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Review

The Role of Oxidative Stress in Parkinson's Disease

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Abstract. Oxidative stress plays an important role in the degeneration of dopaminergic neurons in Parkinson's disease (PD). Disruptions in the physiologic maintenance of the redox potential in neurons interfere with several biological processes, ultimately leading to cell death. Evidence has been developed for oxidative and nitrative damage to key cellular components in the PD substantia nigra. A number of sources and mechanisms for the generation of reactive oxygen species (ROS) are recognized including the metabolism of dopamine itself, mitochondrial dysfunction, iron, neuroinflammatory cells, calcium, and aging. PD causing gene products including DJ-1, PINK1, parkin, alpha-synuclein and LRRK2 also impact in complex ways mitochondrial function leading to exacerbation of ROS generation and susceptibility to oxidative stress. Additionally, cellular homeostatic processes including the ubiquitin-proteasome system and mitophagy are impacted by oxidative stress. It is apparent that the interplay between these various mechanisms contributes to neurodegeneration in PD as a feed forward scenario where primary insults lead to oxidative stress, which damages key cellular pathogenetic proteins that in turn cause more ROS production. Animal models of PD have yielded some insights into the molecular pathways of neuronal degeneration and highlighted previously unknown mechanisms by which oxidative stress contributes to PD. However, therapeutic attempts to target the general state of oxidative stress in clinical trials have failed to demonstrate an impact on disease progression. Recent knowledge gained about the specific mechanisms related to PD gene products that modulate ROS production and the response of neurons to stress may provide targeted new approaches towards neuroprotection.

Keywords: Neurodegeneration, neuroprotection, neuroinflammation, reactive oxygen species, dopamine, mitochondria

INTRODUCTION

The mechanisms responsible for neuronal degeneration in Parkinson's disease (PD) are complex and remain to be fully elucidated. Among the various neuronal types that degenerate in this disease, there is little doubt that the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) is responsible for the characteristic motor symptoms and drives symptomatic therapies [1, 2]. Accumulating evidence indicates that oxidative damage and mitochondrial dysfunction contribute to the cascade of events leading to degeneration of these dopaminergic neurons [3–7]. This is supported by postmortem brain analyses showing increased levels of 4-hydroxyl-2-nonenal (HNE), a by-product of lipid peroxidation [8, 9], carbonyl modifications of soluble proteins [10], and DNA and RNA oxidation products 8-hydroxy-deoxyguanosine and 8-hydroxy-guanosine [11, 12]. The link between oxidative stress and dopaminergic neuronal degeneration is further supported by modeling the motor aspects of PD in animals with toxins that cause oxidative stress including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, 1,1'-dimethyl-4,4'-bipyridinium dichloride (paraquat), and 6-hydroxydopamine (6-OHDA) [13–17]. In addition to PD, several other neurodegenerative disorders including Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis are associated with oxidative stress as well, despite having distinct

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pathological and clinical features [18] suggesting that oxidative stress is a common mechanism contributing to neuronal degeneration [19, 20].

Here we review the role of oxidative stress in the pathogenesis of PD, its effects on cellular homeostasis, the biochemical and molecular events that mediate or regulate neuronal vulnerability, and the role of PD-related gene products in modulating the cellular responses to oxidative stress in the course of neurodegeneration.

THE BIOCHEMISTRY OF OXIDATIVE STRESS

Oxidative stress defines a disequilibrium between the levels of reactive oxygen species (ROS) produced and the ability of a biological system to detoxify the reactive intermediates, creating a perilous state contributing to cellular damage.

ROS can be generated through several pathways such as direct interactions between redox-active metals and oxygen species via reactions including the Fenton and Haber-Weiss reactions, or by indirect pathways involving the activation of enzymes such as nitric oxide synthase (NOS) or NADPH oxidases. As a general principle, the chemical origin of the majority of free radicals requires the activation of molecular oxygen [21]. Examples of ROS include the superoxide anion radical (O_2^{2-}), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2). Superoxide anion, which is produced mainly by mitochondrial complexes I and III of the electron transport chain, is highly reactive and can easily cross the inner mitochondrial membrane, where it can be reduced to H_2O_2. Besides being produced by mitochondria, H_2O_2 can also be generated by peroxisomes [22]. As peroxisomes contain catalase, H_2O_2 is converted to water, preventing its accumulation. However, when peroxisomes are damaged and their enzymes down-regulated, H_2O_2 is released to the cytosol where it contributes to oxidative stress. In the presence of reduced metals such as ferrous iron (Fe^{2+}), H_2O_2 can be converted by the Fenton reaction into the highly reactive hydroxyl radical, the most harmful of all ROS [23].

Besides ROS, evidence also exists for the involvement of reactive nitrogen species (RNS) in mediating nitrosative stress [24]. RNS are generated by the quick reaction of superoxide with nitric oxide (NO), which results in the production of large amounts of peroxynitrite ($ONOO.^{-}$) [25, 26]. NO is produced by NO synthase (NOS) [27], which has three isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS) identified in neurons, and inducible NOS (iNOS) identified in glial cells [27–29]. NO is present within cells and in the extracellular space surrounding dopaminergic neurons produced by either nNOS or iNOS [30]. Additionally, with gliosis, activated glial cells that express iNOS may contribute to increased NO levels [31, 32]. NO inhibits several enzymes including complexes I and IV of the mitochondrial electron transport chain, leading to ROS generation. It also reacts with proteins to form S-nitrosothiols thus altering their function, and with lipids causing their lipid peroxidation [25]. Peroxynitrite, which is oxidatively a more active molecule and a more potent oxidizing agent than NO, can induce DNA fragmentation and lipid peroxidation [25, 26]. Peroxynitrite also induces a dose-dependent impairment in dopamine synthesis independent of dopamine oxidation or cell death [26]. Exposure of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, to peroxynitrite results in nitration of tyrosine residues and modification of cysteines leading to decreased catalytic activity [33, 34]. The role of NO in PD is supported by postmortem brain tissue analyses showing increased expression of iNOS and nNOS in basal ganglia structures using in situ hybridization and immunohistochemical studies [35, 36]. Experimentally in the MPTP model, the gliosis in the SN is associated with significant up-regulation of iNOS [37], while inhibition of nNOS protects against MPTP toxicity [38, 39]. Together, these observations suggest that NO and its metabolite peroxynitrite may play a role in PD.

All organisms have developed adaptive responses to oxidative stress that result in increased production of defensive enzymes, molecular chaperones and antioxidant molecules [40]. Under physiologic conditions, ROS are involved in signaling events mediated by thiol residues in proteins that have the potential to regulate transcription [41]. On the other hand, under conditions of oxidative stress, free radical-mediated oxidative damage occurs at various sites within the cell such as peroxidation of cellular membrane lipids resulting in the generation of toxic products including HNE and malondialdehyde [9], protein oxidation demonstrated by cross-linking and fragmentation as well as carbonyl group formation [10, 42], and DNA and RNA oxidation [11].

ROS PRODUCTION IN THE PD BRAIN

The extensive production of ROS in the brain may provide an explanation for the magnitude of the role that these reactive molecules play in PD. The brain consumes about 20% of the oxygen supply of the body,

and a significant portion of that oxygen is converted to ROS [43]. ROS can be generated in the brain from several sources, both in neurons and glia, with the electron transport chain being the major contributor at the mitochondrial level [44, 45]. Other ROS sources include monoamine oxidase (MAO), NADPH oxidase (NOX) and other flavo-enzymes along with NO, which is abundant in the brain due to the presence of NOS [43]. Considerable experimental evidence suggests that a significant contributor to dopaminergic neuronal loss in the PD brain is ROS, which result from dopamine metabolism, low glutathione (GSH), and high levels of iron and calcium in the SNpc [6]. Additionally, the brain contains high concentrations of polyunsaturated fatty acids, which under oxidative stress conditions result in lipid peroxidation and the generation of toxic products [46].

Dopamine

Dopamine is an unstable molecule that undergoes auto-oxidation to form dopamine quinones and free radicals. This reaction is catalyzed by metals, oxygen or enzymes such as tyrosinase [47]. Other enzymes, such as MAO and catechol O-methyl transferase (COMT) are also involved in dopamine metabolism. MAO-A and MAO-B, located in the outer mitochondrial membrane, degrade excess dopamine in the cytosol by catalyzing its oxidative deamination [48].

