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Review Article

Reprint of: Revisiting oxidative stress and mitochondrial dysfunction in the pathogenesis of Parkinson disease—resemblance to the effect of amphetamine drugs of abuse

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ABSTRACT

Parkinson disease (PD) is a chronic and progressive neurological disease associated with a loss of dopaminergic neurons. In most cases the disease is sporadic but genetically inherited cases also exist. One of the major pathological features of PD is the presence of aggregates that localize in neuronal cytoplasm as Lewy bodies, mainly composed of α-synuclein (α-syn) and ubiquitin. The selective degeneration of dopaminergic neurons suggests that dopamine itself may contribute to the neurodegenerative process in PD. Furthermore, mitochondrial dysfunction and oxidative stress constitute key pathogenic events of this disorder. Thus, in this review we give an actual perspective to classical pathways involving these two mechanisms of neurodegeneration, including the role of dopamine in sporadic and familial PD, as well as in the case of abuse of amphetamine-type drugs. Mutations in genes related to familial PD causing autosomal dominant or recessive forms may also have crucial effects on mitochondrial morphology, function, and oxidative stress. Environmental factors, such as MPTP and rotenone, have been reported to induce selective degeneration of the nigrostriatal pathways leading to α-syn-positive inclusions, possibly by inhibiting mitochondrial complex I of the respiratory chain and subsequently increasing oxidative stress. Recently, increased risk for PD was found in amphetamine users. Amphetamine drugs have effects similar to those of other environmental factors for PD, because long-term exposure to these drugs leads to dopamine depletion. Moreover, amphetamine neurotoxicity involves α-syn aggregation, mitochondrial dysfunction, and oxidative stress. Therefore, dopamine and related oxidative stress, as well as mitochondrial dysfunction, seem to be common links between PD and amphetamine neurotoxicity.

Contents

Abbreviations: α-syn, α-synuclein; ASK1, Apoptosis signaling-regulating kinase 1; CMA, Chaperone-mediated autophagy; DAT, Dopamine transporter; ETC, Electron transport chain; iPS, Induced pluripotent stem; LAMP-2A, Lysosome-associated membrane protein 2A; LB, Lewy body; L-DOPA, *levo*-3,4-dihydroxyphenylalanine; LN, Lewy neurite; LRR, Leucine-rich repeat domain; LRRK2, Leucine-rich repeat kinase 2; MAO, Monoamine oxidase; MAP, mitogen-activated protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrapyridine; MPP^+, 1-methyl-4-phenylpyridinium; mtDNA, Mitochondrial DNA; NAC, Non-amyloid-β component; NMDA, *N*-methyl-d-aspartate; 6-OHDA, 6-hydroxydopamine; PD, Parkinson disease; PINK1, PTEN-induced putative kinase 1; Roc, Ras of complex protein; ROS, Reactive oxygen species; siRNA, Short-interfering RNA; SN, Substantia nigra pars compacta; SNCA, α-synuclein; TH, Tyrosine hydroxylase; Trx1, Thioredoxin 1; Ub, Ubiquitin; UCHL1, Ubiquitin C-terminal hydrolase 1; UPS, Ubiquitin proteasome system; VMAT2, Vesicular monoamine transporter 2; WT, Wild type

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Introduction to Parkinson disease

Parkinson's disease (PD) is a chronic and progressive neurological disease associated with a loss of dopaminergic neurons in the substantia nigra pars compacta (SN), as well as with more widespread neuronal changes that cause complex and variable motor and nonmotor symptoms. PD is the second most prevalent neurodegenerative brain disorder, affecting 1 to 2% of the population above 65 years of age, and its prevalence increases to approximately 4% in individuals above 85 years of age [1,2]. The etiopathogenesis of PD is still not fully understood. In most cases the disease is sporadic: a multifactorial, idiopathic disorder that seems to arise from a combination of environmental exposures and genetic susceptibility. The remaining cases are the result of genetic inheritance. Moreover, 15 to 20% of the patients with PD report a family history of the disease, although monogenic forms of PD are relatively rare [2–„4]. Nevertheless, old age continues to be the main risk factor in the development of the disease [5,6], making it clear that during aging, our cells display a greater degree of dysfunction, leading to cell stress (including decreased capacity to cope with oxidative stress) and greater energy demand. Investigations into postmortem PD brains, particularly in the SN, have consistently demonstrated abnormalities in mitochondrial function and increased levels of oxidative stress [7–„14]. Furthermore, there is evidence of inflammation through microglial activation in the SN, even at the time of death. The finding that α-synuclein (α-syn) is the major component of Lewy bodies (LBs) directed studies on protein metabolism and defects in protein degradation through the ubiquitin (Ub) proteasome system (UPS) and autophagy pathways as contributory factors to PD pathogenesis. These cellular pathways are interconnected, because mitochondrial dysfunction, namely complex I inhibition, leads to increased free radical generation, which further evokes deficits in the respiratory chain. Importantly, the UPS is dependent on oxidative phosphorylation for energy production and oxidatively damaged proteins increase the bulk of substrates to be degraded by the UPS. Moreover, this leads to increased cell dysfunction and a lowered threshold to apoptosis [15], a type of programmed cell death characterized by membrane blebbing, shrinking of organelles, and chromatin condensation and fragmentation [16].

Clinical and pathological aspects

Clinically, PD has often been characterized by the presence of cardinal motor signs, namely resting tremor, rigidity, bradykinesia, and postural instability. One of the main features affecting these patients includes a slowness of initiation of voluntary movement with a progressive reduction in speed and amplitude of sequential motor tasks [17,18]. For a long time, PD was thought to involve a relatively simple neuropathological process primarily centered on the loss of dopaminergic neurons in the SN. This results in the loss of dopaminergic transmission in the striatum, leading to the majority of the classical motor symptoms of PD. The disease becomes evident when approximately 80% of striatal dopamine and 50% of nigral neurons are lost [2,19,20]. However, recent evidence indicates that nonmotor characteristics such as autonomic insufficiency, cognitive impairment, olfactory deficits, sleep disturbance, depression, and psychosis are very common during the course of the disease. The clinical diagnosis of PD is typically based on the presence of cardinal motor features, absence of atypical findings suggestive of an alternate diagnosis, and response to *levo*-3,4-dihydroxyphenylalanine (L-DOPA) [2,21].

In addition to the loss of dopaminergic neurons in the SN, PD is neuropathologically characterized by the presence of LBs and Lewy neurites (LNs) in vulnerable populations of neurons. These are intracytoplasmic insoluble protein inclusions located in either the neuronal cell body or the neuronal processes, respectively. The principal component of LBs and LNs is α-syn, a small protein of 140 amino acids that is predominantly expressed in the neocortex, hippocampus, SN, thalamus, and cerebellum [22]. The pathological definition of PD depends upon the presence of LBs in the SN neurons, although it is clear that pathology is also present outside this area [23,24].

