC2 protein expression in the presence of 10% FBS, consistent with changes in the midbrain following ROT injection. Therefore, mTORC1 appears to be activated by ROT in the presence of sufficient nutrients and growth factors. The exact mechanism by which mTORC1 is activated and mTORC2 inhibited by ROT under this nutrient-rich condition requires additional studies. Control cells treated with 1% BSA also exhibited enhanced p-mTORC1 and reduced p-mTORC2 consistent with a previous study (Peruchetti et al., 2014). Conversely, ROT decreased p-mTORC1 and increased p-mTORC2 expression in the presence of 1% FBS or 1% BSA, changes shown to directly inhibit cell growth, and mitochondrial proteins during nutrient starvation. Our results are in line with another study reporting decreased mTORC1 in PC12 cells following ROT treatment for 24 h (Laplante and Sabatini, 2009). These results suggest that ROT treatment under nutrient shortage (serum starvation) inhibits mTORC1, resulting in lower mitochondrial membrane potential, oxygen consumption, and cellular ATP levels (Schieke et al., 2006).

Studies also suggest that mTORC1 acts as a negative regulator of mTORC2 (Dibble et al., 2009; Xie and Proud, 2014). Another study found that Rheb activated mTORC1 but inhibited mTORC2, while TSC1/2 inhibited Rheb/mTORC1 but activated mTORC2, most likely by overcoming the negative feedback loop (Yang et al., 2006). To further explore the role of mTOR complex signaling pathways in ROT toxicity, we examined expression of Raptor, a required cofactor for rapamycin-sensitive mTORC1 signaling, and Rictor, a regulatory subunit of mTORC2 (Li et al., 2007). ROT treatment decreased the phosphorylation levels of Raptor and Rictor both *in vitro* and *in vivo*, inconsistent with the aforementioned changes in mTORC1 and mTORC2 activity and suggesting that ROT can differentially stimulate or inhibit mTOR complex activities under different nutrient conditions; however, further investigations using specific substrates of these mTOR complexes necessary to examine the underlying mechanisms.

mTORC1 is the major transducer of nutrient signaling for cell growth (Efeyan et al., 2012). Similarly, AMPK senses energy deficiency in the form of an increased AMP/ATP ratio to regulate a myriad of cellular processes (Poels et al., 2009). The substrates ULK1 (ATG1) and ATG13 function downstream of mTORs and AMPK (Hosokawa et al., 2009; Egan et al., 2011). In this study and others (Wu et al., 2011), ROT-induced AMPK phosphorylation at Thr172 in SH-SY5Y cells cultured with 10% FBS medium. Increased p-AMPK was also reported in ROT treated HepG2 cells (Hou et al., 2018). Furthermore, activated ULK1 was shown to directly phosphorylate AMPK (Loffler et al., 2011). In contrast, Sciarretta et al. (2018) reported that ROT decreased mTORC1 and inhibited AMPK-ULK1 under serum starvation. Under starvation, AMPK translocates to the lysosome and both lysosomal AMPK and mTORC1 contribute to autophagy via ULK1/ATG13/FIP200 regulation (Ha et al., 2015). In this study, ROT inhibited mTORC1, AMPK/ULK/ATG13 in cells cultured with 1% FBS or 1% BSA (nutrient starvation). In ROT-injected mouse midbrain, the activation of mTORC1 by ROT inhibited the AMPK/ULK1/ATG13 pathway after 14 days. From the above results, we speculate that the nutrient-sensing molecules mTORC1 and AMPK/ULK1/ATG13 are differently regulated by ROT toxicity *in vitro* and *in vivo*.

The endoplasmic reticulum is involved in protein folding, maintenance of Ca^{2+} homeostasis and cholesterol synthesis, and ER dysfunction is implicated in the pathogenesis of α-syn mediated NDDs (Paillusson et al., 2017). The ER depends on ATP to correct misfolded protein errors, so ROT-induced ATP reduction can lead to ER stress, which in turn initiates the UPR through activation of PERK, and IRE-1α (Jiang et al., 2016). In the present study, ROT enhanced p-PERK Thr981 and IRE-1α expression levels, indicating ROT-induced ER stress, in both SH-SY5Y cells and mouse midbrain and striatum. This enhanced activation of PERK signaling results in a sustained reduction of global protein synthesis, leading to neuronal loss (Liu et al., 2015). In addition, PERK over-activation has been observed in post-mortem brain and spinal cord tissues of NDD patients (Smith and Mallucci, 2016). Taken together, the present study suggests that ROT-induced ER stress may trigger the death of SH-SY5Y cells and DAergic neurons in mice.