Under normal conditions, dopamine levels are regulated through oxidative metabolism by MAO-A, which is mostly localized in catecholaminergic neurons [49]. However, with neuronal degeneration that occurs in PD and aging, MAO-B located in glial cells increases and becomes the predominant enzyme to metabolize dopamine [50–52], which is taken up by astrocytes via sodium-dependent and independent mechanisms [53–55]. The products of MAO-B mediated metabolism of dopamine are 3,4-dihydroxyphenyl-acetaldehyde, an ammonium molecule and H_2O_2. Hydrogen peroxide is highly membrane permeable entering into neighboring dopaminergic neurons where it can react with Fe^{2+} to form hydroxyl radical [56, 57]. In support of the role of elevated MAO-B expression in neurodegeneration, inducing this enzyme in the astrocytes of adult transgenic mice results in selective and progressive loss of nigral dopaminergic neurons [58].

The products of dopamine oxidation, dopamine quinones, can also contribute to neurodegeneration [59]. Dopamine quinones can cyclize to form aminochrome, which is highly reactive and leads to the generation of superoxide and depletion of cellular NADPH. Aminochrome can form adducts with proteins such as alpha-synuclein [60] and is the precursor of neuromelanin, a brain pigment that may contribute to neurodegeneration by triggering neuroinflammation [61]. Postmortem brain analysis has shown significant increase in cysteinyl adducts of dopamine in PD substantia nigra suggesting accelerated oxidation, although this finding maybe related to L-dopa therapy [62].

The transport and storage of dopamine can also contribute to increased ROS production and cellular dysfunction. Normally, dopamine is sequestered in storage vesicles through an active transport process that requires vesicular monoamine transporter 2 (VMAT2) in preparation for the release of the transmitter after depolarization. Thus, VMAT2 keeps cytoplasmic dopamine levels under control preventing ROS generation. As a result, over-expression of VMAT2 confers protection against MPTP toxicity, while dopaminergic neurons with genetic or pharmacological blockade of VMAT2 are more susceptible to toxic insults [63]. In addition, the reuptake of synaptically released dopamine into nigrostriatal terminals requires dopamine transporters (DAT). Perturbations in this step again impact the levels of cytoplasmic free dopamine that is susceptible to be oxidized [64, 65]. As discussed further below, some gene products linked to inherited forms of PD are associated with enhanced dopamine reuptake and impaired storage suggesting that such mechanisms can contribute to the vulnerability of nigral neurons to oxidative stress.

Neuromelanin

Neuromelanin is a polymer pigment synthesized in catecholaminergic neurons in the brain that contains catecholamine-based compounds such as oxidized dopamine, dopamine metabolites as well as proteins and lipids [66, 67]. Although its biological role is not completely understood, several hypotheses have been proposed trying to elucidate its significance to PD. One of these hypotheses suggests that neuromelanin increases cellular vulnerability, purporting a correlation between the proportion of this pigment in different brain regions and neuronal loss [68, 69]. However, this hypothesis cannot be supported since the amount of neuromelanin within individual dopaminergic neurons in the SNpc is similar to that in the ventral tegmental area (VTA), which is relatively resistant to neuronal loss in PD [70–72]. An alternative hypothesis has

been proposed based on changes in the pigment itself rather than the amount, including increased density, modifications in its structure and decreased lipid content [70, 73]. Neuromelanin may also play a role in PD through its interaction with metals since it is an intracellular store for iron [74]. Analysis of neuromelanin in the SN of PD patients has shown an early accumulation and overload of iron, which can potentially result in increased oxidative stress [75–77]. The interaction of neuromelanin with alpha-synuclein has also been suggested as a mechanism for this pigment to modulate neuronal vulnerability. alpha-Synuclein is over-expressed in individual melanized neurons [78–80], and its aggregates redistribute to neuromelanin in the SN early in PD but not in healthy controls [70]. Finally, neuromelanin that leaks from degenerating neurons may contribute to the neurodegenerative process by activating microglia [81].

Glutathione

Analysis of postmortem brain tissue from PD patients shows decreased amount of glutathione (GSH) relative to glutathione disulfide (GSSG) (GSH:GSSG ratio) in the SN compared to controls [82–86]. GSH is a tripeptide consisting of glutamate, cysteine and glycine, with the reactive thiol group of its cysteine residue serving as an effective antioxidant. GSH is synthesized in the cytoplasm but has to be transported to the mitochondria, where it functions as an antioxidant molecule [87, 88]. As oxidative stress induces apoptosis, the mitochondrial status of GSH has become recognized as an important marker in this event. There are also consistent observations that impaired complex I activity leads to increased ROS production and subsequent decrease in GSH levels. This decrease in GSH levels can result from decreased synthesis due to inhibition of glutathione reductase, or from increased levels of glutathione disulfide (GSSG) and altered GSH:GSSG ratio [89]. Conversely, depletion of GSH in the SN results in selective decrease of mitochondrial complex I activity likely via thiol oxidation of critical residues leading to marked reduction in overall mitochondrial function [90–93].

GSH levels are finely regulated in healthy neurons, and alterations from the physiological basal levels can induce cell death. Down-regulation of GSH synthesis in the rat brain has been shown to result in progressive degeneration of nigral dopaminergic neurons. Curiously, over-production of GSH in this model was also associated with cell death [94]. The mechanism of the latter finding remains unclear. In addition, diminished GSH content in SH-SY5Y cells results in inactivation of glutaredoxin 1 (Grx) and exacerbates the sensitivity of cells to L-DOPA-induced apoptosis [95]. Grx1 is involved in glutathionylation, a process characterized by the reversible formation of mixed disulfides between protein thiols and GSH [96]. Under conditions of moderate oxidative stress, proteins can be S-glutathionylated, and this process may protect the cell by preventing the irreversible oxidation of cysteine to cysteine sulfinic and sulfonic acid [97]. In fact, knock-down of Grx in SH-SY5Y cells results in increased apoptosis, supporting the notion that disrupting the regulation of protein glutathionylation may sensitize dopaminergic neurons to apoptosis [95, 98]. Additionally, consistent with the hypothesis of altered thiol-disulfide homeostasis in stressed dopaminergic neurons, mouse brains lesioned with MPTP exhibit decreased activity of isocitrate dehydrogenease (IDH) [97]. This enzyme catalyzes the oxidative decarboxylation of isocitrate to alpha-ketoglutarate, which requires either NAD^+ or $NADP^+$ with the consequent production of NADH and NADPH, respectively. NADPH is an essential reducing equivalent for the regeneration of GSH by glutathione reductase and for the activity of the NADPH-dependent thioredoxin system, both playing important roles in protecting cells from oxidative damage [99, 100]. Thus, IDH contributes to the supply of NADPH needed for GSH production against oxidative damage [101], and the activity of IDH can be important for the regulation of cell survival. Therefore, oxidant-induced deactivation of this enzyme can play a role in PD [43].

Iron

Postmortem brain tissue from PD patients has higher levels of iron in the SN compared to controls [102–105]. The possible association of oxidative/nitrosative iron dysregulation in the neurodegenerative process of PD is also supported by the presence of nitrosylated iron regulated protein 2 (IRP) in Lewy bodies in the SN [106, 107]. Iron is an important element for almost all cell types, including brain cells. It is an essential cofactor for proteins involved in the normal function of neuronal tissues, such as the non-heme iron enzyme TH required for the synthesis of catecholamine neurotransmitters [108]. Iron ions can generate ROS since ferric iron (Fe^{3+}) and ferrous iron (Fe^{2+}) can react with superoxide and hydrogen peroxide, respectively, in a chain reaction generating the highly reactive hydroxyl free radical,

which together with dopamine oxidation can trigger neurotoxicity [109, 110].

In normal brains, iron displays a heterogenous pattern of regional distribution with greater abundance in the globus pallidus, putamen and SN of the basal ganglia [111]. However, with aging and degenerative processes such as PD, there is an abnormal, progressive deposition of iron and increased free iron concentration in the SNpc [102]. Although several studies confirm this increase, the stage of the disease at which iron changes occur is controversial. One aspect of the debate is whether elevated iron levels represent a cause or the result of neuronal destruction [102, 110]. In the early presymptomatic stage of PD, no significant increase in iron content in the SN is detected, as opposed to symptomatic PD patients. This supports the notion that elevation of iron content may be a secondary event in the neuronal degeneration. An alternative explanation is that elevated iron content in symptomatic disease may accelerate and worsen the neuronal degeneration via oxidative stress-mediated pathways [112]. On the other hand, iron levels can be enhanced by oxidative stress through several pathways including increased release of iron from ferritin by superoxide anion, from heme proteins such as hemoglobin and cytochrome c by peroxidase, and from iron-sulfur proteins by peroxynitrite [91, 113, 114]. Experimentally, iron accumulation can occur as an early event in dopaminergic neuronal loss since exposure of neonatal mice to iron results in Parkinson-like neurodegeneration with age. These animals exhibit striatal dopamine depletion by 12 months of age, develop progressive nigral neuronal loss by 24 months of age, and become more vulnerable to toxic injury [115]. Additionally, feeding a high iron diet to one month old weanling mice or stereotaxic infusion of iron into the SNpc of rats results in significant increase in iron levels in the striatum, associated with decreased levels of total glutathione (GSH and GSSG) and increased levels of hyroxyl radical [116]. On the other hand, intramuscular injection of the iron chelator desferrioxamine significantly lowers iron levels in the brain and has a neuroprotective effect against iron and MPTP in mice [117]. These results collectively suggest that increases in midbrain iron levels can be a contributor to the neurodegeneration associated with PD [116, 117].