Sporadic and familial forms of PD

Sporadic and inherited forms of PD share pathological, biochemical, and clinical features, with dysfunction of mitochondria, increased oxidative stress levels, and associated molecular pathways representing a link between the two forms of PD, as well as the natural aging process [6]. Environmental factors were long thought to be the principal cause of PD, particularly after the influenza pandemic of 1918, when a significant number of individuals developed postencephalitic parkinsonism. Infectious agents in the environment were then suspected to be the causal factors [25,26]. This environmental theory was subsequently supported by the identification of *N*-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) in the early 1980s, whose metabolite 1-methyl-4-phenylpyridinium (MPP^+) is responsible for the selective degeneration of the nigrostriatal pathways, by inhibiting mitochondrial complex I of the respiratory chain and subsequently increasing oxidative stress levels in dopaminergic neurons. Moreover, MPP^+ was shown to bind vesicular monoamine transporters (VMATs) and redistribute vesicular dopamine to the cytosol [27]. Within the cytosol, MPP^+ may also interact with various cytosolic enzymes, namely xanthine oxidase, aldehyde dehydrogenase and lipoamide dehydrogenase [28]. MPTP leads to a parkinsonian syndrome in

rodents (namely mice, whereas rats are generally resistant to systemic MPTP), primates, and humans [29,30]. A similar pathogenic mechanism was suggested for rotenone, a common naturally occurring botanical pesticide, which also acts as an inhibitor of the mitochondrial respiratory chain complex I and leads to a failure of the mitochondrial energy supply of the cell. Betarbet and co-workers [31,32] showed that chronic treatment with rotenone was sufficient to cause typical features of PD, including loss of dopaminergic neurons and appearance of α-syn-positive inclusions in rodents. The involvement of oxidative damage caused by mitochondrial dysfunction after rotenone treatment was also demonstrated in in vitro models, suggesting specific neuronal death by rotenone [33]. In this regard, a large number of environmental situations and agents have been identified, including farming and rural life, industrial chemicals, metals, and pesticide exposure; however, no conclusive relationships between individual studies have been found [26,34,35]. Factors decreasing the risk of developing PD can also provide valuable clues to understanding its etiology. Evidence that cigarette smoking and caffeine and tea intake may reduce the risk for PD development appears clear, but there is still uncertainty over the exact roles of these factors in the disease [36,37].

Over the past 2 decades, the scientific view of PD etiology has dramatically changed. Although most patients with idiopathic or late-onset PD (90% of all cases) do not seem to have inherited the disease, a positive family history is frequently associated with a high risk of developing PD. Studies in families with rare Mendelian inheritance (5–10%), for which several causative genes have been identified, show protein aggregation and abnormal handling of misfolded proteins by the UPS, together with oxidative stress, mitochondrial dysfunction, and alterations in autophagy–lysosomal pathways and kinase signaling pathways, suggesting that all play a major role in the pathogenesis of PD [26].

Molecular genetic analysis of PD families in the past years, in particular the mapping and the subsequent cloning of genes that cause inheritable forms of this disorder, has provided important insights into the mechanisms underlying PD pathology. Particularly, it has been shown that PD is not a single clinical entity, but rather a heterogeneous group of diseases with various associated pathologies and a variable spectrum of clinical symptoms and signs. Furthermore, some familial forms include atypical clinical features, such as early onset, onset with dystonia, or the early occurrence of dementia and dysautonomia. Alternatively, PD may result from a combination of genetic predisposition and environmental factors or, moreover, from common genetic variants in genes identified in monogenic forms that have been found to confer a risk of developing the sporadic disease.

Understanding of the mechanisms underlying the initiation and progression of PD came with the identification of mutations in the gene encoding α-syn (SNCA, PARK1 locus) in an Italian family (the Contursi kindred) and the evidence that α-syn is the major component of LBs [26,38–„40]. Since then, a total of 18 loci have been indicated through linkage analysis (PARK1–15) or genomewide association studies (PARK16–18) [41–„53]. Mutations within 11 genes of these loci have conclusively been demonstrated to cause familial parkinsonism in either autosomal dominant or recessive forms [2,54], including PARK1 and PARK4 (SNCA), PARK2 (parkin), PARK5 (ubiquitin C-terminal hydrolase L1 (UCHL1)), PARK6 (PTEN-induced putative kinase 1 (PINK1)), PARK7 (DJ-1), PARK8 (leucine-rich repeat kinase 2 (LRRK2)), PARK9 (ATPase type 13A2), PARK11 (Grb10-interacting GYF protein 2), PARK13 (Omi/Htra2 (HTRA2)), PARK14 (phospholipase A2 group VI), and PARK15 (F-box protein 7) [26]. Furthermore, common polymorphisms within two of these genes (SNCA and LRRK2) are now well associated with risk factors for common sporadic PD [2,55], which suggests that the contribution of genetics to PD may be more relevant than previously thought.

Spread of pathology and neuronal circuits affected

Research in the past 25 years has confirmed that although the lesion in the SN is a key hallmark of the pathological confirmation of PD, the pathological lesions are much more extensive and involve a number of pathways in the brain stem and areas of the neocortex [18,56,57]. It is currently recognized that early neuronal loss occurs in other regions involved in motor control [58,59] and in neurons of the mesocortical system [60]. The involvement of other neuronal populations takes place later in PD or only in certain clinical phenotypes and includes neuronal loss in the cholinergic basal forebrain [61], in the hypothalamic hypocretin system [62,63], and in the upper brain-stem serotonin system [64]. Neuronal loss is very restricted to areas containing LBs, such as the amygdala, the dorsal motor nucleus of the vagus nerve, the locus coeruleus, and the neocortex [65–„68]. Although the pathology of PD affects several neuronal systems, it does not cause substantial brain tissue loss [69,70], because cell loss is restricted to only certain neuronal populations, including the SN [19].

Various types of studies suggest that PD is slow in progressing through the nervous system, with restricted pathology occurring before the characteristic symptoms. In fact, the onset of PD is so gradual that it is frequently difficult to assess a patient when the disease first emerges, and by the time the diagnosis is made, pathology is likely to be widespread [18]. Many of the earliest symptoms are commonly misinterpreted, because some are linked to the normal aging process; these include depression, rheumatism, loss of elasticity, and other secondary symptoms, namely fatigue, autonomic dysfunction, constipation, or sleep disturbances, leading to insufficient information to make a definitive diagnosis. Indeed, the major symptoms associated with PD seem to appear after a significant neuronal cell loss.

