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-Synuclein and Mitochondrial Dysfunction Induced ER Stress Leading to Parkinson's Disease

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α-Synuclein and Mitochondrial Dysfunction Induced ER Stress Leading to Parkinson's Disease

Abstract

Parkinson's disease is, at the neuropathological level, characterized by the death of dopaminergic neurons. PD pathogenesis is multifactorial, there are several mechanisms that malfunction leading to the death of dopaminergic neurons. The mitochondria are vital for the normal functioning of a cell, they are the primary energy generator for the cell. In addition to their energy metabolism role, mitochondria are involved in many cellular processes, such as the stress response and cell death pathways. As a result, mitochondrial impairment and the genes that are related to its functionality are linked to progressive neurodegeneration. Considering parkin, PINK1, DJ-1, and LRRK2 gives insight into how PD develops and potential therapeutic options. Mitochondrial dysfunction works in conjugation with another classic PD characteristic, the accumulation of misfolded proteins. ER stressed caused by the accumulation and formation of aggregates induces a cellular defense mechanism, UPR; however, chronic ER stress impairs many of the protective functions that the cell possesses leading to cell death. Individually mitochondrial dysfunction and ER stress have been implicated in PD, but their interaction is not entirely understood. Furthermore, the role of key mitochondrial related PD genes in mitochondria-ER stress resulting in dopaminergic neurodegeneration has not been fully integrated in literature. This review attempts to reconcile these key mechanistic events.

Introduction

Parkinson's disease (PD) was first described as "shaky palsy" in 1817 by Dr. James Parkinson. It is a chronic, progressive, neurodegenerative disease with motor and nonmotor features. The disease is characterized by the loss of dopaminergic neurons and the subsequent deficiency of dopamine in the striatum [7]. The motor symptoms that manifest is resting tremors, bradykinesia, and muscle rigidity. These motor symptoms have been grouped into a symptom complex called *parkinsonism* [10]. Research has shown that pathophysiological changes that underlie PD may start before the onset of the motor issues. A number of nonmotor symptoms present before the motor, such as sleep disorders, depression, and cognitive changes [11]. There are different classifications of PD, familial and sporadic. A hallmark of sporadic PD is the presence of proteinaceous aggregates in Lewy Bodies. However, there have not been consistent findings that LBs play a part in familial PD. Misfolded proteins usually trigger a cellular response in the endoplasmic reticulum (ER) called the Unfolded Protein Response (UPR). The formation of these Lewy bodies suggests that some impairment in the cell's normal protein degradation mechanism.

The cause of sporadic PD is not as simple as some aggregates of a protein, it has been determined to be a multifactorial disorder that consists of several types of proteins and dysfunction in many different biochemical pathways. The different proteins that have been identified to have a role in the development of LBs are a-Synuclein, ubiquitin, and molecular chaperones [16]. Environmental factors and genetic susceptibility need to also be considered. Research into PD genes has shown that mutations in mitochondrial genes are responsible for monogenic familial forms. The unique biochemical environment that the mechanism of oxidative

respiration makes the mitochondria vulnerable for genetic mutations. The death of dopaminergic neurons that is emblematic of parkinsonism is intertwined in many cell systems. There are many pathways that a cell can take to remove a stressor or neurotoxic substances and dysfunction in more than one of them can compound leading to PD. Within mitochondrial dysfunction there are several proteins that are involved in pathogenesis, such as α-Synuclein, parkin, PINK1, and DJ-1. Genetic markers are the latest factors to be considered for the etiopathogenesis of PD, especially the genetic mutations in the mitochondria [7].

1. Parkinson's Disease

1.1 Pathophysiology

Parkinson's disease is a disorder of the extrapyramidal system, it includes the motor structures of the basal ganglia. PD is characterized by the loss of dopaminergic function and diminished motor function leading to the clinical manifestations of the disease [11]. The striatal dopamine loss has been identified as the major cause of the major symptoms of PD. However, the nonmotor symptoms suggests that other neurotransmitters are involved, such as the glutamatergic, cholinergic, serotonergic, and adrenergic systems. Neuromodulators adenosine and enkephalins have also been proposed as having a role [10]. Further evidence suggests that PD may start in the dorsal motor nucleus of the vagal and glossopharyngeal nerves and in the anterior olfactory nucleus, indicating pathogenesis in the brain stem that continues to higher cortical levels. The histopathological features of PD include the loss of pigmented dopaminergic neurons and the presence of Lewy bodies (LBs) [12]. Degeneration of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) occurs progressively, which then project to the striatum. Patients experience motor symptoms of PD after they have lost 50%-80% of the dopaminergic neurons. Two types of dopamine receptors are responsible for the motor component of the extrapyramidal system, D_1 (excitatory type) and D_2 (inhibitory type). This system includes the basal ganglia, which incorporates the internal globus pallidal segment (GPi) of the ventral striatum, and the substantia nigra pars reticulata (SNpr). Dopamine loss in PD patients causes increased activity in the GPi/SNpr circuits, which leads to GABA dysfunction and thalamus inhibition. The end result of thalamus inhibition is its inability to adequately activate the frontal cortex, resulting in the decreased motor activity [13]. However, PD is a complex neurodegenerative disease that involves several biochemical pathways, and it is the interaction of these pathways that lead to neuropathology of the disease [65].

1.2 Lewy Bodies

A major feature of PD is the presence of LBs. LBs are described as intracellular cytoplasmic aggregates made up of proteins, lipids, and other materials. In patients with PD, LBs are found in the dopaminergic neurons in the substantia nigra as round bodies with radiating fibrils. It is suggested that the formation of LBs may be secondary to refractory proteolytic processes involving abnormal breakdown or overproduction influenced by genetic mutations [12]. Gene mutations involving α-Synuclein have been found to aggregate and form insoluble fibrils associated with LBs; α-Synuclein has now been identified as a potential therapeutic target for PD therapy [14]. The formation of LBs involves excess production of a misfolded form of ubiquitin protein. Ubiquitin has a role in protein recycling. The accumulation of ubiquitin occurs

as a result of the malfunction of the ubiquitin proteasome system (UPS) [10]. However, there are many mechanisms that lead to the malfunction of the UPS. Mitochondrial dysfunction, ER stress, and proteotoxic stress, to name a few. One of the proteins that link the formation of LBs with the mitochondria is the LRRK2 protein, which has been found to be present in LBs [96]. LBs appear to play a role in the neurodegeneration that is characteristic of PD. The lesion patterns are consistently seen at various stages of the disease. These patterns are located in the dorsal nucleus, medulla, and pons, and may support early premotor features (olfactory and rapid eye movement) of PD [15]. LB formation is not likely to be the cause of one malfunction or aggregation of one protein. There is a large network of interacting mechanisms that contribute to the homeostasis of the dopaminergic neurons. However, deficiencies in one area of maintenance may lead to a cascade of neurotoxic damage resulting in inadequate protein removal that is emblematic of LBs.