Calcium

The regulation of intracellular Ca^{2+} is a metabolically expensive process that requires the actions of ATP-dependent pumps [118] and, therefore, results in increased mitochondrial activity and concomitant increased ROS generation. Studies with primary mesencephalic dopaminergic neurons in culture have shown that the opening of L-type Ca^{2+} channels leads to increased mitochondrial oxidant stress in dendrites [119]. In addition, the formation of alpha-synuclein aggregates exacerbates this effect in perinuclear and dendritic compartments [119].

Differences in the regulation of Ca^{2+} homeostasis may explain the greater susceptibility of dopaminergic neurons in the SNpc compared with their counterparts in other brain regions. In contrast to nigral neurons, VTA dopaminergic neurons, which are relatively spared in PD [71, 72], have much lower density of the L-type calcium channel $Ca_v1.3$ [120], do not manifest Ca^{2+} oscillations, and express high levels of the Ca^{2+}-buffering protein calbindin [71]. Instead VTA neurons use HCN/Na^+ channels for pacemaking. Thus, cytoplasmic dopamine levels are higher in SN neurons than in VTA dopaminergic neurons [121]. These findings suggest that the differences in calcium signaling could contribute to increased susceptibility of SN neurons and consequently cell death [121].

Lipids

The brain has high concentrations of polyunsaturated fatty acids, such as docosahexaenoic acid and arachidonic acid, compared with other organs. As these fatty acids are highly unsaturated, oxidative stress makes them susceptible to lipid peroxidation, which is one of the major outcomes of free radical-mediated injury [122]. Lipid peroxidation results in structural damage of membranes, compromising their integrity and consequently cell viability. As some of the lipid peroxidation products are chemically reactive, they are believed to be the major effectors of tissue damage [123]. In fact, lipid peroxidation can perpetuate in the presence of free reactive iron, since iron can react with lipid hydroperoxides to generate alkoxyl radical, which in turn can react with polyunsaturated fatty acids, the substrate for lipid peroxidation [124].

4-Hydroxyl-2-nonenal (HNE) is a highly reactive lipophilic alpha, beta-alkenal, which can form stable adducts with thiol or amine groups on proteins. HNE can also activate members of the caspase family and cause DNA fragmentation, leading to apoptosis [125]. Additionally, HNE decreases GSH levels due to its rapid consumption via GSH peroxidase and the high reactivity of HNE with sulfhydryl groups [126].

Thus, the high propensity of polyunsaturated fatty acids for peroxidation under oxidative stress conditions can result in neuronal damage and contribute to PD progression. In support of this possibility, levels of lipid peroxidation products including HNE and malondialdehyde are increased in the SN of PD patients, while polyunsaturated fatty acids are decreased [124]. Elevated HNE levels are also detected in the cerebrospinal fluid of these patients [9, 127].

MITOCHONDRIAL DYSFUNCTION

Although substantial evidence points to the presence of oxidative stress in PD, it is not entirely clear whether accumulation of ROS is a primary event or a consequence of other cellular dysfunctions. Since mitochondria play a dual function as source and target of ROS, compelling evidence suggests that mitochondrial dysregulation plays a critical role in the pathogenesis of PD. Mitochondria are dynamic organelles with many functions. Besides their role in energy generation, they are closely involved in calcium homeostasis, stress response and cell death pathways. Therefore, impairment of mitochondrial function leads to cellular damage and is related to neurodegeneration [4].

The electron transport chain is the major source of ROS, since during reduction of oxygen to water a small percentage of superoxide anion is produced [128–130]. The process of electron transfer creates a proton gradient across the inner mitochondrial membrane that drives the synthesis of ATP through ATP synthase (complex V). Complexes I, II, III and some dehydrogenases of the tricarboxylic acid (TCA) cycle may also generate superoxide anion [128, 131]. In turn, manganese superoxide dismutase (MnSOD) can convert superoxide to H_2O_2 [132, 133].

Complex I (NADH:ubiquinone oxidoreductase) catalyses the first step in the mitochondrial electron transport chain. It extracts energy from the oxidation of NADH and transfers it to ubiquinone, generating ubiquinol. Ubiquinol is a membrane-soluble carrier that releases a pair of electrons to Complex III [134]. Complex II (succinate-coenzyme Q reductase) makes the link between the TCA cycle and the electron transport chain, releasing electrons to Complex III through ubiquinol [135]. Complex III (ubiquinone-cytochrome c oxidase) contributes to the proton gradient through the reduction of cytochrome c by oxidation of ubisemiquinone and the pumping of protons from the mitochondrial matrix into the intermembrane space [136]. When there is a decrease in electron transfer, molecular oxygen can capture electrons from Complex III, resulting in superoxide anion formation [128, 137, 138].

Mitochondrial dysfunction was first linked to PD upon the recognition of MPTP-induced parkinsonism among some drug abusers, and the finding of significant dopaminergic neuron loss in their SN at post-mortem analysis [139]. MPTP crosses the blood-brain barrier and is taken up by astrocytes where it is metabolized into 1-methyl-4-phenylpyridinuim (MPP^+) by MAO-B and released into the extracellular space. MPP^+ is a substrate for the dopamine transporter and is taken up selectively into dopaminergic neurons where it inhibits Complex I of the mitochondrial electron transport chain [15]. Several groups have reported decreased Complex I activity and ubiquinone in the SN of PD patients, abnormalities that may lead to neuronal degeneration [140–142]. In addition, gene expression profiling in dopaminergic neurons from PD patients showed down-regulation of genes encoding mitochondrial proteins, among others, providing further evidence for mitochondrial dysfunction in PD [143]. Unexpectedly, complex I deficiency has also been observed in the platelets and skeletal muscle of PD patients [144–146], although not all reports agree about the latter likely due to methodological differences [147, 148]. Nevertheless, it seems clear that mitochondrial function is impaired in PD at different levels ranging from organelle biogenesis, mitochondrial fusion/fission, to mitophagy. Additionally, some of the inherited PD-linked proteins play a significant impact in this process [3, 5, 7, 149–154]. For example loss of ATP13A2, a P-type ATPase associated with an autosomal recessive atypical parkinsonian syndrome is associated with increased mitochondrial mass resulting in increased oxygen consumption and increased ROS production in cultured cells [155]. More about the role of mitochondria in PD pathogenesis is reviewed below under PD-linked proteins.

UBIQUITIN-PROTEASOME SYSTEM (UPS)

The ubiquitin-proteasome system (UPS) is the main pathway through which cells degrade and remove damaged and unwanted proteins [156]. During oxidative stress, the efficient clearance of these unwanted materials by the proteasome is considered as a defense mechanism, since degradation lessens the threat of oxidized proteins forming toxic aggregates. In addition, the amino acid products released in the process of

degradation, which may become oxidized and thereby function as ROS scavengers, can protect vital cellular components from oxidation [157–159]. Mutations in the genes for parkin and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), which are linked to PD and are components of the UPS, indicate a role for UPS in PD pathogenesis [156]. UPS is also involved in the degradation of defective mitochondria, thus, minimizing the production of oxidative free radicals [160, 161]. Additionally, alpha-synuclein, the key component of Lewy bodies, is a substrate of the UPS [161], while oxidatively damaged and aggregated alpha-synuclein impairs proteasomal function [162, 163]. Inhibition of mitochondrial Complex I also impairs proteasomal activity through oxidative modification of proteasome components besides increasing the production of oxidatively damaged proteins [164]. In the SNpc of PD patients, evidence has been presented for impaired UPS with structural proteasome alterations including loss of the alpha-subunit, the component that regulates and stabilizes the proteasome complex ([165, 166]. In culture, cell death induced by proteasome inhibitors leads to a cascade of events involving increased oxidative and nitrosative stress, as well as damage to and alterations of mitochondrial function [167]. Thus, increased oxidative stress can cause UPS dysfunction, which in turn exacerbates the vulnerability of nigral dopaminergic neurons in PD [168].