The neuropathological distribution of the insoluble inclusions (LBs) constituted by misfolded α-syn throughout the brain has gained importance over time. This is due to the work developed by Heiko Braak and colleagues, who proposed a theory for the progression of PD [71,72], based on LB pathology progression through cell-to-cell contact. They examined brains from patients with confirmed diagnoses of neurodegenerative disorder and made several observations, paying special attention to the development of PD neuropathology. In brain sections derived from autopsied PD patients, they detected an apparent correlation between the amount of α-syn deposits and the stage of the disease [71]. Moreover, based on these initial observations, Braak's team performed anatomical neuropathological studies to characterize the cerebral progression of α-syn-positive LBs and LNs, as the disease process continued. This led to the establishment of an association between LB pathology and clinical symptoms [71–„73] and to the development of the Braak staging concept for PD, which includes six stages that represent presymptomatic (before the motor symptoms) and symptomatic phases [71].

In stage 1, Braak et al. [73] suggested that LB pathology appears outside the SN. These initial α-syn-immunoreactive inclusions are found within the olfactory system, causing autonomic and olfactory deficits. In stage 2, the LBs and LNs are more widespread within the medulla oblongata, including monoaminergic areas. During these first stages, the individuals do not present any perceptible motor symptoms [73]. Stage 3 is characterized by the progression of Lewy pathology into the brain stem. The α-syn-positive deposits are typically found in the midbrain and basal forebrain and later spread into the SN. In stage 4, cell loss into the SN is evident, as well as α-syn immunoreactivity in the cerebral cortex. At this time, patients may display the first symptoms consistent with parkinsonism. Finally, in end stages 5 and 6, few cells remain positive for neuromelanin in the SN, and α-syn-positive pathology begins to invade the neocortex. At these phases, the motor symptoms are clearly severe and cognitive dysfunction becomes evident [20,72,74–„76]. In addition, Braak and

colleagues [72] proposed that projection neurons with no or limited myelination were particularly susceptible to the deposition of α-syn, suggesting that PD progression as described above might evolve via these neuronal pathways, affecting more sensitive neurons before reaching the less vulnerable cells.

Recent studies demonstrating that misfolded aggregated α-syn can be transferred across synapses and spread within postsynaptic cells give strength to the concept of cell-to-cell transfer [18,77]. However, the most controversial aspect of the Braak PD staging is identifying where LB pathology begins in the brain, because other studies on similar populations suggest that 7–8% of patients did not present medullary LBs [78,79]. Furthermore, the lack of correlation between Braak staging and clinical severity has also been seen as problematic as the clinical deficits associated with PD seem to be more related to the degree of dopaminergic cell loss than to the severity of Lewy pathology.

Oxidative stress in sporadic PD

The etiology of sporadic PD is unknown, although mitochondrial dysfunction and oxidative and nitrosative stresses have been implicated in the mechanisms associated with PD pathogenesis [80,81].

Oxidative stress is characterized by a redox imbalance between the generation of free radicals or other reactive species and antioxidant defenses, and it may be related to changes in mitochondrial function and protein clearance [82]. There is considerable evidence for the involvement of mitochondrial dysfunction in sporadic PD, because multiple genes for which mutations or polymorphisms increase the risk of PD are linked to mitochondrial function [83]. Moreover, mitochondrial toxins were described to induce a phenotype similar to PD [84].

Analysis of biochemical markers for oxidative damage in tissue samples from patients or in postmortem brains provided direct evidence of oxidative stress in PD. Increased levels of lipid peroxidation were found in the SN, as suggested by decreased levels of polyunsaturated fatty acids (substrates for lipid peroxidation) and increased levels of malondialdehyde [85] and 4-hydroxynonenal [86]. The nucleic acid oxidation product 8-hydroxyguanosine is also elevated in the neurons affected in the disease, compared to surrounding brain regions in the PD brain, as well as in comparison to the SN of age-matched controls [87,88]. Lipid and DNA oxidation was also found to be systemically elevated in PD [82]. Oxidative and nitrative posttranslational modifications have been identified on proteins that may affect the disease progression [81]. This includes increased levels of oxidized protein carbonyls in the SN of PD patients [89,90] and nitration of tyrosine residues within LBs in the PD brain [91,92]. Increased 3-nitrotyrosine levels have also been detected after systemic administration of the PD-inducing toxin MPTP in baboons [93] and mice [94]. Along with the increase in oxidative damage, decreased levels of the antioxidant glutathione were found in the SN of PD patients [95–97].

Sources of oxidative damage in PD

The preferential loss of nigral neurons in PD, compared to other nearby catecholaminergic neurons, has been associated with the presence of neuromelanin, because neurons that contain this pigment are the most affected in the disease [98,99]. Neuromelanin appears to result from the autoxidation of dopamine, in a process that generates reactive oxygen species (ROS) [100] The presence of high amounts of iron in PD SN [101], which contribute to the production of free radicals via the Fenton-Haber Weiss reaction, has also been pointed out as a cause for selective degeneration of these neurons. In this reaction, hydrogen peroxide (H_2O_2) generated during normal metabolism, during electron transport in mitochondria, or by pathogenic mechanisms can be subsequently converted to the extremely toxic hydroxyl radical ($^{\bullet}OH$), which may elicit cellular damage, lipid peroxidation, and eventually apoptosis [102].

Under normal physiological conditions, excess iron can be sequestrated in ferritin and neuromelanin, and in this respect neuromelanin has been described to be neuroprotective [103]. Thus, elevated levels of iron observed in the SN of PD patients may reflect a dysfunction of brain iron homeostasis. Alternatively, the excess in iron content may be due to brain iron deposition associated with aging [104].

Oxidative stress in PD has also been suggested to be a consequence of complex I dysfunction [83]. Complex I impairment seems to be important for the pathogenesis of PD because exposure to inhibitors of this complex, such as MPP^+ or rotenone, reproduces the clinical symptoms of PD observed in human subjects [105,106]. Complex I is the largest of the electron transport chain (ETC) complexes, consisting of 46 subunits, 7 of which are encoded by mitochondrial DNA (mtDNA) [107], and the major site of superoxide production in the ETC [108]. Complex I activity and expression are decreased in the SN [109–„112] and cortex [113] of PD patients to a greater extent than would be expected from normal aging [107]. Oxidized, functionally impaired, and misassembled complex I subunits have been reported in PD [114]. Moreover, complex I dysfunction was reported in skeletal muscle and platelets of PD patients [112].