2. α-Synuclein

2.1 α-Synuclein Structural Properties

α-Synuclein is a 140 amino acid protein found in the brain. It is present primarily in both the soluble and membrane-associated fractions of the brain [1]. α-Synuclein is a part of the synuclein family along with the β and γ forms. These three forms of synuclein are neuronal proteins that typically localize at the presynaptic terminal under normal physiological conditions [2]. In an aqueous solution the protein has no defined structure. α-Synuclein is composed of three different regions, an amino terminus that has apolipoprotein lipid-binding motifs, a central hydrophobic region, and a carboxyl terminus that is highly negatively charged [2]. It has been described as a "natively unfolded protein". α-Synuclein's unfolded structure is determined by its relatively low hydrophobicity and high net charge [1]. The polarity and charge of α-Synuclein allows the protein to interact with water to a higher degree. The protein typically is an aqueous solution and its interaction with water reduces the hydrophobic effect, maintaining the "unfolded structure". However, α-Synuclein does conform to particular structures under particular conditions, it adopts α-helical structures when binding to negatively charged lipids and has β-sheet rich structures when incubated for prolonged periods of time [2]. Changing the protein environment in such a way to increase the hydrophobicity and/or decrease the net charge has been shown to induce some partial folding [1]. There are several methods that can be employed to try and modulate the environment around α-Synuclein to induce different structural patterns. The high negative charge at physiological neutral pH can be neutralized by acidifying the solution, and the hydrophobicity can be increased with higher temperature [6]. Later experiments confirmed that the when the pH was reduced to 3.0 or the temperature was increased, α-Synuclein gained a more ordered secondary structure, became more compact, and developed a semblance of a tightly packed nucleus [6]. The relative compactness of α-Synuclein under the non-physiological conditions was a result of clustering due to the hydrophobic effect of the polar residues of the protein [2]. Because the structure of α-Synuclein is natively unfolded, there is partial misfolding of α-Synuclein in certain regions for functional purposes.

2.2 α-Synuclein Function

The function of α-Synuclein is not entirely understood, but many of the functions that are proposed relate to its structure and the interactions that it makes [1-3]. Many different functions have been suggested to be attributed with α-Synuclein, including synaptic vesicle release and trafficking, fatty acid binding, physiological regulation of certain enzymes, transporters, and neurotransmitter vesicles, as well as neuronal survival [1]. Neuronal survival is of vital importance when discussing α-Synuclein in the context of neurodegenerative disease, particularly Parkinson's. There are three groups in the Synuclein family, α, β, and γ, knockout of these genes in vertebrates leads to neuronal dysfunction [1,4]. This indicates that α-Synuclein plays a role in stabilizing long-term operation of the nervous system. β-Synuclein was shown to protect against α-Synuclein degeneration leading to neurodegenerative diseases [2]. α-Synuclein has also been shown to physically interact with at least 30 proteins, underlying its important role in cell to cell signaling [1,2]. Diseases that results from α-Synuclein pathology typically affect neurophysiology, indicating that the functions of α-Synuclein have to do with brain function. α-Synuclein can potentially be a down regulator for the enzyme tyrosine hydroxylase (TH). TH activity modulates the production of dopamine and its control at cellular levels. The interaction between α-Synuclein and TH is not direct [4]. Tyrosine hydroxylase is normally phosphorylated when active; however, through the action of α-Synuclein and its interaction with PP2A, a phosphatase, TH is dephosphorylated at the Ser40 residue and is inactivated [4,5]. This stops the enzyme from converting tyrosine to L-dopa a precursor for dopamine.

2. 3 α-Synuclein Dysfunction

There are two structural classes of amyloid fibrils: those derived from folded proteins and those derived from intrinsically disordered protein. These amyloid fibrils are formed from the folded proteins by either the refolding mechanism or by the gain-of-interaction model [8]. α-Synuclein belongs to the second class of amyloid fibrils, it is intrinsically disordered amyloid protein that forms fibrils by converting some or all of the polypeptide into well-defined, beta-sheet rich secondary structure. The beta-sheets easily form a cross-beta structure, in which the beta-sheets are arranged in parallel, with highly ordered amino acid side chain patterns exposed on the surface of the beta-sheets [8,9]. The sidechains from the beta-sheets interdigitate in a self-complementary manner to give rise to highly ordered steric zippers. The formation of steric zippers is not unique to α-Synuclein fibrils, but the morphology of α-Synuclein fibrils exhibit a distinct structural polymorphism. These differences come from the unique structure of α-Synuclein and the interactions its sidechains have with the environment [2]. Changes in the buffer solution seem to be factor in the rate of folding and aggregation, like pH, temperature, ion concentration, and external variables such as agitation or toxins. The potential of α-Synuclein to form this beta-sheet structure is reminiscent of the beta-sheets of β-amyloid, which provides a pathogenetic basis for two neurodegenerative diseases [1]. In Parkinson's disease α-Synuclein is heavily expressed within Lewy bodies, in particular, in the "halo" of the inclusions [53,54]. α-Synuclein fibrils form the basis for mature LBs present in synucleinopathies, however in the process of fibril formation, intermediates forms develop. The early intermediate forms are soluble and have different oligomeric characteristics, such as sphere-like, ring-like, and string-like. These protofibrils eventual coalesce into fibrils and become insoluble [55]. The insoluble fibrils combine and cannot be removed, which leads to the generation of α-Synuclein aggregates, this aggregation is the main pathogenic component of α-Synuclein [2]. Research into the