NEUROINFLAMMATION

Several lines of evidence support a role for neuroinflammation in the pathophysiology of PD mediated mainly by activated microglia [169]. Microglia are phagocytic cells, components of the innate immune system of the central nervous system, that usually have a resting phenotype but become activated upon brain injury or immune challenge [169]. Activated microglia are an important source of superoxide and nitric oxide, which in turn contribute to oxidative and nitrative stress in the brain microenvironment. They can also promote neurodegeneration by producing other potentially toxic agents such as glutamate and tumor necrosis factor-alpha [44, 169–171]. In addition, astrogliosis within the SN leads to local microglia activation [58]. But microglia also have a dual role in the brain, acting as neuroprotective cells through the elimination of endogenous or exogenous substances, and they have high levels of GSH and glutathione peroxidase, which act to protect them from toxic levels of H_2O_2 [172]. Notably, activated microglia and T lymphocytes have been detected in the SN of patients with PD along with an increase of pro-inflammatory mediators in the brain and cerebrospinal fluid [29, 173–175]. Post-mortem studies have also revealed the presence of inducible NO synthase (iNOS) in activated microglia of PD nigra [29]. Moreover, microglial activation is observed in *in vitro* and *in vivo* models using toxins such as MPTP, rotenone or 6-OHDA, as well as with lipopolysaccharide (LPS) [176–180]. Dopaminergic neuronal death releases oxidized proteins, lipids and DNA in the extracellular space that are recognized as damaged molecules by microglia causing their activation. Microglial activation in turn leads to increased cytokine formation, increased production of reactive oxygen and nitrogen species, and decreased secretion of trophic factors responsible for the normal maintenance of neuronal viability [176–180]. Thus, neuronal death aggravated by microglia further induces the activation of microglial cells, creating a neurotoxic vicious cycle [169]. Since the midbrain contains more microglial cells compared to other brain regions, these cells would be particularly damaging to dopaminergic neurons [181].

Although microglia activation and inflammatory changes are generally considered as a consequence of neuronal destruction, the finding of human leukocyte antigen (HLA) as a risk factor for PD in Genome-Wide Association Studies (GWAS) raises the possibility for a more general pro-inflammatory state in this disease as a primary cause of neuronal loss in some cases or at least increasing PD risk as a disease modifier genotype [182, 183]. Interestingly, even peripheral inflammation, for example induced by the injection of carrageenan into the rat paw, reportedly exacerbates LPS-induced inflammatory changes in the SN along with greater dopaminergic neuron loss [183]. Several studies have suggested a link between the innate inflammatory response of the central nervous system and the peripheral immune system [184]. Increased concentration of neuroinflammatory markers, such as IL-2, IL-6, TNF-alpha, osterpontin and RANTES/chemokine (C-C motif) ligand 5 has even been detected in the serum of PD patients [184], but these findings need to be validated. Nevertheless, these preliminary results raise the hypothesis that the loss of dopaminergic neurons induced by a genetic insult or environmental toxin can be exacerbated by a mild to moderate peripheral inflammation [185]. All these observations suggest that neuroinflammation along with its consequences including oxidative stress is a critical component of the pathogenesis of PD.

PHYSIOLOGICAL AND EXTERNAL DETERMINANTS OF OXIDATIVE STRESS IN PD

Besides genetic determinants that exacerbate oxidative stress described below [186], other factors that play a role in the redox status of an organism are recognized as risk factors for the development of PD. These include aging and environmental factors such as exposure to toxins.

Aging

Age is the main risk factor for PD, with an exponential increase of the disease occurrence above the age of 65 [187–189]. Besides the presumed decades needed for misfolding of pathogenic proteins to reach a critical threshold to incur neuronal damage, age associated impairment of mitochondrial function and consequent increased ROS production appear to be important aspects in neurodegenerative disorders that develop later in life [186, 189–191]. In aged and PD afflicted brains, high levels of mitochondrial DNA (mtDNA) deletions are found in pigmented neurons of the substantia nigra [192–195], and ROS-mediated damage can result in mutations in the mitochondrial genome [196–198]. This leads to the expression of mutant forms of the electron transport chain subunits and mitochondrial transfer RNAs essential for translation [199], collectively exacerbating ROS production. This scenario would create a vicious cycle of further injury to mtDNA and other mitochondrial components, thus, increasing ROS production [192–194, 200]. Additionally with aging, mitochondrial function diminishes concomitant with changes in their morphology and decreased number [201], and age-related decreased autophagy results in the accumulation of defective mitochondria [202, 203].

Toxins

Epidemiologic studies corroborate an association between exposure to pesticides frequently used in agriculture with the increased risk of developing PD [204–207]. Sera from PD patients have higher levels of these substances than controls [204, 205], and organochlorine insecticides are detected at higher levels in the PD substantia nigra than in non-PD brains [208]. The risk may be increased after exposure to multiple pesticides compared to exposure to any one alone [209, 210]. In addition, consumption of pesticide-contaminated well-water may increase the risk of PD [211]. Although these toxins act through different mechanisms, they share a common feature of increased oxidative stress due to increased ROS production. For example, the herbicide paraquat, which is linked to increased risk of PD [210, 212–215], undergoes redox cycling and is reduced by NADPH before being oxidized by an electron acceptor to produce superoxide [216]. The pesticide rotenone can freely cross cellular membranes and accumulates in mitochondria, where it inhibits Complex I by impairing oxidative phosphorylation [16]. The results of these epidemiologic studies support a contribution of oxidative stress to the pathogenesis of dopamine neuronal loss in PD.

THE ROLE OF PD-RELATED PROTEINS IN OXIDATIVE STRESS

The discovery of genes linked to familial forms of PD, such as alpha-synuclein, parkin, DJ-1, PINK-1 and Leucine-rich repeat kinase 2 (LRRK2) has yielded important insights into the molecular pathways in the disease pathogenesis and highlighted previously unknown mechanisms by which oxidative stress contributes to the disease (Fig. 1). Some of these mechanisms are complex, involve various cell biologic processes, and are modulated by several of these proteins. These discoveries are also beginning to help interpret some of the biochemical defects in the PD brain.

alpha-Synuclein

alpha-Synuclein is a natively unfolded protein that can associate with vesicular and membranous structures and plays a role in synaptic vesicle recycling, storage and compartmentalization of neurotransmitters [217–219]. Mutations and multiplication of the SNCA gene are linked to dominantly inherited PD and increase the propensity of the protein to aggregate [220–224]. Fibrils of alpha-synuclein are present in Lewy bodies [225], and the formation of aggregates is associated with increased oxidative or nitrosative stress [107, 226, 227]. Oxidative conjugation of dopamine with alpha-synuclein inhibits the transition of alpha-synuclein from protofibrils to mature fibrils, leading to the potential accumulation of cytotoxic soluble protofibrils in dopaminergic neurons. The addition of antioxidants has the ability to reverse the formation of these adducts, suggesting that catechol oxidation can contribute to the accumulation of alpha-synuclein protofibrils [91, 228].

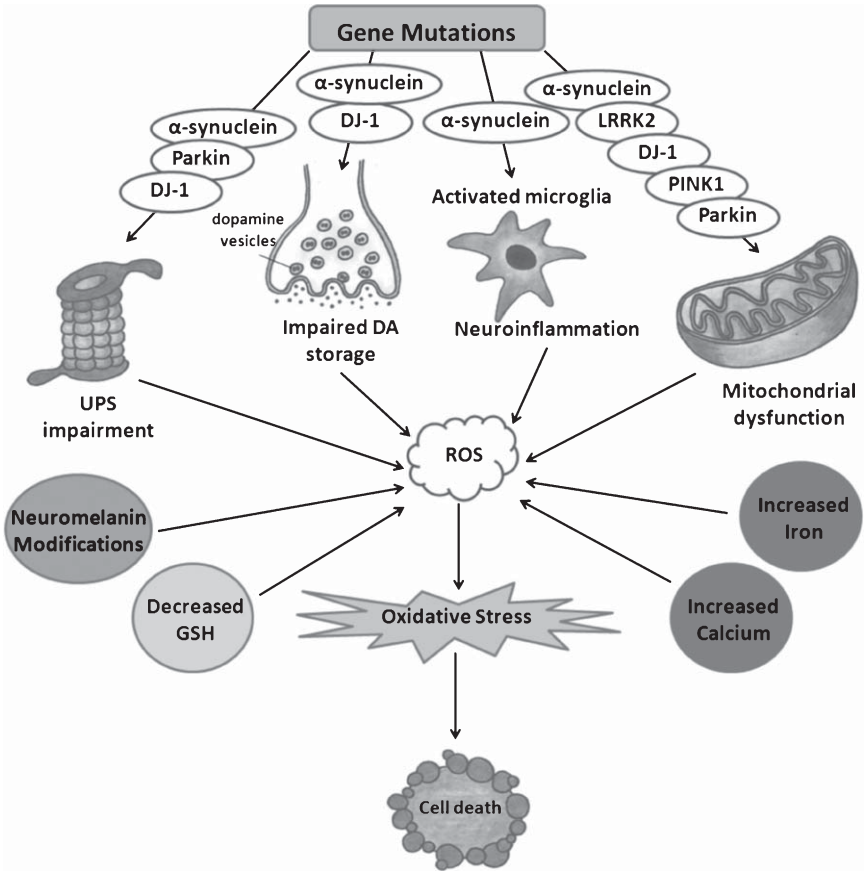

Fig. 1. Mechanisms leading to oxidative stress in PD and the role of PD-related gene products in this process. DA = dopamine; ROS = reactive oxygen species; UPS = ubiquitin-proteasome system; GSH = glutathione.