SN is also particularly vulnerable to toxic effects caused by nitric oxide ($^{\bullet}NO$), leading to neurodegeneration of dopaminergic and catecholaminergic cells, probably because of the presence of high concentrations of 6-hydroxydopamine (6-OHDA) in this structure, which readily reacts with $^{\bullet}NO$, generating dopamine semiquinones (described below) and increasing intramitochondrial and cytosolic peroxynitrite ($ONOO^-$) formation [83]. Indeed, NO is known to readily react with superoxide anions ($O_2^{\bullet -}$) to form the highly toxic $ONOO^-$, which induces cellular damage through protein nitration, lipid peroxidation, and DNA fragmentation [115]. Apart from the recognized $^{\bullet}NO$ control of complex IV activity and cell respiration, both $^{\bullet}NO$ and $ONOO^-$ have been reported as direct inhibitors of complex I, probably because of S-nitrosylation and Fe-nitrosation of complex I subunits [116]. Tyrosine nitration, protein oxidation, and damage to iron–sulfur centers with sustained complex I inhibition are associated with increased generation of $O_2^{\bullet -}$ by complex I [83].

Dopamine and oxidative stress

Even though neuronal alterations observed in PD are not restricted to dopamine neurons, the dopamine neurons of the nigrostriatal pathway are the major site of neuronal degeneration in PD [99]. Selective degeneration of the dopamine neurons of the SN suggests the possibility that dopamine itself may contribute to the neurodegenerative process [117]. Moreover, the loss of dopaminergic neurons in the SN, leading to the degeneration of the nigrostriatal pathway and resulting in the marked reduction in striatal dopamine, seems to be crucial for the development of the clinical features of the disease, as demonstrated by the remarkable response of most of the motor symptoms to the dopamine precursor L-DOPA (dopamine replacement therapy). Thus, dopamine may play a role in the selective degeneration of this brain area in PD. Under normal conditions, dopamine is synthesized from tyrosine by tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, and aromatic amino acid decarboxylase. Once formed, dopamine is safely stored in high-millimolar concentrations in synaptic vesicles after uptake by

VMAT2 [118]. Impairment of vesicular storage of dopamine, which may be due to the presence of α-syn protofibrils, oxidative stress, or weak base compounds such as methamphetamine (further described below) [119], leads to increased dopamine levels in the cytoplasm. Concordantly, α-syn overexpression was shown to increase cytosolic catecholamine concentration [120]. MPP^+ interaction with VMAT and redistribution of vesicular dopamine to the cytosol also cause intracellular dopamine oxidation [27]. Under conditions of oxidative stress, dopamine has the potential to form reactive metabolites by enzymatic and nonenzymatic mechanisms, which may further contribute to mitochondrial dysfunction and oxidative damage, accelerating dopaminergic cell death in PD. Dopamine may be metabolized intracellularly by monoamine oxidase (MAO), a mitochondrial enzyme present in the cytoplasmic side of the outer mitochondrial membrane, and aldehyde dehydrogenase, producing 3,4-dihydroxyphenylacetic acid and H_2O_2. MAO exists in two isoforms, MAO-A and MAO-B, which have different substrate specificity. Both isoforms oxidize dopamine, but in humans dopamine is preferentially oxidized by MAO-B, whereas in rodents it is predominantly oxidized by MAO-A [121].

In addition, autoxidation of the catechol ring of dopamine produces $O_2^{\bullet -}$ and H_2O_2, which may react with transition metal ions, such as iron, via the Haber-Weiss Fenton reactions, producing the highly toxic hydroxyl radical $^{\bullet}OH$. This $O_2^{\bullet -}$ may also easily react with $^{\bullet}NO$, forming the highly toxic $ONOO^-$.

The autoxidation of dopamine also produces the electron-deficient dopamine quinones or semiquinones, which is facilitated by the presence of transition metal ions [117]. Dopamine quinones readily react with cellular nucleophiles, such as the reduced sulfhydryl group on protein cysteinyl residues, and covalently modify protein structure [117]. These cysteinyl residues are often at the active site of proteins and, thus, covalent modification by dopamine quinones often results in inactivation of protein function, which may result in compromised cell survival. This mechanism may play a role in the degenerative process in PD [122]. Dopamine was found to modify proteins associated with the genetic forms of PD, such as α-syn [123], parkin [124], LRKK2, or UCHL1 [125]. Other proteins that are modified by dopamine oxidation include TH [126], superoxide dismutase 2, mitochondrial creatine kinase, mitofilin, and mitochondrial heat-shock protein 70 [125]. α-Syn was shown to interact with dopamine-quinones, stabilizing α-syn protofibrils [127] and promoting α-syn aggregation; interestingly, this process may also prevent dopamine-quinones from producing greater oxidative damage to dopaminergic neurons [128]. Excess α-syn also seems to potentiate the production of ROS by endogenous dopamine and cell death, involving the formation of soluble α-syn–14-3-3 protein complexes [129]. Moreover, direct binding and functional coupling of α-syn to the dopamine transporter (DAT; responsible for the reuptake of this neurotransmitter from the synaptic cleft) was reported to accelerate dopamine uptake, increasing dopamine levels in the cytosol and dopamine-induced apoptosis [130].

ROS are physiological activators of adenosine triphosphate-sensitive K^+ (KATP) channels, causing K^+ efflux and membrane hyperpolarization, which play a role in the physiological function of dopamine neurons, controlling dopamine release in the striatum. Dopamine neurons in the ventral tegmental area may be less sensitive to KATP channel activation than SN neurons, because of increased levels of uncoupler protein 2, which provides partial constitutive impairment of their respiratory chain and an intrinsic capacity to buffer mitochondrial ROS [131].

Oxidative damage is also known to impair ubiquitination and degradation of proteins by the proteasome [132]. Dopamine oxidation contributes to additional factors implicated in PD, such as protein aggregation, endoplasmic reticulum stress, and lysosomal dysfunction [117]. Thus, the combination of mitochondrial dysfunction, oxidative stress, and dopamine oxidation probably increases the vulnerability of dopaminergic neurons to degeneration in PD [133].

Excitotoxicity leading to mitochondrial dysfunction and oxidative stress

There are several lines of evidence suggesting a toxic effect of glutamate in the neurodegeneration of dopaminergic neurons in the SN [107], as evidenced by the protection conferred by *N*-methyl-D-aspartate (NMDA) receptor antagonists, such as memantine [134] and MK-801 [135–138], against MPP^+ neurotoxicity. Amantadine, another NMDA receptor antagonist, is currently used to treat PD in humans [139, for review]. Glutamate toxicity, or excitotoxicity, has been pointed out as an alternative source of oxidative stress in PD [107,140]. The firing patterns of cortical glutamatergic projections to the basal ganglia seem to be altered in PD. The glutamatergic projections to the subthalamic nucleus become overactive in PD and pharmacologic blockade of subthalamic neurotransmission (to the SN) has antiparkinsonian symptomatic effects and may also help to protect the remaining dopamine neurons of the SN from excitotoxic degeneration [140].