different variants of α-Synuclein have shown that there are species that are more vulnerable to the generation of fibrils. A53T and A30P α-Synuclein adopt the protofibril conformation than their wild-type counterparts and among the two, A53T more readily converts into a mature fibril [56]. Dopamine and its metabolites act as inhibitors for the conversion of protofibrils into their mature forms. The loss of the dopaminergic neurons in the pathogenic process of PD decreases availability of these substances and the increased presence of protofibrils [57]. There are several proposed mechanisms that α-Synuclein aggregates can be neurotoxic. Populations of α-Synuclein can induce toxicity directly and/or mediate toxicity through various mechanisms using proteins involved in different cellular processes [50]. The neurotoxicity of α-Synuclein and its aggregates can be grouped into three classes: mechanical disruption of cellular compartments/processes, toxic gain of function, and toxic loss of function [51]. A common method that disruption occurs is permeation of cellular membranes by the amyloid aggregates. α-Synuclein binds to lipid membranes and disrupt membrane bilayers by utilizing pore-like channels [52]. α-Synuclein gain of function is associated with PD pathogenesis stemming from experiments that showed a dose-dependent correlation of α-Synuclein accumulation to the PD phenotype. Features of the disease were shown in when WT overexpression of α-Synuclein was induced [2]. However, it is generally assumed that α-Synuclein gain of function is inherent within the normal protein, but a toxic component arises when the concentration exceeds a certain level. This was evident when comparing α-Synuclein knockout mice with mice that overexpressed the protein and there was no overt neuropathological or behavioral phenotype [58,59]. Perhaps the most pertinent location that α-Synuclein exerts its pathogenic effects would be at the synapse. While there has been evidence to suggest that α-Synuclein induces neurotoxicity in cells, the exact mechanism it uses is fragmented. The effects that are observed at the synapse are loss of presynaptic proteins, decrease of neurotransmitter release, redistribution of SNARE proteins, enlargement of synaptic vesicles, and inhibition of synaptic vesicle recycling [60]. α-Synuclein has been shown to utilize a pore-forming mechanism to disrupt cellular compartments, one compartment that α-Synuclein may target at the synapse are the synaptic vesicles. These pores leak out dopamine into the cytosol and its build up leads to oxidative stress induced cell death [52,61]. α-Synuclein also mimic the characteristics of other mitochondrial toxins, it plays a role in the downregulation of complex I. α-Synuclein has been shown to reside, in some quantities, within mitochondria [62]. The effects that α-Synuclein has on the mitochondria have cell wide implications and induce a variety of other protein dysfunction. α-Synuclein appears to induce mitochondrial fragmentation and may be a casual factor in mitochondrial dysfunction and death [63]. Possibly through this mechanism of fragmentation α-Synuclein also appears to induce excessive mitophagy, leading to inappropriate mitochondrial removal [64]. These effects place the mitochondria under stress, especially inhibiting complex I function, leading to oxidative stress, generating reactive oxygen species and eventual neuronal death.

3. Mitochondrial Dysfunction

3.1 Characteristics

The mitochondria are the site of ATP synthesis through the electron transport chain. The transport of electrons involves movement through 4 complexes and a series of coupled redox

reactions. These reactions provide the energy to pump hydrogen ions from the mitochondrial matrix into the intermembrane space, thus creating an electrochemical gradient. This gradient has an electrical potential that used to drive the conversion of ADP to ATP [7]. Complex I is the primary gateway for electrons to enter the respiratory chain [17]. Complex I is responsible for the electron transfer of NADH to ubiquinone and this action is coupled to the movement of four protons from the mitochondrial matrix to the intermembrane space [18]. Because mitochondria action is closely linked with oxygen and hydrogen, (in the form of protons, which can be acidic) it is an important source of reactive oxygen species (ROS) [18,19]. Complex I, and to a lesser degree complex III, are the primary sites of ROS generation. They are responsible for transferring a single electron to oxygen thus creating a superoxide anion. This superoxide anion is the proximal ROS, mainly produced in the mitochondrial matrix. It is quickly converted to hydrogen peroxide (another ROS) by MnSOD. In the presence of Fe2+, hydrogen peroxide can be converted into a highly reactive hydroxyl radical [20,21]. This increased concentration of ROS and/or the cell's inability to remove it, results in oxidative damage to mtDNA, proteins, lipids, and interferes with the redox signaling pathways. Oxidative damage to the mtDNA causes mutations that further damage components of the respiratory chain leading to a continuous cycle of oxidative stress [20]. Mitochondrial DNA (mtDNA) is a double-stranded circular genome of about 16.6 kb, and it replicates independently from the cell cycle and nuclear DNA replication [7]. mtDNA encodes for 13 proteins, all of which are components for the respiratory chain complex; seven subunits are for complex I, one subunit for complex III, three subunits of complex IV, and two for ATP synthase. mtDNA codes for 22 tRNAs and two rRNAs that are used to create the proteins [22]. However, these are not all of the proteins that mitochondria require. Mitochondria require about 1500 different proteins, a majority of mitochondrial proteins are encoded by nuclear DNA, translated in the cytoplasm and imported into the import machinery [23]. Thus, there are many genes and mechanisms that can be affected by mutations and cause mitochondrial dysfunction. Typically, mutations in the mitochondrial genome are symptomatic in postmitotic cells that have a high energy demand, such as neurons [22]. mtDNA is characterized by an increased vulnerability to mutations, it contains less efficient DNA repair mechanisms and lacks protective histones; as stated before, mitochondria generate reactive oxygen species and it is speculated that mtDNA's proximity to the respiratory chain favors mtDNA damage [24]. These mutations can proliferate through clonal expansion either in daughter mitochondria or daughter somatic cells, until the mutations reach a certain threshold and respiratory deficiency occurs [25]. Dopaminergic neurons from the substantia nigra have shown an age-dependent increase in somatic mtDNA, shown through long range PCR and quantitative real time or single molecule PCR. Levels of mtDNA deletions were slightly higher in dopaminergic neurons of PD patients when compared to age-controlled models [7]. A link between mtDNA and neurodegeneration has been further substantiated by genetic mouse models. A knockout of TFAM in midbrain dopaminergic neurons caused reduced mtDNA expression, respiratory chain deficiency, and neuronal cell death, leading to progressive L-dopa-responsive impairment of motor functions [26]. α-Synuclein was the first gene that was described to be associated with familial PD, since then four other genes have conclusively been linked to autosomal recessive or dominant parkinsonism. Parkin, PINK1, DJ-1 are associated with autosomal recessive and LRRK2 with autosomal dominant [7].