Additional *in vitro* and *in vivo* experiments support the notion that increased oxidative stress in the brain may contribute to alpha-synuclein aggregation [226]. For example, incubation of recombinant alpha-synuclein with cytochrome c in the presence of H_2O_2 [162], or exposure of cells in culture to H_2O_2 and ferrous iron, MPP^+, NO and superoxide promotes alpha-synuclein aggregation [226, 229, 230]. Additionally, oxidative stress can cause nuclear membrane modifications and alpha-synuclein translocation to the nucleus where it can form complexes with histones leading to its oligomerization into insoluble fibrils [231–233]. *In vivo* studies with rats also demonstrated alpha-synuclein aggregation following systemic exposure to the pesticides rotenone [234] or dichlorvos [235]. Additionally, alpha-synuclein transgenic mice exposed to paraquat exhibit increased proteinase-K-resistant alpha-synuclein inclusions [236].

Besides the impact of oxidative stress in promoting alpha-synuclein aggregation, the over-expression or misfolding of alpha-synuclein, and particularly its mutant variants, is associated with increased ROS production [237, 238]. As a result, experiments with cultured cells have demonstrated that over-expression of wild-type or mutant alpha-synuclein increases sensitivity to dopamine toxicity [237, 239, 240]. Similar observations have been made *in vivo* whereby mice over-expressing mutant or wild-type alpha-synuclein exhibit increased sensitivity to MPTP and 6-OHDA [241–243]. Conversely, mice lacking alpha-synuclein demonstrate marked resistance to MPTP and other mitochondrial toxins such as malonate and 3-nitropropionic acid [244, 245]. Notwithstanding the shortcomings of over-expression studies, these results suggest that ROS promotes alpha-synuclein aggregation, which in turn increases ROS, creating a vicious cycle leading to neurodegeneration.

Several mechanisms have been proposed to explain the increased oxidative stress in the brain in the context of alpha-synuclein misfolding. One possible mechanism can be increased cytosolic catecholamine concentrations, which result from the disruption of

vesicular membrane integrity by pore forming alpha-synuclein protofibrils [246], thus, exacerbating the toxicity of oxidized catechol metabolites [247, 248]. alpha-Synuclein may also play a role in synaptic vesicle recycling through its interaction with phospholipase D2 (PLD2) localized primarily in the plasma membrane [249]. Misfolded or mutated alpha-synuclein has been suggested to result in dysregulated vesicle recycling through PLD2 related mechanisms, leading to reduced number of vesicles available for dopamine storage [250]. In addition to alpha-synuclein disrupting vesicular membrane integrity and recycling, it can also interact with dopamine transporter on the plasma membrane causing clustering of the transporter and greater reuptake of this neurotransmitter into the neuron [251]. These processes that can lead to increased cytosolic dopamine concentrations by alpha-synuclein are implicated in the relative susceptibility of dopaminergic neurons in PD [247, 248].

alpha-Synuclein can also bind to the inner mitochondrial membrane where it associates with complex I, decreasing its activity, and impairing mitochondrial function [238, 252–259]. This interaction is supported by *in vivo* studies demonstrating alpha-synuclein in the mitochondrial membrane of dopaminergic neurons of the mouse brain [254], and in the mitochondrial fraction of human dopaminergic neurons, whereby levels in the SN and striatum are higher in post-mortem PD brains than control subjects [258]. In neuroblastoma cell lines, iron overload leads to the aggregation of alpha-synuclein with clumped mitochondria [231]. Functional studies have shown that the binding of alpha-synuclein to mitochondria is followed by cytochrome c release, increased calcium and ROS levels resulting in cell death [260]. The release of cytochrome c has been suggested to be the primary event that leads to apoptotic cell death in alpha-synuclein induced oxidative stress [261]. alpha-Synuclein can also increase mitophagy, which is an important cellular homeostatic process to remove aged and dysfunctional mitochondria and protect cells against high levels of ROS. However, it is also hypothesized that excessive mitophagy may remove functional mitochondria resulting in bioenergetic failure [262]. Structural and functional abnormalities of mitochondria have been observed with the over-expression of mutant or wild-type alpha-synuclein and have been associated with oxidative stress [263, 264]. Additionally, alpha-synuclein can impact mitochondria indirectly. Under conditions of oxidative stress, it localizes to the nucleus and binds to the promoter of peroxisome proliferator-activated receptor gamma-coactivator-1 alpha (PGC1α) gene leading to down-regulation of PGC1α-target genes and consequently disturbing mitochondrial morphology and function [265].

The neurotoxicity of alpha-synuclein can also be mediated by the activation of microglia leading to increased ROS generation [252, 266–269]. Microglial BV-2 cell line incubated with conditioned media from SH-SY5Y cells over-expressing wild-type or mutant alpha-synuclein release proinflammatory cytokines such as TNF-alpha and IL1-alpha [270, 271]. Similarly, exposure of primary microglial cells to recombinant aggregated or mutated alpha-synuclein results in increased activated microglia and ROS production followed by the secretion of TNF-alpha, IL6 and IL-1beta [266, 272, 273] and the activation of intracellular pathways such as ERK1/2 and p38 MAPK [272]. Neuron/glia co-culture experiments have also shown exacerbation of dopaminergic neuronal toxicity that is made worse with increasing percentage of microglial cells in the plate [274–276]. Mutant alpha-synuclein produces greater insult in this regard than wild-type protein [274]. alpha-Synuclein also promotes microglia activation and ROS production induced by LPS treatment [276]. Similar results have been observed in *in vivo* models, where mice over-expressing wild-type alpha-synuclein exhibit a neuroinflammatory response with microglial and astrocytic activation and neurotoxicity [266, 277–280]. One proposed mechanism for alpha-synuclein induced microglial activation is through the release of misfolded alpha-synuclein from dying dopaminergic neurons into the nigral parenchyma. In this regard, evidence has been presented for oligomeric alpha-synuclein as an agonist for toll-like receptor 2 (TLR2) and the importance of this signaling pathway in microglia activation [281]. Additionally, alpha-synuclein induced microglial activation is partially attenuated with microglia from mice lacking the scavenger receptor CD36, suggesting a role for this pattern recognition receptor in this process [266]. Further, microglial activation appears to require alpha-synuclein phagocytosis and NADPH oxidase activation, since knocking down this enzyme results in attenuated ROS release from microglia and protects dopaminergic neurons in primary mesencephalic neuron-glia co-cultures when challenged with alpha-synuclein [276].

The ability of alpha-synucleiln to activate microglia is exacerbated when it is nitrated, a process that increases its propensity to aggregate and become resistant to proteolysis [273, 276, 282]. Besides the

potential role of nitrated alpha-synuclein in the activation of the brain's innate inflammatory response, this protein can also activate the adaptive immune response with increased leukocyte infiltration [244, 279, 283]. The more persistent neuroinflammatory response seen in transgenic mice carrying the human alpha-synuclein A53T mutation following systemic LPS administration compared with wild-type mice is associated with the accumulation of nitrated alpha-synuclein and dopamine neuron degeneration [284].

Iron is among the factors that accelerate the propensity of alpha-synuclein to aggregate [162, 261, 285–288]. In addition, the two have a synergistic action in neurotoxicity through the nuclear factor erythroid 2-related factor (Nrf2) and heme oxygenase-1 (HO-1) pathway. In SK-N-SH neuroblastoma cells, ferrous iron down-regulates Nrf2 and HO-1, and this effect is exaggerated when alpha-synuclein is overexpressed, resulting in increased cell toxicity. On the other hand, knocking down alpha-synuclein prevents down-regulation of Nrf2 and HO-1 induced by ferrous iron and protects cells against ferrous iron-induced cell death [289]. The presence of a putative iron-responsive element (IRE) in the 5'-UTR of the alpha-synuclein mRNA raises the possibility of iron-dependent translational control of expression as well. This is based on the similarity between the sequences present in the alpha-synuclein mRNA and the IRE present in the ferritin 5'-UTR. Accordingly, HEK293 cells treated with the iron chelator desferrioxamine have decreased alpha-synuclein mRNA levels [290]. Therefore, iron may regulate alpha-synuclein levels and aggregation via the IRE/ iron regulatory protein system (IRP) and may impact oxidative stress through this pathway as well.