Excitotoxicity is caused by a massive influx of extracellular calcium resulting from the overactivation of the NMDA receptor. Cytoplasmic calcium activates several calcium-dependent enzymes involved in the degradation of proteins, phospholipids, and nucleic acids, many of which can elicit the generation of endogenous ROS or reactive nitrosative species [16, for review]. Activation of these pathways may lead to necrotic cell death, involving mitochondrial dysfunction, membrane breakdown, cytoskeletal alterations, and $^{\bullet}NO$-derived free radicals, or apoptosis.

Impairment of energy metabolism in PD, resulting from mitochondrial dysfunction, may affect the maintenance of the resting potential, thus increasing glutamate release, which exacerbates NMDA receptor activation. This may in turn induce a rise in cytosolic calcium and mitochondrial uptake of calcium, generating free radicals. Thus, mitochondrial dysfunction associated with the loss of calcium homeostasis and enhanced cellular oxidative stress has long been recognized to play a major role in cell damage associated with excitotoxicity [16]. Under normal conditions, calcium taken up by mitochondria can physiologically increase ATP generation by activating matrix dehydrogenases [141]. However, an increase in mitochondrial calcium can promote ROS and $^{\bullet}NO$ generation and promote the loss of cytochrome *c* due to the mitochondrial permeability transition, which can result in increased mitochondrial ROS release [142]. Mitochondrial buffering of calcium may be impaired in PD, as suggested by reduced carbonyl cyanide *m*-chlorophenylhydrazone-releasable calcium in PD cybrids [143]. Mitochondrial dysfunction can further induce α-syn oligomerization in PD cybrids via ATP depletion-driven microtubule depolymerization and ROS-driven protein oxidation [144].

Oxidative stress in familial PD

As described earlier, heritable cases of PD constitute early onset forms of the disorder [145–147]. Previous studies have shown evidence that the products of PD-associated genes have crucial effects on mitochondrial morphology, function, and oxidative stress [148].

Autosomal dominant forms of PD

Dominant mutations often act through a gain-of-function mechanism. To date, at least two genes, SNCA and LRRK2, have been shown to cause autosomal dominant PD, with a mutation frequency of 5% [26].

α-synuclein (SNCA)

SNCA (also known as PARK1) was the first gene identified in a large Italian family (Contursi kindred) segregating a pathogenic missense mutation—A53T—causing dominantly inherited PD and LB pathology [38,39]. Since then, two groups of disease-causing mutations have been described in the α-syn gene: (1) three missense point mutations (A53T, A30P, and E46K) [39,54,149,150] (Fig. 1) and (2) whole-locus multiplications, including duplications and triplications, leading to a pathogenic overexpression of the wild-type (WT) protein.

Human α-syn was first identified as the precursor protein for the non-amyloid-β component (NAC) of Alzheimer disease amyloid plaques [151]. Structurally, this small protein of 140 amino acids is characterized by: (1) a highly amyloidogenic domain (NAC), which itself presents a high propensity to aggregation and to form oligomeric structures and insoluble fibers [152]; (2) seven imperfect repeats (KTKEGV) at the N-„terminal, which form an amphipathic α-helical domain when the protein interacts with lipid-containing membrane microdomains [153]; and (3) an acidic C-terminal region [154] (Fig. 1).

Predominantly localized to presynaptic nerve terminals, α-syn can also be found in other neuronal compartments such as the cytosol, mitochondria, and nucleus. Although its physiological function is still unknown, it has been proposed to play a role in the integration of presynaptic signaling and neuronal plasticity [155]. Prior findings are consistent with these roles, with α-syn appearing to control synaptic vesicle fusion and recycling. Most data indicate that α-syn inhibits synaptic transmission in an activity-dependent manner, as observed in α-syn-deficient mice, exhibiting accelerated recovery of dopamine release when presented with multiple stimuli [156]. In addition, α-syn is also predicted to have specific effects in dopaminergic neurons, where it may negatively regulate the activity of TH, as well as the activity of DAT [157]. Products resulting from the interaction between dopamine and α-syn have been widely explored to address whether this interaction may produce toxic forms of α-syn [158]. Dopamine is prone to oxidation and its quinone derivatives react with proteins, in general via a covalent modification of a cysteine (as described above). α-Syn, however, does not possess a cysteine residue in its structure. Data from Norris et al. [159] suggest an ionic interaction between dopamine-quinone and residues 125–129 of α-syn. Dopamine modification of α-syn seems to maintain small α-syn oligomers in a reactive protofibril conformation by inhibiting progression to forming less reactive aggregates and this effect was more evident for α-syn mutant forms [160,161].

Fibrillization and aggregation of mutant α-syn play a central role in neurodegenerative mechanisms, because of either primary alterations in the peptide sequence or increased levels of physiological protein, possibly exceeding existing pathways of degradation. An increase in proteins that are determined to be degraded by the UPS and impairment of the UPS function are contributive factors to proteolytic stress due to accumulation and aggregation of proteins in cytosol. How α-syn is degraded is still a matter of debate, but it is likely that both UPS and the lysosomal degradation pathway intervene in regulating the steady-state levels of the protein under normal physiological conditions. Degradation of α-syn by the proteasome [162,163] and the formation of inclusions in neuronal cells treated with proteasome inhibitors have been described [164,165]. On the other hand, the involvement of autophagy and lysosomes has also been demonstrated [166–„169]. Evidence has been shown for mutant, and possibly excessive normal, α-syn facilitating its pathological accumulation by impairing the function of protein-degradation processes [170–„173]. Cuervo et al. [170] identified, in the α-syn sequence, the pentapeptide motif required to target cytosolic proteins to lysosomal degradation via chaperone-mediated autophagy (CMA). In cellular models and rodents, α-syn was shown to interact with lysosome-associated membrane protein 2A (LAMP-2A), a transmembrane receptor of substrates for lysosomal degradation, giving evidence to the theory that CMA is involved in its clearance [170,174]. Accordingly, LAMP-2A gene silencing slowed the degradation of monomeric and oligomeric species of α-syn in primary neurons of the ventral midbrain [174]. CMA, however, was not the only pathway for α-syn degradation. Vogiatzi et al. [174] also reported that clearance of WT α-syn was in part mediated by macroautophagy. The study involved the selective macroautophagy inhibitor 3-„methyladenine, which led to a considerable increase in the steady-state levels of α-syn in PC12 cells and in primary cortical and ventral midbrain neurons, suggesting that dysfunction of this degradation process could also contribute to the gradual accumulation of endogenous WT α-syn