3.2 Parkin

The gene encoding the E3 ubiquitin ligase parkin (PARK2) was identified as a causative gene for familial early onset parksonism [27]. Parkin's structure has been difficult to gain insight into, due to its high cysteine content. This high cysteine content (35 of 465 amino acid residues) rendering parkin prone to misfolding and preventing the native folding of soluble recombinant parkin [29]. The natively folded protein contains a ubiquitin-like domain (UBL) at the N-terminus and an RBR domain near the C-terminus, consisting of two RING finger motifs, RING1 and RING2, that flank the cysteine-rich in between RING finger domain [28,30]. The RBR domain is conserved among a specific class of proteins that have E3 ubiquitin ligase activity [7]. Parkin belongs to this RBR class of E3 ubiquitin ligase, which combines features of RING and HECT E3 ligases to attach the ubiquitin to substrates. RING ligases are used as scaffolds to facilitate the direct transfer of ubiquitin from a ubiquitin charged E2 to the substrate, while HECT ligases utilize a thioester intermediate with ubiquitin using a catalytic cysteine residue. RING and HECT are the two defined classes of ubiquitin ligases, however, it has been identified that parkin utilizes a RING/HECT hybrid mechanism [28]. The two critical regions, active cysteine in RING2 (C431) and the E2-binding site in RING1, are blocked by autoinhibition [31,32]. This autoinhibition is mediated by two structural elements: RING0 buries the catalytic C431 in RING2 by sharing a hydrophobic interface with RING2. The other structural element is a flexible linker between the IBR and RING2 at opposite sides of the molecule. This linker forms a helix that serves as the repressor element of parkin (REP). REP binds to RING1 and prevents the interaction between RING1 and E2 containing ubiquitin [28]. A well-known function of E3 ubiquitin ligases is its targeting substrate proteins for proteasomal degradation by the covalent attachment of ubiquitin [28]. Parkin serves as a neuroprotective protein. Parkin protects cells against a wide range of stressors, including mitochondrial dysfunction, excitotoxicity, endoplasmic reticulum stress, proteasome inhibition, and overexpression of α-Synuclein [30,33]. It is hypothesized that parkin mediates its activity by at least three mechanisms, promoting the removal of damaged mitochondria via mitophagy, increasing proteasomal degradation of toxic substrates, and/or modulating nondegradative ubiquitin signaling within cell death or viability pathways [28]. One of the roles that parkin has, as it relates to the mitochondria, is its prevention of mitochondrial swelling and cytochrome *c* release [34]. In addition, parkin protects mtDNA from oxidative damage and stimulates mtDNA repair [35].

3.2.1 Parkin Dysfunction

Studies revealed that pathogenic mutations in these ubiquitin ligases, especially parkin, result in the accumulation of parkin substrates. The concentrated aggregates cause neurotoxicity and the death of dopaminergic neurons [7]. A particular study analyzed mitochondrial features in tissues of parkin mutant patients, specifically measuring complex I and IV activity in isolated leukocyte mitochondria. Among patients with parkin mutations and sporadic PD patients, there was a significant decrease (about 60%) in complex I activity; however, decreases on complex IV activity was only observed in sporadic PD patients [36]. The decreased function of these complexes leads to impaired energy production in the mitochondria and metabolic stress throughout the cell. Cultured fibroblasts from parkin patients showed functional mitochondrial defects. Membrane potential decreased by 30%, complex I activity by 45%, ATP production by 58% and an increase in rotenone-induced mitochondrial fragmentation [37]. In a separate study parkin mutant fibroblasts were characterized by a 22% reduction in the mtDNA copy number

and increased vulnerability to mtDNA damages from oxidative stress [35]. Parkin has a specific role in the targeting of substrate proteins for degradation. Parkin has an intrinsic vulnerability to misfolding, this in addition, to its association with mitochondria and oxidative stress, makes it susceptible to loss-of-function. Evidence from cellular and animal models suggests that parkin mutations leads to the accumulation of neurotoxic parkin substrates can induce cellular dysfunction and degeneration of dopaminergic neurons, indicative of PD pathogenesis [28]. One example of this accumulation comes from a mouse model, where transgenic overexpression of aminoacyl-tRNA synthetase complex-interacting multifunctional protein-2 (AIMP2) leads to age-dependent selective loss of dopaminergic neurons and motor deficits [38]. Another parkin substrate, parkin-interacting substrate (PARIS), showed the same selective degeneration of dopaminergic neurons when accumulated. PARIS transcriptionally represses a peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). PGC-1α is an important mediator of mitochondrial biogenesis and controls general mitochondrial maintenance. PARIS normal action is the stimulation of mitochondrial biogenesis by serving as a coactivator for various transcription factors. In this model, PARIS accumulation from parkin loss of function interferes with mitochondrial metabolism [39].

3.3 PINK1

PTEN-induced kinase 1 (PINK1) is a 581-residue protein, containing an N-terminal mitochondrial targeting sequence, a transmembrane helix, a serine/threonine kinase domain, and a C-terminal domain with unknown function [40]. Mutations in PINK1 is one of the factors responsible for autosomal recessive PD. No crystal structure of PINK1 has been established, which makes it difficult to pinpoint the exact mechanism that it utilizes. However, it contains a conserved serine/threonine domain that resembles other kinase domains [41]. The protein shares the most structural features with the calmodulin-dependent kinase family. The kinase domain contains N- and C-terminal lobes, consisting of smaller subdomains, seen in most kinases. However, PINK1has three loop inserts in the N-terminal lobe. These loop inserts are some of the mutation sites for PD [42]. PINK1's kinase domain also contains an activation loop with two serine residues, these residues play a role in the activation of the protein kinase when phosphorylated. The activation loop also contains Arg407, which is a potential site of interaction with the phosphorylated serine and a PD mutation site [40]. One of the mechanisms that is used to regulate kinase activity is by homo-dimerization of the kinase. It was established that wildtype PINK1 exists as a dimer [72,73]. PINK1 is activated in cells upon mitochondrial membrane depolarization, but the exact mechanism is unknown. It is synthesized outside of the mitochondria and imported through the outer and inner membrane of the mitochondria for post-translational modification [43]. Mitochondrial import of PINK1 is driven by the inner membrane proton gradient, thus membrane depolarization leads to the accumulation of unprocessed PINK1 at the outer mitochondrial membrane [44]. PINK1's position at the outer mitochondrial membrane allows its kinase domain to face the cytoplasm (when unprocessed) and enable it to phosphorylate cytosolic and outer membrane substrates. PINK1 can act as a "surface receptor" linking its activity to the condition of the mitochondria and detect damaged mitochondria [40,45]. Animal studies in Drosophila indicated that PINK1 plays a role in the maintenance of mitochondria, and its role is closely linked to parkin [46,47]. As a function of its maintenance of mitochondria, PINK1 was shown to regulate HtrA2, a mitochondrial protease that plays a role in mitochondrial homeostasis and regulating mitochondrial morphology [48,49]. One of the