Maintaining physiologic levels of alpha-synuclein in neurons is important for their survival. Chaperone-mediated autophagy (CMA) clears aggregated alpha-synuclein and prevents its accumulation. However, post-translational modifications of alpha-synuclein impair its degradation by CMA. Studies with cultured cells and isolated lysosomes have shown that dopamine-modified alpha-synuclein is not only poorly degraded by CMA but also blocks the degradation of other substrates that utilize this pathway, thus, causing considerable cellular stress [291].

A critical process that leads to the progressive neuropathology in PD is the prion-like trans-neuronal propagation of alpha-synuclein [292, 293]. The endocytosis of alpha-synuclein into neurons triggers abnormal protein aggregation leading to a cytotoxic cascade that culminates in mitochondrial dysfunction and cell death demonstrated in primary human fetal enteric neurons [294]. Notably, HNE induces alpha-synuclein oligomerization, enhances its translocation to vesicles and its release from cells, and hence promotes alpha-synuclein oligomers to be transferred across cells [295].

The dual pathogenetic mechanisms whereby the biology of alpha-synuclein is altered by oxidative stress while oxidized or nitrated alpha-synuclein has greater neurotoxicity create a feed forward state of progressive neuronal death seen in PD.

Parkin

Parkin is a cytoplasmic and nuclear protein that functions as an E3 ubiquitin ligase, and the loss of this activity due to mutations is associated with autosomal recessive early-onset PD [296–298]. Parkin plays a role in neuroprotection against several insults, including alpha-synuclein toxicity and oxidative stress, and it is crucial for dopamine neuron survival [299].

Several reports have provided a link between parkin and oxidative stress. SH-SY5Y cells overexpressing wild-type parkin have decreased ROS levels and are relatively protected against apoptosis induced by dopamine or 6-OHDA [300]. On the other hand, expression of mutant parkin is associated with increased levels of protein carbonyls, lipid peroxidation and nitrated proteins [301]. One possible way for parkin to exert neuroprotection against oxidative stress is via its role in the clearance of damaged mitochondria, which would generate ROS. Under physiologic conditions, the majority of parkin is localized in the cytosol, but upon oxidative stress it translocates to depolarized mitochondria, inducing their autophagic elimination [154, 302]. In this process, parkin ubiquitinates multiple mitochondrial substrates, including proteins involved in mitochondrial fusion [303, 304], leading to their degradation by the UPS and mitophagy [305]. *Drosophila* expressing mutant Parkin exhibit age-dependent degeneration of dorsomedial dopaminergic neurons with aberrant morphology [306–308]. These flies also have swollen mitochondria with disruption and disintegration of the cristae, which are associated with muscle dysfunction [307]. And in mice, alpha-synuclein mediated mitochondrial dysfunction is further exacerbated in parkin knockout background [309].

The E3 ligase activity of parkin is impaired by post-translational modifications induced by oxidative or nitrosative stress [310, 311]. For example, in dopaminergic neuronal MES23.5 cells stably expressing human

parkin, dopamine treatment modifies parkin, decreasing its solubility and inactivating its E3 ubiquitin ligase function [310]. In addition, S-nitosylated parkin has been detected in HEK 293 cells transfected with parkin and treated with the NO source S-nitrosoglutathione [310]. Moreover, in the brains of PD patients, evidence has been presented for the presence of nitrosylated parkin, which had poor solubility [311]. These observations suggest that parkin can indirectly regulate the oxidant stress level of cells through regulating the UPS and contributing to mitochondrial quality control, and that its activity can also be impacted by such stress.

PINK1

PTEN-induced putative kinase 1 (PINK1) is a mitochondrially targeted serine-threonine kinase, whose mutations are linked to autosomal recessively inherited PD [312–314]. PINK1 mutations are associated with loss of its kinase function [152] and, thus, its enzymatic activity appears to play an important role in neuroprotection. Accumulating evidence suggests PINK1 as a key regulator of mitochondrial quality control, supporting preservation of mitochondrial respiration through cristae stabilization, phosphorylation of chaperones and possibly regulation of mitochondrial transport or mitophagy [312–315].

Mitochondria are recognized as the main target organelle for normal PINK1 function. PINK1 deficiency results in shortening, swelling and fragmentation of mitochondria in cultured cells [150, 316–319] associated with loss of mitochondrial enzyme activity, particularly that of Complex I [151, 320–323]. In addition, knocking down PINK1 in SH-SY5Y cells results in decreased mitochondrial DNA synthesis followed by loss of mitochondrial membrane potential and decreased ATP production [320]. On the other hand, over-expressing wild-type PINK1 in neuronal cell lines protects against staurosporine-induced mitochondrial cytochrome c release and subsequent apoptosis through caspase 3 activation, while PINK1 mutants lack this protective effect [314] and enhance oxidative stress-induced cell death [324]. Further, induced pluripotent stem cells (iPSC) from PINK1 mutant human subjects have increased vulnerability to MPP^+ and hydrogen peroxide [325].

The impact of PINK1 deletion on mitochondria is also demonstrated *in vivo*. Mice lacking the PINK1 gene exhibit an increase in the number of larger mitochondria in the striatum with no change in total mitochondria numbers. These morphological changes are associated with impaired mitochondrial respiration in the striatum but not in cerebral cortex, suggesting specificity of this defect for the nigrostriatal dopaminergic circuitry. PINK1 knockout mice also show increased sensitivity to oxidative stress [151], since mitochondria isolated from the brains of these animals exhibit increased cellular stress induced by H_2O_2 compared with mitochondria isolated from wild-type mice [151]. Together, these results suggest that loss of PINK1 function results in mitochondrial dysfunction, increasing susceptibility to oxidative stress-induced cell death. Accordingly, measures that enhance the antioxidant potential of cells can mitigate the consequences of PINK1 deficiency. For example, *Drosophila* over-expressing human SOD1 or treated with vitamin E are protected from dopaminergic neuron loss induced by PINK1 inactivation [326]. Evidence for oxidative stress and compensatory changes has also been demonstrated in fibroblasts and lymphoblasts obtained from PD patients homozygous for a PINK1 mutation. These cells have increased lipid peroxidation as shown by increased malondialdehyde levels, slightly decreased Complex I activity and a trend to superoxide elevation. This profile is accompanied by compensatory induction of two antioxidant defenses, mitochondrial superoxide dismutase and the glutathione pathway with elevated levels of glutathione reductase and glutathione-S-transferase [327].

PINK1 can also modulate mitochondrial function and oxidative stress through its functional interactions with other PD linked proteins. The best studied interaction is the PINK1/parkin pathway. PINK-1 mutant flies share a similar phenotype as parkin mutants [152, 328], and over-expression of parkin can rescue the mitochondrial defects of PINK-1 mutant flies [152, 328, 329]. Parkin can also protect PINK1 deficient mice against MPTP [330]. Mechanistically, PINK1 and parkin work together for the selective clearance of damaged mitochondria through mitophagy [303]. When mitochondria are damaged, parkin translocates to mitochondria to initiate mitophagy. However, if PINK1 is knocked down by RNAi, both the translocation of parkin and mitophagy are abrogated indicating that PINK1 is required for the recruitment of parkin to damaged mitochondria [303, 331].

Besides its functional interaction with parkin, PINK1 can also act as a neuroprotective protein against oxidative stress through its interaction with DJ-1 [332]. Over-expression of DJ-1 and PINK1 synergistically protects SH-SY5Y cells against MPTP-induced cell death; this effect is lost in the presence of A39S mutant DJ-1 and P399L mutant PINK1 [332]. *In vivo*,

viral vector mediated expression of DJ-1 mitigates the increased sensitivity of PINK1 deficient mice to MPTP [330]. Similarly, ubiquitous up-regulation of DJ-1 in *Drosophila* can ameliorate the phenotype of PINK1 deletion [333]. Another *Drosophila* model that used RNAi mediated down regulation of PINK1 and muscle specific expression of DJ-1 failed to demonstrate this rescue effect [329, 334]. Collectively, however, the available information suggests that DJ-1 acts either downstream of PINK1 or in a pathway that is parallel with the PINK1/parkin pathway [333, 335]. The precise mechanism of this synergy between DJ-1 and PINK1 in protecting cells and maintaining their homeostasis in an oxidative environment remains unclear.