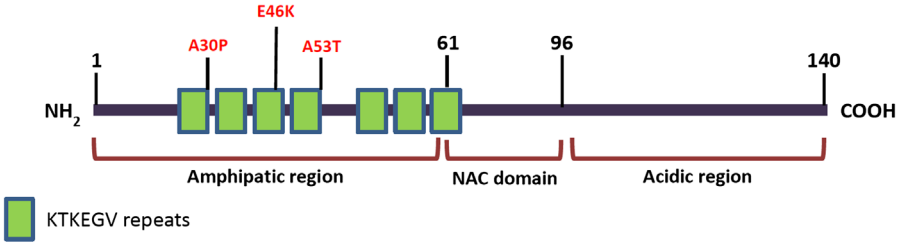

Fig. 1. Schematic representation of the α-syn protein. α-Syn is a 140-amino-acid protein with seven imperfect repeats (KTKE GV) localized in the N-„terminal. It has three distinct regions: the amphipathic region in the N-„terminal, a central hydrophobic NAC (non-amyloid-β component) domain, and an acidic C-„terminal region. The three pathogenic missense mutations are represented in red. Numbers above the protein line indicate the limits of each region. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

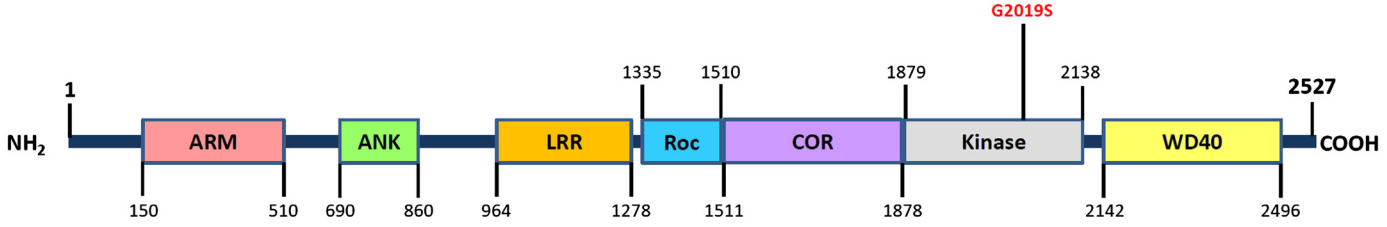

Fig. 2. Schematic representation of the LRRK2 protein. LRRK2 is a 2527-amino-acid protein, constituted by several conserved domains: ARM (armadillo), ANK (ankyrin repeat), LRR (leucine-rich repeat), Roc (Ras of complex proteins: GTPase), COR (C-terminal of Roc), kinase domain, and WD40. Numbers below and above the protein line indicate the limits of each domain. The single most common mutation occurring in LRRK2, G2019S, is present in the kinase domain of the protein and is represented in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in sporadic PD. Furthermore, oxidative stress linked to mitochondrial dysfunction may also increase misfolded proteins and therefore lead to aggregation of α-syn and subsequent death of dopaminergic neurons [175]. Thus, α-syn aggregation itself may also contribute to increased oxidative stress levels, leading to a vicious cycle in the cell [176]. Pesticides such as paraquat and maneb were found to lead to proteasomal dysfunction and nitrative/oxidative damage causing an upregulation and subsequent fibrillization of recombinant α-syn [177,178]. MPTP, a known selective dopaminergic neurotoxin that inhibits mitochondrial complex I activity (as mentioned above), creates an oxidative stress environment that enhances α-syn aggregation and consequent death of dopaminergic neurons [176]. Moreover, Dauer and colleagues [179] investigated the toxic effect of MPTP in mice lacking the SNCA gene and concluded that these animals were resistant to MPTP, giving support to a critical role for α-syn in the pathogenesis of toxin-induced dopaminergic neuron death. A recent study performed by Kalivendi et al. [180] demonstrated that oxidant-induced alternative splicing of α-syn plays a crucial role in the mechanism of dopamine neuron cell death contributing to PD. The authors provided evidence that the parkinsonian mimetic MPP^+ is able to induce alternative splicing of α-syn, leading to the deletion of exon 5, which resulted in the generation of a 112-aa protein, instead of the 140-aa WT α-syn. This process was deleterious to dopamine cells, because the expression of the 112-aa form of α-syn exhibited a more pronounced effect on proteasomal dysfunction and enhanced cell death, compared to WT α-syn. Although α-syn function is not yet defined, there seems to be a reciprocal relationship between the activity of this protein and mitochondrial function. As described before, oxidative damage to α-syn affects its aggregation—an effect that partially explains the cellular toxicity of the protein. α-Syn contains an N-terminal mitochondrial-targeting sequence [181], and cytosol acidification or α-syn overexpression can lead the protein to localize to mitochondria [182,183], resulting in complex I impairment and increased ROS production [181], increased protein tyrosine nitration, and decreased mitochondrial transmembrane potential [184]. In addition, it was shown that human embryonic kidney cells that overexpress α-syn show enhanced susceptibility to cell death and have lower ATP levels compared to control cells [183]. In brain-stem neurons of mice that overexpress human A53T mutant α-syn, there is evidence of degenerating and dysmorphic mitochondria with DNA damage [185]. Furthermore, in both SH-SY5Y neuroblastoma cells and isolated rat mitochondria, α-syn seems to induce mitochondrial release of cytochrome *c*, increased mitochondrial calcium and NO, and oxidative modification of mitochondrial components. These findings suggest a pivotal role for mitochondria in oxidative stress and apoptosis induced by α-syn [186]. In a model of aging yeast, functional mitochondria are required for α-syn toxicity [187].

In addition to all these facts, it has been recognized that α-syn is one of the most abundant components of LBs; α-syn can aggregate under a variety of posttranslational modifications, namely phosphorylation at serine-129, ubiquitination, C-terminal truncation, and nitration [40,188–„193]. Whether these modifications contribute to progressively conducting the protein into insoluble fibrils and modulating toxicity is still controversial.

Leucine-rich repeat kinase 2 (LRRK2)

Mutations in LRRK2 represent the most common known cause of familial PD and are inherited in autosomal dominant form, but with a significant variation in penetrance. The resulting disease phenotype is identical to that of sporadic PD, except for a slightly lower age at diagnosis [194], which has increased the interest in this gene. The LRRK2 gene encodes a large multidomain protein with 2527 amino acids, the physiological function of which is currently unknown. However, there are numerous functional domains associated with this protein, including a leucine-rich repeat (LRR) domain, a Ras of complex protein (Roc) GTPase domain followed by its associated C-terminal of Roc domain, a kinase domain of the tyrosine kinase-like subfamily homologous to other mitogen-activated protein kinase kinase kinases, and a C-terminal WD40 domain [195] (Fig. 2). This combination of motifs is highly conserved in vertebrates [196]; however, LRRK2 is unusual, because it encodes two distinct enzymes, kinase and GTPase, in the same molecule. Initial biochemical studies on LRRK2 suggested that this protein is able to undergo phosphorylation and to phosphorylate generic substrates such as moesin and myelin basic protein [197–„200] and that the kinase activity is mildly regulated by the GTPase domain [201–„204]. The presence of multiple protein-interaction domains, namely armadillo, ankyrin, LRR, and WD40 (Fig. 2), suggests that LRRK2, in addition to its predicted protein kinase GTPase activities, might work as a multiprotein signaling complex.