proteins that is phosphorylated by PINK1 is TRAP1 (TNFR-associated protein 1; heat shock protein 75). TRAP1 phosphorylation is essential for PINK1's protective activity against oxidative stress [67]. A functional link between PINK1 and HtrA2/Omi has also been established. HtrA2/Omi is a mitochondrial serine protease that is activated by apoptotic stimuli and released into the cytosol, where it interacts with inhibitor of apoptosis proteins. HtrA2/Omi is most likely phosphorylated by the p38 kinase pathway, but some direct interaction with PINK1 is needed to facilitate the transfer of the phosphate [68]. This supports the notion that PINK1 provides protection from apoptotic cell death in certain stress conditions [66]. It is difficult to look at PINK1 function without considering its interaction with parkin. This relationship was first established when looking at the remarkable phenotype similarities that PINK1 and parkin mutant flies shared. PINK1 mutants also show reduced ATP and mtDNA concentration leading to cell death. Experiments in flies showed that parkin overexpression suppressed the PINK1 mutation phenotype; however, PINK1 overexpression did not rescue parkin loss-of-function. This provides evidence that PINK1 and parkin act along the same pathway, where parkin is downstream of PINK1 [69,70,71].

3.3.1 PINK1 Dysfunction

Mutations in PINK1 are the second most common cause of the autosomal recessive, early-onset parkinsonism after parkin mutations [66]. Most of the mutations that cause disease are missense mutations at the kinase domain and interfere with its kinase activity. Similar to many other kinase proteins, PINK1 mutations cause deficiency in many different pathways and thus its effects on mitochondrial function and morphology are multifaceted. There are decreases in mitochondrial membrane potential, complex I and IV activity, ATP production, mitochondrial import and mtDNA levels, increases ROS production, and abnormal mitochondrial morphology [7]. Experiments generating Flag-tagged wild type L347P- or E417G-adenovirus to infect SH-SY5Y cells, tested to see how mutant PINK1 impairs mitochondrial respiration and ATP synthesis. Post infection results observed a statistically significant defect in O_2 consumption rate. The impaired electron transport chain also demonstrated that cells expressing these mutations had statistically significant reductions in mitochondrial ATP synthesis [73]. ATP is used as an energy source throughout the cell and as it pertains to PD, in the proteasomal activity. PD pathology is typically characterized by the presence of insoluble protein inclusions of α-Synuclein. These inclusions are often positive for polyubiquitin, a product of the ubiquitin proteasome system. The UPS is a tightly regulated pathway to target proteins by the 26S proteasome and with most proteases, it requires ATP [74]. PINK1 mutants' impairment of the ATP synthesis was found to have effects on proteasome function. L347P-PINK1 and E417G-PINK1 led to decreased degradation of a proteasome substrate. Results also showed that loss of PINK1 led to increased SDS-insoluble aggregates in both the wild type α-synuclein and the human disease form (A53T- α-synuclein) [73].

3.4 DJ-1

The DJ-1 gene encodes for a 189-amino acid protein. This protein has been associated with rare cases of early onset autosomal recessive PD [75]. DJ-1 is associated with the ThiJ/PfpI family, which has chaperone activity, and shares structural similarities with *E. coli* chaperone Hsp31 [76]. In humans, DJ-1 forms a homodimer with a six-strand parallel beta-sheet sandwich by

alpha-helical arrangements. DJ-1 belongs to the protein superfamily but has key differences that contribute to its unique mechanism. DJ-1 contains a distorted catalytic triad for protease activity and its active site is blocked by an additional C-terminal helix. The dimer that it forms also varies from other members of the superfamily [77]. The catalytic triad varies at residues E18. C106, and H126, but the variation at C106 was interesting because it appeared to form a strained main-chain conformation and was easily oxidized [78]. This information is pertinent as it pertains to DJ-1 because of its role as a redox-activated chaperone [79]. DJ-1 is expressed throughout the body and has diverse biological roles in male fertility, oncogenesis, and neuroprotection. Like parkin, DJ-1 primarily localizes in the cytosol but also exists in the nucleus and mitochondria [80]. This subcellular localization relates to signal transduction, transcriptional regulation, and during stress, mitochondrial dysfunction [77]. DJ-1 is a regulator of antioxidative gene induction, during oxidative stress, DJ-1 expression increased the transcription and enzymatic activity of glutamate cysteine ligase (GCL), the rate limiting enzyme of glutathione biosynthesis. Glutathione plays a central role in maintaining cellular redox status and protecting cells from oxidative injury. In the presence of A53T mutant α-Synuclein, DJ-1 activates the molecular chaperone Hsp70 to block α-Synuclein aggregation and toxicity [81]. DJ-1 can be SUMOylated (SUMO = small ubiquitin-like modifiers) by PIASx-alpha, a SUMO ligase, to fully activate DJ-1. SUMOylated DJ-1 influences regulation in the vital p53 pathway by repressing p53 transcriptional activity thus decreasing Bcl-2 associated X protein expression and prevent ultraviolet induced cell death [82]. DJ-1 plays an indirect role in general mitochondrial maintenance through its synergistic transcriptional activities with PGC-1α. However, DJ-1 does not directly interact with PGC-1α, rather it stimulates PGC-1α activity through pyrimidine tract-binding protein-associated splicing factor (PSF). PSF binds to PGC-1α and suppresses its transcriptional activity. DJ-1 prevents this repression by inhibiting the SUMOylation of PSF [83]. DJ-1 displays direct cytoprotective functions against mitochondrial ROS with a quenching effect. DJ-1 oxidation is needed to activate the protein and an in vitro assay showed that DJ-1 eliminated hydrogen peroxide by oxidizing itself [84]. However, DJ-1 may exert its antioxidative effects to larger degree by influencing antioxidative gene regulation. DJ-1 stabilizes Nrf2, a master regulator of antioxidant transcriptional responses, by associating with its inhibitor Keap1 and preventing ubiquitination [85].