Mitochondria-associated ER membrane abnormalities have been described in cellular models of a number of NDDs, including PD (Cali et al., 2013). GRP75 is essential for maintaining physical contact between the ER and mitochondria, thereby facilitating Ca^{2+} exchange and transfer through ER-bound IP_3R and mitochondrial VDAC1 (Honrath et al., 2017). Expression of GRP75 was increased in SH-SY5Y cells by ROT treatment but decreased in midbrain and striatum of C57BL/6J mice. As previously reported, GRP75 overexpression in human DAergic cells enhanced vulnerability to ROT-induced cytotoxicity (Jin et al., 2006), but *in vivo* GRP75 overexpression reduced infarct size and protected against mitochondrial damage in a rat middle cerebral artery occlusion model of stroke (Xu et al., 2009) and a rat model of intracerebral hemorrhage (ICH) (Lv et al., 2017). GRP75 expression was also decreased in the mitochondrial fraction isolated from the SNpc of PD patients compared to controls (Jin et al., 2006). The above results suggest that GRP75 may be either beneficial or harmful in different pathogenic contexts, although the exact mechanisms are still unknown (Shi et al., 2008). One possible explanation is provided by reports that GRP75 interacted with MAM-associated α-syn in a DAergic cell line with strong membrane attachment affinity due to a high lipid:protein ratio (Zabrocki et al., 2008; Fantini and Yahi, 2011).

The MAM tethering proteins mitofusions Mfn1 and Mfn2 are dynamin-related GTPases responsible for membrane fusion via a large cytosolic GTPase domain embedded in the outer mitochondrial membrane (Rojo et al., 2002; Suarez-Rivero et al., 2016). Mfn2 heterodimerizes with Mfn1 to link ER and mitochondria to regulate organelle tethering (de Brito and Scorrano, 2008; Chen et al., 2012). Our study suggests that α-syn can affect mitochondrial morphology and modulate mitochondrial dynamics through reduction of Mfn1- and Mfn2-dependent tethering in SH-SY5Y cells and mouse brain (Xie and Chung, 2012). Moreover, these findings identify MAM interface and inter-organelle contact disruption as novel mechanisms of ROT-induced α-syn toxicity.

Many studies rely on artificially overexpressed or recombinantly tagged proteins to investigate the underlying signaling mechanisms in PD. However, in the present study, we compared ROT-induced toxicity among cultures under different nutrient availability conditions and *in vivo* to further elucidate the potential mechanisms of α-syn neurotoxicity. As expected, ROT decreased TH and concomitantly increased α-syn accumulation, indicating degeneration of dopaminergic neurons both *in vitro* and *in vivo*. The nutrient-sensing mTOR and AMPK pathways appear to form a negative feedback loop that regulates the extent of ROT toxicity. More importantly, the present study implies that ER and MAM tethering proteins are also intimately involved in ROT-induced neurodegeneration. From these findings, we conclude that ROT-treated cell culture systems and mouse models are limited for recapitulating the clinical and pathological phenotypes of PD. We also conclude that serum concentration in culture medium can greatly influence the effects of ROT on cell growth, oxidative stress, and the various underlying signaling pathways. Further research *in vitro* and *in vivo* is necessary to establish stronger links between ROT-induced pathogenic mechanisms and human PD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

All animal experiments were approved by the Ethical Committee of Animal Research of the DGIST, Daegu, South Korea accordance with international guidelines.

AUTHOR CONTRIBUTIONS

MR and Y-IL conceived and designed the study, and wrote the manuscript. MR performed the major experiments. Y-JH helped to perform the animal experiments. MR analyzed the data. All authors read and approved the manuscript 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/fnins.2019.01028/full#supplementary-material

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