The single most common mutation occurring in LRRK2, G2019S, is present in the kinase domain of the protein and augments the kinase activity [200]. This mutation is responsible for both familial (4% worldwide) and apparently sporadic (1% worldwide) PD cases and has a very low frequency in healthy populations (< 0.1% worldwide) [194,205–„207]. Particularly, most patients with the G2019S mutation exhibited α-syn-positive LBs as in sporadic PD [206,208,209]. Tong et al. [210] showed that accumulation and aggregation of α-syn and ubiquitinated proteins occurred in aged LRRK2 germ-line deletion mice with great loss of LRRK2. The autophagy–lysosomal pathway was impaired together with an increase in apoptotic cell death, inflammatory response, and oxidative stress.

Although LRRK2 is mainly localized to the cytoplasm, approximately 10% of the protein is associated with the outer mitochondrial membrane [200], raising an important question of whether mutant LRRK2 kinase increased activity might directly affect mitochondrial function. Furthermore, impaired mitochondrial function and an increased susceptibility to apoptosis were observed in ex vivo models of fibroblasts from PD patients carrying the G2019S mutation in the LRRK2 gene or based on induced pluripotent stem (iPS) cells that carry the G2019S mutation [211,212]. In addition, dopaminergic neurons derived from G2019S-iPS cells showed an increase in key oxidative stress response genes and pathogenomic accumulation of α-syn [212].

These studies support an involvement of LRRK2 in mitochondrial dysfunction and impaired protein degradation pathways with α-syn accumulation and speculation on the kinase activity of the protein, which seems to be required for cellular toxicity and neuronal death.

Autosomal recessive forms of PD

Most recessive alleles result in the absence of the encoded protein or inactive protein and consequently in a loss of function. Homozygous or heterozygous mutations in the recessive genes parkin (PARK2), PINK1 (PARK6), and DJ-1 (PARK7) are unequivocally associated with a heritable early age at onset, in most cases before age 40, and no atypical signs [213–„215]. Furthermore, autosomal recessive PD is characterized by: (1) slowly progressive disease course; (2) excellent response to L-„DOPA, despite early L-DOPA induced dyskinesias; and (3) minimal cognitive decline and dysautonomia.

Parkin

Mutations in the parkin gene at PARK2 are the most frequent known cause of early onset (< 40–50 years) PD (10–20% worldwide; 50% of recessive familial forms) [216,217]. Pathological changes include significant loss of dopaminergic neurons in the SN and milder changes in the locus coeruleus. Initially LBs were thought to be absent

in the brain [218], but LB-positive cases have been reported in a reduced number of patients with parkin mutations [219].

The parkin gene encodes a protein of 465 amino acids that contains an N-terminal ubiquitin-like domain followed by three RING finger domains (RING 0–2) separated by an in-between-ring domain localized at the C-terminal part. Like other RING-finger proteins, parkin acts as a Ub E3 ligase. It has an important role in the function of Ub, which is primarily involved in the targeting of aggregation-prone substrates for degradation by the UPS, by conferring substrate specificity [220]. Many ubiquitination substrates have been proposed for parkin, including the aminoacyl-tRNA synthetase cofactor, p38, and a rare 22-kDa glycosylated form of α-syn [221–„223]. Parkin is predominantly a cytosolic protein, but also colocalizes to synaptic vesicles, the Golgi complex, the endoplasmic reticulum, and the mitochondrial outer membrane [223–„227]. Many PD-linked point mutations alter WT parkin cellular localization, solubility, or propensity to aggregate [228–„230]. Other mutations, including insertions or deletions, lead to parkin loss of function, specifically loss of its ligase activity, resulting in accumulation of toxic substrates and degeneration [220]. However, possibly the most relevant activity of parkin is related to its neuroprotective function against a variety of pathogenic factors. Particularly, this neuroprotection is given by delaying mitochondrial swelling and rupture and the subsequent cytochrome *c* release and caspase 3 activation, as shown in cells overexpressing parkin [227]. As mentioned above, parkin can associate with the mitochondrial outer membrane, suggesting a direct and local protective effect [231]. The protein is involved in the regulation of transcription and replication of mtDNA in proliferating cells, stimulating this organelle's biogenesis—an effect blocked by parkin short-interfering RNA (siRNA) knockdown [231]. Furthermore, in a parkin knockout mouse line, a reduced number of mitochondrial oxidative phosphorylation proteins, decreased mitochondrial respiratory capacity, age-dependent increased oxidative damage, and increased protein and lipid peroxidation were reported [232], leading to nigrostriatal damage. Changes in mitochondrial morphology have also been observed, but this led only to disruption of complex I function in nigral mitochondria and did not result in cell death [233]. Thus, mitochondrial defects and an increased susceptibility to oxygen radical damage were also reported in a parkin knockout model of *Drosophila,* suggesting that abnormalities in parkin ubiquitination function might be secondary in the course of pathogenic events [234,235]. In vitro studies of a PARK2-knockdown SH-SY5Y neuroblastoma cell line showed apoptotic cell death and high levels of autoxidized forms of L-DOPA and dopamine, suggesting that parkin might have important antioxidant properties [236]. Rothfuss and coworkers [237] confirmed that the association between parkin and mtDNA led to protection from oxidative stress and stimulation of mitochondrial genome repair. These functions were damaged in parkin-deleted human fibroblasts [237].

Overall, functional parkin seems to be crucial in the maintenance of mitochondrial antioxidant defenses and protection of mtDNA.

PINK1

PINK1 gene (PARK6) mutations are the second most common cause of autosomal recessive, early onset parkinsonism [147]. Although age at onset for PINK1-related PD is usually in the fourth or fifth decade, clinical features are similar to late-onset PD, with slow progression, excellent response to L-„DOPA, and, in some cases, dementia [238–„241]. Nigrostriatal neuronal loss has been observed in postmortem brains of PD patients with PINK1 mutations [242].