3.4.1 DJ-1 Dysfunction

DJ-1 became associated with neurodegeneration when it was discovered as the PARK7 gene causing autosomal-recessive juvenile parkinsonism. The first described large genomic deletion that impaired the DJ-1 coding region was L166P, which causes severe destabilization of the protein. The severe L166P mutation disrupts the alpha-helix G of the structurally important C-terminal helix-kink-helix motif and functional loss of DJ-1 [86,87]. The presence of L166P DJ-1 resulted in the native isoform of DJ-1 being doubled. The mutation blocks dimerization and the monomer form are more rapidly degraded, leaving less functional DJ-1. The interaction with DJ-1 and GCL is essential in protecting the cell from oxidative stress. Mutations in DJ-1 causes a decrease in glutathione which results in oxidative stress and increased cytotoxicity. Experiments confirmed that L166P DJ-1 showed no protection against protein oxidation when compared with controls. It is also important to note that increased glutathione was specifically linked to increased survival of both primary dopamine neurons and dopamine cell lines. This impaired glutathione recruitment by DJ-1 mutation may assist the development of PD in patients with

other pathogenic factors [81]. DJ-1 mutation at Lys130 was unable to regulate androgen receptor activity, Lysine 130 seems to be SUMOylated and the modification seems to be necessary for full activity [88]. Oxidative stress by ROS rendered DJ-1 incapable of inhibiting SUMOylation on PSF and thus PGC-1α activity was reduced [83]. DJ-1 mutant cells (R89Q, D149A, and L166P) had significantly increased levels of DLP1, a mitochondrial fission protein. Changes in mitochondrial morphology was found to have an influence on mitochondrial function. In cells with DJ-1 mutant variants, there was an increase in the ROS levels and a decrease in ATP and mitochondrial membrane potential. This dysfunction was also replicated in well-differentiated neurons (rat E18 primary cortical neurons), where samples containing the mutant variants saw abnormal mitochondrial morphology and increased mitochondrial fragmentation [89].

3.5 LRRK2

The Leucine-rich repeat kinase 2 (LRRK2) gene encodes a large multidomain protein composed of 2527 amino acids. The functional domains that the protein consist of are a kinase domain that is a part of the mixed lineage kinase family, a Roc domain with similarity to the Ras/GTPase superfamily, a COR domain, a WD40-repeat domain, and leucine rich repeats [90]. Purified LRRK2 has been shown to be dimeric, allowing for self-interaction. The two monomers lie in a head-to-tail orientation with large sections of the molecules interacting to stabilize the dimer [91,93]. It has also been argued that either the Roc domain or the COR domain provide the primary interaction motif [92]. The N- and C- terminal regions of LRRK2 contribute to the interactions around the central portion of the molecule that binds GTP. Further research is needed to see what contributes to LRRK2's self-interaction. What has been established is that in the dimerization process, one of the posttranslational modifications that occurs is autophosphorylation. Autophosphorylation has been observed to activate LRRK2 serine/threonine kinase activity [93]. However, the kinase domain in the structure provided by [91] shows that it is positioned towards the solvent, meaning that it may not be strictly a *cis*-acting event [92]. LRRK2 is known to phosphorylate a variety of proteins, one potential substrate is the Rab family of small GTPases. In particular, Rab10, which regulates intracellular vesicular transport. This protein family functions as a molecular switch in the tethering, docking, fusion, and motion of intracellular membranes [94]. Rab GTPase refers to a family of proteins and while Rab10 has this set of functions, Rab32 was shown to interact with mitochondria. LRRK2 can bind to the outer mitochondrial membrane and about 10% of overexpressed LRRK2 was associated with the outer mitochondrial membrane [7]. Rab32 also has roles in trafficking, they help assemble complexes used for fission and fusion. Rab32 localizes to the mitochondria and contributes to mitochondrial fission [95]. LRRK2 morphology regulation is not limited to the mitochondria, there is also evidence that LRRK2 is involved in neuronal maintenance [99].

3.5.1 LRRK2 Dysfunction

Pathogenic PD LRRK2 mutations predominantly map to the kinase and the Roc-COR (GTPase) domains. Other PD risk factors in coding mutations are found in the WD40 domain. The mutations in the catalytic Roc-COR and kinase domains of LRRK2 are a common cause of familial PD. LRRK2 missense mutations seem to be the most commonly known genetic cause of PD. The mutations seem to induce an increase in LRRK2 kinase activity and a decrease in the GTPase activity. Cell and animal models support the model that LRRK2 mutations affect

vesicular trafficking, autophagy, protein synthesis, and cytoskeletal function. It has been established that G2019S mutation increases LRRK2 kinase activity (in autophosphorylation and phosphorylation of substrates) and that toxicity is dependent on this increased activity, suggesting a gain of function mechanism [7,96]. Studies with mutant LRRK2 at the GTPase domain P-loop reveal that a function Roc GTPase domain is necessary for kinase activity, suggesting that GTP binding may regulate kinase activity [97]. In a C. elegans model dopaminergic neurons were particularly sensitive to mutant LRRK2. Specifically, the G2109S variant increased the vulnerability of dopaminergic neurons compared to wild type [98]. Overexpression of G2019S led to a dramatic reduction in neurite length and branching, as well as overall neurite growth. At the later time points the cells expressing the mutants had decreased neuron survival [99]. This change in morphology may have to do with LRRK2's physical and function interaction with protein translation machinery. PD-linked LRRK2 mutations altered protein synthesis and are toxic [96]. Pathogenic LRRK2 influences mRNA translation by negatively regulating miRNA activity [100]. LRRK2 dysfunction also impedes vesicle trafficking, leading to neuronal dysfunction. G2019S was reported to over phosphorylate endophilin A, which is required for the endocytosis of synaptic vesicles. Hyper phosphorylating endo A resulted in impaired vesicle recycling [101]. Mutant LRRK2 associates with autophagic vesicles and multivesicular bodies in the human brain, this causes a disruption of autophagy in midbrain dopamine neurons leading to eventual neuronal death and locomotor deficits. LRRK2 is typically degraded using the UPS or CMA, but the mutant variant impedes the CMA and inhibits the uptake of other substrates. This is potentially mechanism of toxicity. Like α-Synuclein, LRRK2 mutations exist in LBs [96]. This link between α-Synuclein and LRRK2 in neuropathology is supported by evidence that overexpressed LRRK2 promotes aggregation and toxicity of α-Synuclein in A53T mice, while LRRK2 KO blocked toxicity [102]. The stimulatory action of pathogenic LRRK2 on mRNA translation could be an explanation for increased α-Synuclein or pathogenic LRRK2 may promote oligomerization of α-Synuclein on the lysosomal surface and impair its uptake through chaperone-mediated autophagy and subsequent degradation [103,104].