DJ-1

DJ-1 is an evolutionarily conserved neuroprotective protein that regulates anti-oxidant, anti-apoptotic, and anti-inflammatory pathways [336]. It accomplishes these beneficial effects through several mechanisms including through acting as an ROS quencher, transcriptional co-activator and molecular chaperone [337–339]. It is expressed ubiquitiously in both neurons and glial cells [340]. Mutations in the DJ-1 gene are linked to autosomal recessive, early-onset PD [341].

The role of DJ-1 as a neuroprotective protein has been established in several models using toxins that mimic the oxidative stress in PD. A number of *in vitro* and *in vivo* studies have established that over-expression of wild-type DJ-1 is protective against H_2O_2, 6-OHDA, rotenone or MPTP, while DJ-1 mutants lack this effect [337, 342–347]. On the other hand, knocking down DJ-1 exacerbates the cell death induced by oxidative stress, as well as in response to other insults such as endoplasmic reticulum (ER) stress, proteasome inhibition and calcium entry [342, 348, 349]. Accordingly, transgenic mice expressing a redox-sensitive optical probe targeted to the mitochondrial matrix show, as a consequence of calcium influx, increased mitochondrial oxidative stress, which is exacerbated by knocking out DJ-1 [350]. As expected, DJ-1 knockout mice are more susceptible to MPTP and 6-OHDA than wild-type mice [344, 351, 352], although they do not exhibit dopamine neuronal degeneration at basal conditions [353–355]. Conversely, over-expression of DJ-1 in mice ameliorates MPTP-induced nigral neuronal loss [344, 351, 352]. Similarly, microinjection of DJ-1 protein into the medial forebrain bundle of the rat 6-OHDA model of PD dramatically improves the phenotype, including motor behavior, striatal dopamine and tyrosine hydroxylase content, and nigral neuronal count [356].

DJ-1 deficiency has also been modeled in *Drosophila melanogaster* and *Caenorhabditis elegans*. The fruit fly has two DJ-1 orthologs, DJ-1alpha and DJ-1beta, with DJ-1alpha being predominantly expressed in the testis, and DJ-1beta ubiquitously expressed [333, 357–359]. Mutant flies with both homologs deleted display no loss of dopaminergic neurons at basal conditions but have increased sensitivity to oxidative stress induced by paraquat or rotenone [333, 357–359]. Double mutant flies also have defective mitochondrial respiration, ATP production and shortened lifespan [333]. DJ-1beta loss-of-function mutant flies exhibit increased number of enlarged mitochondria, which confers increased sensitivity to H_2O_2 and paraquat [360]. These mutants also have increased H_2O_2 levels in their mitochondria associated with protein damage and alteration of different cellular pathways [361]. Surprisingly, despite the restricted expression pattern of DJ-1alpha, knocking it down by transgenic RNA interference results in the accumulation of ROS in post-mitotic neurons, hypersensitivity to oxidative stress and degeneration of dopaminergic and photoreceptor neurons [362]. DJ-1alpha is also up-regulated in DJ-1beta mutant flies and apparently sufficient to confer protection against paraquat [363]. On the other hand, over-expression of DJ-1beta protects DJ-1alpha/beta knockout flies from oxidative stress, whereas over-expression of C104A DJ-1beta (homologous to human C106A) cannot [357]. Similarly, as described above under the section about PINK1, up-regulation of DJ-1 rescues the phenotype of PINK1 deleted flies in a Cys104 dependent manner [333], further pointing to the anti-oxidant function of DJ-1 in this protection. Consistent with these findings, knocking down DJ-1 in nematodes exacerbates the sensitivity of worms to rotenone-induced death [364].

Several mechanisms can mediate the protective effects of DJ-1 against oxidative stress. As an ROS quencher, DJ-1 shifts to a more acidic form upon oxidative stress by oxidation of its Cys106 and translocates from the cytoplasm to mitochondria as well as nucleus [365, 366]. Whether Cys106 oxidation is essential for the localization of DJ-1 to mitochondria in response to oxidative stress is controversial [346, 365, 366]. But Cys106 oxidation to cysteine sulfinic acid is necessary for DJ-1 to exert its cytoprotective activity and to bind to the DNA-binding region of other proteins [367]. Further oxidation, however, is believed to render it inactive [368]. Levels of oxidized DJ-1 in the SN

of mice increase dose-dependently following MPTP administration [369]. Further, significant elevation of Cys106-oxidized DJ-1 has been reported in erythrocytes of untreated PD patients [370], and more acidic isoforms of DJ-1 have been detected in extracts of frontal cortex from patients with sporadic PD compared to control subjects [371].

Besides quenching ROS, DJ-1 enhances cellular glutathione levels through increased expression of the enzyme glutamate cysteine ligase. This effect is lost when glutathione synthesis is inhibited, but is reversible by adding exogenous glutathione [372]. DJ-1 may also act as an oxidative-stress-induced chaperone inducer through up-regulating HSP70, which binds to and stabilizes alpha-synuclein, preventing its aggregation and subsequent cell death [368, 373, 374]. Accordingly, over-expression of DJ-1 in dopaminergic cells inhibits protein aggregation and cytotoxicity caused by alpha-synuclein [372]. DJ-1 can also increase the expression of vesicular monoamine transporter-2 (VMAT2) [347, 375]. Since VMAT2 keeps cytoplasmic dopamine levels in check by storing the transmitter in synaptic vesicles, DJ-1 decreases intracellular ROS levels and enhances the resistance of cells against dopamine toxicity. Reducing DJ-1 levels has the opposite effect [347]. Thus, DJ-1 can confer neuroprotection through up-regulating VMAT2 by enabling the rapid and efficient transport of dopamine from the cytoplasm to vesicles. DJ-1 also contributes to mitochondrial quality control. Its loss of function is associated with reduced mitochondrial membrane potential and fragmentation [376], while over-expression of wild-type DJ-1 is associated with elongated mitochondria along with enhanced resistance to oxidative insults [335, 376–378]. ROS scavengers rescue this phenotype [379]. Accordingly, mitochondria isolated from DJ-1 deficient mice produce more ROS compared with controls, and lymphoblasts derived from DJ-1 mutant PD patients exhibit abnormal mitochondrial morphology [379].

Another proposed mechanism for the antioxidant function of DJ-1 is through the stabilization of Nrf2 by preventing the association of this transcription regulator with its inhibitor Keap1 [380]. Over-expression of DJ-1 in SH-SY5Y cells results in increased Nrf2 protein levels, promotes its translocation to the nucleus and enhances its recruitment onto the antioxidant response element (ARE) in the promoter of the redox regulator thioredoxin-1 (Trx1). Knocking down Nrf2 abolishes DJ-1 mediated Trx1 induction and cytoprotection against hydrogen peroxide [381].

DJ-1 acts as a potent inhibitor of the Daxx/Apoptosis Signal Regulating Kinase 1 (ASK1) cell-death signaling pathway [382]. Under basal conditions, DJ-1 present in the nucleus interacts with Daxx preventing the translocation of this death protein to the cytoplasm and its interaction with ASK1 following insults including oxidative stress. This process would block the activation of ASK1 and hence cell death [382]. Additionally, DJ-1 controls ASK1 activity by modulating the interaction between ASK1 and Trx1. ASK1 is bound to Trx1 under basal conditions, but the two dissociate upon oxidant insult allowing ASK1 activation and subsequent cell death. Wild-type DJ-1 interacts with ASK1 blocking its oxidant-induced dissociation from Trx1, while the PD linked L166P mutant cannot. Cys106 of DJ-1 is required for its ability to regulate ASK-1/Trx1 interaction [383]. Consistent with the death signaling activity of ASK1, MPTP challenge of cultured cells and mice results in activation of this kinase, while knocking out ASK1 in mice ameliorates MPTP toxicity and suppresses the neuroinflammatory response in the SNpc and striatum [384]. Collectively, these results indicate that the DJ-1-ASK1 pathway plays an important role in the anti-oxidant response.

LRRK2

LRRK2 is a large multi-domain protein linked to autosomal dominant PD. Disease associated mutations are identified in almost all its domains but are commonest in its kinase domain leading to increased kinase activity [153, 385–392]. As kinase activity is required for cell-death, it is suggested that LRRK2 enzymatic activity plays an important role in the pathogenesis of PD [391, 392].

LRRK2 is predominantly a cytoplasmic protein, although it can also be associated with the outer mitochondrial membrane, raising the possibility that the increased kinase activity of mutant LRRK2 might directly affect mitochondrial function [153, 393]. In agreement with this hypothesis, increased LRRK2 activity leads to neuronal death via mitochondrial-dependent apoptosis, while lack of LRRK2 has a protective effect on mitochondrial dysfunction [394]. Over-expression of wild-type or mutant LRRK2 with enhanced kinase activity in various cell lines or primary neurons leads to mitochondrial fragmentation and dysfunction associated with increased ROS generation and increased susceptibility to H_2O_2 [149, 395, 396]. Further, iPSC cells from LRRK2 mutant human subjects exhibit increased vulnerability to MPP^+ [325].