4. ER Stress Signaling

One of the main functions of the ER is to initiate protein folding in the secretory pathway. The mechanism is complex and dynamic, consisting of a network of protein chaperones, foldases, and co-factors at the ER lumen to catalyze the folding and maturation of proteins [112]. The ER plays a vital role in cellular protein quality control by extracting and degrading proteins that are not correctly folded or assembled into native complexes, this process is known as ER-associated degradation (ERAD), checks that only properly folded and assembled proteins are transported to the final destination [113]. The accumulation of diffusible oligomer or large aggregates of misfolded proteins is a key characteristic of many neurodegenerative diseases. Fluctuations in protein homeostasis leading to these accumulations is a major factor underlying the pathology of brain disease-linked proteins. This suggests that perturbation in ER function is a common event in PD. Decreasing protein aggregation or reducing the effects of downstream signaling are thought to be relevant mechanisms for therapy [105]. Misfolded proteins exert stress onto the endoplasmic reticulum (ER) which is mitigated by the actions of the unfolded protein response (UPR). The ER stress response is aimed to protect the cells against toxic build-up of misfolded proteins. Several conditions can incite the UPR, such as, hypoxia, glucose deprivation, oxidative

stress, viral infection, high fat or cholesterol, and mutations in specific proteins The UPR is a complex signal transduction pathway that modulates the expression of proteins. These proteins are involved in a wide range of functions including folding, quality control, protein entry into the ER, and organelle biogenesis. The UPR regulates transcription and translation of genes that mediate these functions to re-establish homeostasis and ER function. The ER is associated with UPR through stress receptors on the ER membrane. The receptors transduce information about the protein folding status in the ER lumen to the nucleus and cytosol by regulating expression of transcription factors and other proximal signaling substrates [106,113]. There are three main types of ER resident transmembrane signaling proteins that operate as stress sensors that activate UPR signaling responses. These sensors include double stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF-6), and inositol requiring kinase 1 (IRE-1) [105]. UPR is initiated by the binding of the ER chaperone BiP/GRP78 to misfolded proteins. Under normal conditions, BiP/GRP78 forms a complex with three other key proteins at the ER membrane, PERK, transcription factor ATF-6, and endoribonuclease IRE-1. By binding BiP/GRP78 to the misfolded protein, PERK, ATF-6, and IRE-1 are released from BiP/GRP78 and activated. PERK homodimerizes and autophosphorylates to phosphorylate eukaryotic initiation factor 2-alpha (peIF2alpha). The role of peIF2alpha is to decrease the frequency of mRNA translation initiation and reduce the overall protein load on the ER; however, it also increases the translation of specific mRNAs, such as activating transcription factor 4 (ATF4). ATF4 activates transcription of genes involved in amino acid metabolism and transport, redox reactions, and ER stress induced apoptosis [107]. IRE-1 is a serine/threonine protein kinase and endoribonuclease that regulates the unconventional splicing of the mRNA for transcription factor X-Box Binding protein-1 (XBP-1). XBP-1 translocates to the nucleus and controls the expression of UPR-related genes that function in protein quality control, folding, the ERAD system, and ER and GA biogenesis. IRE-1 has other functions in cell signaling, initiating the activation of alarm pathways mediated by Apoptosis Signal-regulating Kinase 1 (ASK1) and c-Jun-N terminal kinase (JNK) pathway. This pathway is the result of prolonged ER stress [105].

4.1 ER Stress Defense Mechanisms

Misfolded proteins play a central role in neuronal cell loss in PD. These proteins may inhibit synaptic function, interfere with signal transduction pathways, cause dysfunction of protein degradation through the ubiquitin proteasome system (UPS), and cause eventual cell death [108]. An important effect of the UPR is the up regulation of ERAD components. ERAD maintains ER homeostasis by eliminating misfolded proteins, protein subunits that fail to assemble into their native complexes, and proteins whose levels must be acutely regulated in response to metabolic needs [113]. ERAD can be broken down into three interdependent components: 1) recognition of the protein target and its association with the appropriate luminal chaperones, 2) association with ERAD ubiquitylation machinery, and 3) retrotranslocation into the cytosol for degradation by proteasomes [114]. Mammalian ERAD is complex and contains many redundancies, this causes different ERAD pathways to overlap; however, the overall mechanism involves ubiquitin ligases that attach polyubiquitin chains to proteins as signal for degradation [108]. UPR is initially activated in PD pathogenesis for neuroprotection, to remove the toxic unfolded proteins; however, the prolonged ER stress and UPR activation overwhelms the protective machinery, leading to cell death [108]. CEBP homologous protein (CHOP) is a proapoptotic transcription

factor that is induced through the PERK-eIFalpha pathway. CHOP is activation is induced by peIFalpha recruitment of ATF4, CHOP affects the expression of genes favoring apoptosis in response to ER stress [111]. Autophagy has been proposed as a protective mechanism to overcome neurodegeneration and is modulated by ER stress. Autophagy is induced by many of the same conditions as ER stress and is a catabolic process that mediates the degradation of proteins and dysfunctional organelles. It can be divided into three main types: 1) macroautophagy, 2) microautophagy, and 3) chaperone-mediated autophagy (CMA). While autophagy is considered to be non-selective in the degradation of bulk cytoplasmic components, in some cases it displays substrate specificity. Selective degradation of mitochondria (mitophagy) is particularly important due to its increased association with PD pathogenesis through the PINK1-parkin pathway. α-Synuclein in its native form is degraded using CMA, the protein contains a 15 amino acid sequence that consists of overlapping variations of the KRERQ CMA recognition motif. The chaperone HSC70 recognizes the motif and binds to α-Synuclein. α-Synuclein then binds to the lysosomal-associated membrane protein type 2A (LAMP-2A) at the lysosomal membrane. The CMA receptor along with the chaperone transports α-Synuclein into the lysosome for degradation [117].