A proposed mechanism for the increased vulnerability of LRRK2 mutant cells to oxidative stress is via the kinase-dependent interaction between LRRK2 and dynamin-like protein (DLP1), which facilitates DLP1 translocation to mitochondria and subsequent mitochondrial fission [149, 397]. Another mechanism is through the interaction of LRRK2 with peroxiredoxin 3 (PRDX3), which is a mitochondrial member of the antioxidant family of thioredoxin peroxidases. Mutations in the LRRK2 kinase domain increase phosphorylation of PRDX3 leading to decreased peroxidase activity, increased ROS production, and increased cell death. Notably, postmortem analysis of brains from PD patients carrying the G2019S mutation in the kinase domain of LRRK2 has shown marked increase in phosphorylated PRDX3 compared to normal brains [398].

Animal models used to study the effect of LRRK2 against oxidative stress have shown contradictory results, perhaps suggesting different pathways in LRRK2-mediated neuroprotection. *Caenorhabditis elegans* over-expressing human wild-type LRRK2 in neurons survive longer in response to rotenone or paraquat than wild-type worms, while PD-linked LRRK2 mutants that have increased kinase activity or even kinase dead mutant provide less protection than wild-type LRRK2 [399]. Knock down of the endogenous ortholog of LRRK2 in the worm reduces survival associated with mitochondrial impairment. LRRK2 mutations may also selectively exacerbate the vulnerability of dopaminergic neurons to oxidative stress [399] as these neurons are more influenced by the model than the survival of the whole worm [399]. Similarly, *Drosophila* expressing human wild-type but not mutant LRRK2 are protected against rotenone [400], while flies expressing mutant LRRK2 are more sensitive to H_2O_2 [401]. Furthermore, adding curcumin to flies' diet protects them against H_2O_2 and increases their survival [401]. A proposed mechanism for this protective effect is increased expression of LRRK2 [402]. Despite the limitations of over-expression paradigms, one can conclude from these results that LRRK2 has an important role in mitochondrial function and in the capacity of the cell to keep oxidative stress in check. Contrary to worm and fly models, mouse models do not support a mitochondrial pathway for LRRK2 neuroprotection. This is because LRRK2 knock-out mice have not yielded the expected results as they have a normal dopaminergic system and do not exhibit significant differences in their susceptibility to MPTP when compared with wild-type mice [403]. These observations in mice may be due to compensatory changes during ontogeny [403].

THERAPEUTIC IMPLICATIONS OF OXIDATIVE STRESS IN PD

Knowledge gained about the link between oxidative stress and the pathogenesis of PD has two therapeutic implications. The first is whether currently used drugs have any impact on this process, either positive or negative. And the second is the potential to develop new drugs that might be able to mitigate oxidative stress and by doing so slow disease progression. Extensive investigations into the first question have shown no solid evidence for clinically relevant impact, while the success of the second scenario remains elusive.

PD remains an incurable disease despite repeated efforts to test potential neuroprotective strategies. The gold standard treatment, L-dopa, is transformed to dopamine in dopaminergic neurons by dopa-decarboxylase raising some concerns about the potential toxicity of long term L-dopa therapy. But these concerns were largely laid to rest from the ELLDOPA trial (Earlier versus Late Levodopa), which showed that L-dopa in combination with a peripheral decarboxylase inhibitor is the most effective treatment for PD, with its early use ameliorating the motor symptoms over time [404, 405]. Thus, despite the toxicity of dopamine in culture, there is no evidence for L-dopa having such deleterious effects in the human brain.

Dopamine agonists have been suggested to have neuroprotective and antioxidant effects [406]. For example, pramipexole protects MES 23.5 cells against hydrogen peroxide, dopamine and L-dopa induced toxicity [407]. It also reduces lipid peroxidation and minimizes injury to the SN of MPTP lesioned mice [408]. The antioxidant effect of pramipexole independent of its dopamine receptor agonist property can be responsible for its neuroprotective action [409]. It can reduce the levels of oxygen radicals produced by MPP^+ both when incubated with SH-SY5Y cells and when perfused into the rat striatum [410]. Similarly, ropinirole protects mice against 6-OHDA by increasing glutathione levels [411, 412]. However, in several clinical trials using the dopamine agonists ropinirole and pramipexole, no significant advantage was seen in slowing progression of motor symptoms although imaging studies suggested a benefit of the agonists over L-dopa [413–425]. This is believed to be due to the drugs affecting the imaging studies rather than true neuroprotection.

MAO-B inhibitors prevent MPTP toxicity to nigral dopaminergic neurons in animal models through inhibition of MPP^+ generation [426]. Both clinically used

agents, selegiline and rasagiline, have symptomatic antiparkinson effects [48, 427–430], which have confounded interpretation of clinical trials designed with the goal to study their potential neuroprotective effects. For example, the DATATOP study tested the effects of selegiline and found a significant delay in the time until enough disability developed to require the initiation of L-dopa therapy. But this was attributed to the symptomatic effect of selegiline rather than true neuroprotection [431]. Similarly, early use of rasagiline had greater improvement in motor disability compared with later introduction of the drug [432, 433]. But again questions about the symptomatic effect of the drug and about the delayed-start study design have made it difficult to provide conclusive evidence for neuroprotection by MAO-B inhibitors [432, 434–436].

Among agents that might alleviate oxidative stress, creatine is an antioxidant involved in the inhibition of the opening of the mitochondrial permeability pore and affects mitochondrial energy production [437–439]. In mice, creatine has been shown to protect against MPTP-induced dopaminergic depletion in the SN [440]. Two phase II studies of creatine in PD patients have yielded contradictory results, one failing to show efficacy [441] while the other reporting about 40% less worsening of motor Unified PD Rating Scale scores at one year [442]. In a follow up phase II NET-PD futility study, creatine use at 18 months did not show safety concerns, but the phase III trial was recently terminated due to lack of statistically significant difference from placebo [443, 444].

Other attempts to use compounds with antioxidant properties in PD have included tocopherol (vitamin E), vitamin C, Coenzyme Q10, docosahexaenoic acid (DHA), Ginkgo biloba, or polyphenols found in green tea [431, 445–451]. None of these have yielded convincing evidence for neuroprotective efficacy. In addition to selegiline, the DATATOP study studied the effects of the antioxidant tocopherol in patients with early PD and found no impact on disease progression [431]. Recently, ketogenic diet has been suggested as a potential therapy in neurological diseases, since the mitochondrial Complex II activator D-beta-hydroxybutyrate, a ketone body, has been shown to increase cellular GSH content, decrease intracellular ROS production induced by H_2O_2, inhibit apoptosis and enhance cell viability [452]. Although this diet has not been studied in animal models of PD yet, a caloric restriction diet conferred resistance to MPTP in mice, rats and rhesus monkeys [453–455]. Interestingly, an uncontrolled study of five patients with PD placed on a ketogenic diet for 28 days reported a 43% improvement in their Unified PD Rating Scale scores [456].

CONCLUSION

Although the mechanisms involved in the pathogenesis and progression of PD are not fully understood, there is overwhelming evidence that oxidative stress plays an important role in dopaminergic neuronal degeneration. Since the maintenance of redox potential is an important factor for neuronal survival, it is not surprising that any disruption in this potential might interfere with other biological processes in the cell, ultimately leading to cell death. It is also likely that the interplay between these various mechanisms contribute to neurodegeneration in PD as a feed forward scenario where primary insults lead to oxidative stress, which damages key cellular pathogenetic proteins and disrupts lipid membranes that in turn cause more ROS production. In the brain, ROS comes mainly from dopamine metabolism, mitochondrial dysfunction and neuroinflammation. Therefore, the protective mechanisms involved in the regulation of these processes have been an area of considerable research focus in recent years. In addition, the study of PD-related proteins in combination with experimental studies using animal models has yielded important insights into the molecular pathways of neuronal degeneration and highlighted previously unknown mechanisms by which oxidative stress contributes to PD. The results obtained so far have been used to design therapeutic approaches. However, despite encouraging results in animal models, a number of attempts in clinical trials to date have failed to demonstrate an impact on disease progression. The incomplete representation of the disease phenotype in the various animal models, the time of treatment onset, heterogeneity of the disease subtypes all likely contribute to this dearth of therapeutic progress. Failures from anti-oxidant compounds and strategies tested to date should guide future newer approaches.

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CONFLICT OF INTEREST

None.

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