4.2 ER Stress Factors

Activation of UPR was investigated with immunohistochemistry for pPERK and peIF2alpha. Results found that both proteins were found in the neurons of PD patients but not controls. A connection between pPERK and α-Synuclein was also found, in PD cases there was a strong colocalization in neuromelanin containing neurons in the substantia nigra. Interestingly, pPERK was only observed in neurons containing α-Synuclein, but not all neurons containing α-Synuclein had pPERK present. This suggests a functional connection between α-Synuclein pathology and the occurrence of ER stress [108]. α-Synuclein can induce ER stress with a variety of mechanisms. Overexpression of A53T α-Synuclein leads to the formation of cytoplasmic aggregates and disrupts the UPS [109,110]. The cell's impaired ability to degrade proteins leads to a buildup of misfolded proteins in the ER and making the cell more vulnerable to ER stress [108]. Additional experiments have demonstrated that the earliest defect following α-Synuclein expression is a block in ER to Golgi vesicular trafficking. The inhibition of ER to Golgi trafficking induces ER stress through the accumulation of cargo vesicles, triggering the buildup of immature proteins at the ER [115,116]. The various mutant forms of α-Synuclein vary in the degree to which they inhibit the lysosomal/CMA degradation pathway and thus have different levels of toxicity [117]. A53T and A50P mutants bind tightly to the LAMP-2A receptor but cannot be transported across the lysosomal membrane [118]. These mutants act as receptor inhibitors, preventing other CMA substrates from binding, resulting in a complete block of CMA and a higher degree of toxicity. Blocking CMA activity with mutant α-Synuclein not only results in the direct buildup of toxicity in the neuron through the formation of aggregates, but it also prevents the protective activity of myocyte enhancer factor 2D (MEF2D). MEF2D is a transcription factor in neuronal survival. CMA degradation regulates MEF2D activity and when CMA is inhibited, an inactive form of the protein is present in the cytosol but not the nucleus. This inactive form cannot bind DNA and α-Synuclein prevents Hsc70 from binding to MEF2D. α-Synuclein not only promotes neuronal death by forming aggregates, but it also promotes cell death by inhibiting cell survival proteins [117,119]. As was mentioned before, parkin is involved in both the ubiquitin-proteasome pathway and cell death protection. Parkin dysfunction not only

exerts the pressure onto cells through its own inaction, but it causes a higher demand from the ER stress mechanisms like UPS. This leads to prolonged ER stress and cell death that follows it. This is backed up by the finding that Pael-R, a parkin substrate, triggers ER stress *in vivo* and *in vitro*. Furthermore, the loss of DJ-1 induces ER stress and proteasome inhibition [112,120,121].

5. Conclusion

Although Parkinson's disease is the second most prevalent neurodegenerative disease behind Alzheimer's disease, prevention and treatment of PD remains uncompleted. PD is characterized by the accumulation of misfolded proteins and the death of dopaminergic neurons; however, cell death is the end result of numerous interconnected pathways. Considering only one pathway for a potential therapy gives a fragmented image of the solution. This is the current predicament towards finding a solution. Recent research has identified genes that are associated with PD pathogenesis, specifically their function and more importantly dysfunction. These PD-associated genes (parkin, PINK1, DJ-1, LRRK2) have provided new insights into the biochemical pathways that lead to disease progression. While these genes are not encoded by mtDNA, their function is closely related to mitochondrial homeostasis. Mutations in any one of these genes produces either a dysfunctional protein or a misfolded protein. Due to the nature of the mitochondrial environment and the oxidative respiratory chain, DNA is prone to mutations. Dysfunctional proteins damage the mitochondria causing complications. Complex I impairment generates ROS, causing a positive feedback loop that propagates oxidative stress. Manifold facets of neuron biology are affected in PD, the primary effector of dysfunction could be any of these genes and much more work is needed to figure out how the pieces of the puzzle fit together, and, in this analogy, even mitochondrial dysfunction is only a part of the puzzle. The results of mitochondrial-associated protein misfolding leads to a cascade of dysfunction throughout the cell.

α-Synuclein has also been recently explored as a therapeutic target. Lewy bodies are a cardinal feature of the PD and α-Synuclein aggregates have been found present in high concentrations within the inclusions. LBs are the accumulation of misfolded and aggregated proteins, especially the ubiquitin protein. This protein is widely used across cells as tag for degradation. The increased concentration of misfolded protein generates ER stress and generally, the cell would utilize the Unfolded Protein Response to mediate protein dysfunction through the Ubiquitin Proteasome System. The accumulation of ubiquitin and misfolded proteins indicates dysfunction within the cells' normal degradation system. This problem is compounded when mutations in genes that cause mitochondria dysfunction, such as parkin, a ubiquitin ligase, are introduced. The inability of parkin to tag its substrates for degradation leads to a buildup of proteins causing further ER stress. The accumulation of these stressors induces chronic ER stress, resulting in dopaminergic neuron death.

5.1 Future Directions

Recent research has targeted α-Synuclein therapy, its dysfunction is a factor in many of the pathways towards PD. Several potential solutions have been suggested to mitigate the influence of α-Synuclein. One is to find a molecule that stabilizes the intrinsically disordered conformation of α-Synuclein or block its aggregation potential to disaggregate the aggregates back down to the monomeric state. Alternatively, look for chemical compounds that can either

clear toxic misfolded proteins or protect neurons from the effect of toxic proteins [122]. Finally, and somewhat counterintuitively is the rapid promotion of protein aggregates. Recent evidence has shown that the rapid formation of large inclusions might have a neuroprotective role, and the molecules that stimulate the formation of these large protein aggregates could be used as a therapeutic means for affected neurons [123].

Considering the important role of mitochondria in energy metabolism, calcium homeostasis, cellular quality control pathways and cell death regulation, it is vital to consider that mitochondrial dysfunction highly contributes to dopaminergic neuron vulnerability [7]. These neurons with high mitochondrial dysfunction exhibit a high oxidative burden, thus antioxidants should be considered as a treatment target. Another interesting area of research is the role of calcium in mitochondria homeostasis, and mitochondria's role in regulating calcium homeostasis. An imbalance in calcium buffering seems to be causally related to the selective degeneration of dopaminergic neurons [124].

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