PINK1 is a putative serine/threonine kinase, constituted by 581 amino acids, which is expressed ubiquitously. Homozygous missense and nonsense mutations affecting the kinase domain, as well as insertions, deletions, and truncation of the protein, have been observed, all of them supposedly impairing the kinase activity [243,244].

Because of its N-terminal mitochondrial targeting sequence, PINK1 is localized to the mitochondrial intermembrane space and bound to mitochondrial membranes. However, as PINK1 was also identified as a component of LBs in patients with sporadic PD [242], its final destination within mitochondria is not entirely defined.

The physiological role of PINK1 in mitochondria is thought to include the phosphorylation of mitochondrial proteins in response to cellular stress and the protection against mitochondrial dysfunction [203]. There is evidence that WT PINK1, in contrast to mutant PINK1, may protect neurons from stress-induced mitochondrial dysfunction and apoptosis [245]. Furthermore, loss of PINK1 function in human cell lines produced morphological changes in mitochondria and impaired energy metabolism, showed by a decreased mitochondrial membrane potential [243]. Cells isolated from individuals with a PINK1 mutation that causes a G309D substitution demonstrate reduced complex I activity and evidence of increased oxidative damage compared with cells from age-matched controls [246]. Furthermore, PINK1 deficiency in a *Drosophila* model results in the loss of dopaminergic cells, as well as increased susceptibility to oxidative stress and reduced ATP levels [247]. These mutants also showed reduced mitochondrial mass with disorganized morphology. Moreover, PINK1 seems to exert neuroprotective properties, as shown by Petit et al. [248], in which WT, but not mutant PINK1, attenuated staurosporine-induced apoptosis and reduced mitochondrial cytochrome *c* release when overexpressed in SH-SY5Y cells. Also, silencing of PINK1 increased susceptibility to MPP^+ or rotenone [248]. In this context, decreased expression of PINK1 in human dopaminergic neurons led to reduced long-term survival, along with morphological/structural mitochondrial abnormalities and higher levels of oxidative stress [249].

PINK1 and parkin

PINK1 mutants share many phenotypic features with parkin mutants [250,251]. Therefore, it was suggested that the two proteins could act in common pathways, with parkin working downstream of PINK1, because transgenic expression of parkin rescued all PINK1 impairments, but not the contrary [243,250,251]. Goldman et al. [252] reported that PINK1 and parkin both play a role in the mitochondrial targeting of autophagy, a process by which the cells degrade intracellular material through lysosomes. Mitophagy, which is the mitochondrial-specific form of this process, is important in the control of damaged or stressed cellular organelles. The process appears to be upregulated after alterations in mitochondrial membrane permeability [253]. Parkin is selectively recruited from the cytosol to the mitochondria with low membrane potential, where it mediates the degradation of the impaired organelle [254]. PINK1 works as the biochemical signal that allows parkin to identify dysfunctional mitochondria, whose levels are regulated by voltage-dependent proteolysis [255]. The E3 ligase function of parkin, which is constitutively inhibited, is activated upon PINK1-dependent mitochondrial localization [256]. Therefore, defective mitophagy due to a lack of parkin recruitment to impaired mitochondria by PINK1 causes the accumulation of dysfunctional mitochondria and subsequent enhanced ROS levels and proapoptotic proteins [257]. In addition, overexpression of PINK1 promotes mitochondrial fission, whereas inhibition of the protein causes excessive fusion [258,259]. Fibroblasts from PD patients carrying homozygous PINK1 mutations had truncated mitochondria networks compared to control fibroblasts, when cultured in medium with low glucose or in the presence of galactose to favor mitochondrial energy metabolism over glycolysis [243,260]. Moreover, in

fibroblasts from patients with parkin gene mutations or subjected to siRNA-mediated parkin gene silencing, an increase in mitochondrial interconnectivity was observed, measured as branching, with no differences in organelle length, compared to control cells [261]. After treatment with the complex I inhibitor rotenone, these fibroblasts from PD patients revealed significantly shorter mitochondria, which was consistent with the hypothesis that parkin-deficient cells are more susceptible to insult-induced fission. These observations suggest that the PINK1/parkin pathway may indeed change the balance of mitochondrial dynamics toward fission under basal conditions, but favor a higher degree of functional connectivity of the organelles under conditions of energy depletion or high energy demand. Interestingly, mitochondrial branching was more evident after mitochondrial complex I inhibition in control fibroblasts, which may indicate that increasing mitochondrial connectivity may represent a protective response of cells to an energy crisis [261].

Piccoli and colleagues [262] also performed studies on fibroblasts from a patient with a PINK1 mutation and observed a low mitochondrial respiratory activity and increased oxygen radical production due to complex I inhibition, demonstrating clinically what has been shown experimentally.

DJ-1

Mutations in the DJ-1 gene, also known as PARK7, are the least common cause of autosomal recessive PD (approximately 1% of early onset PD). The DJ-1-related phenotype, with early onset and slow disease progression, closely matches that of patients with parkin or PINK1 mutations, but genotype/phenotype correlations could not be precisely performed, because of the small number of DJ-1 patients [146]. The DJ-1 gene encodes a protein of 189 amino acids, belonging to the peptidase C56 family of proteins [263]. It is a cytoplasmic protein, but under stress conditions can also translocate into the mitochondria and nucleus [146,264]. WT DJ-1 appears to exert an antioxidant function [265–„268], and deletions and point mutations in the DJ-1 gene cause a loss of its physiological function and, therefore, lead to neurodegeneration in rare families [146,269–„271]. Knockdown of DJ-1 by siRNA in the SH-SY5Y human neuroblastoma cell line enhanced susceptibility to several oxidative insults, including H_2O_2, MPP^+, and 6-OHDA [270]. Reciprocally, DJ-1 overexpression in these cells resulted in increased resistance to these insults and reduced intracellular ROS formation [272]. This protection seems to be selective against environmental oxidative stress in vivo, as shown in DJ-1 null *Drosophila* treated with paraquat and rotenone [273]. Thus, DJ-1β-deficient flies displayed a locomotor deficit that was exacerbated by oxidative stress [274]. Furthermore, the levels of DJ-1 modification increase with age, also leading to significant increments in oxidative stress and inactivation of its own function [275]. DJ-1-deficient mice demonstrated hypersensitivity to MPTP and this was observed by increased dopaminergic neuronal loss and striatal denervation [276]. In embryonic cortical neurons, an increased sensitivity to oxidative stress and proteasomal inhibition has also been demonstrated, ending in apoptotic cell death [276,277]. All impairments were reversed by restitution of DJ-1 expression. DJ-1 is normally activated by an oxidative cytoplasmic environment [278]. Shendelman and colleagues [278] described a role for DJ-1 as a redox-sensitive molecular chaperone, which was able to inhibit α-syn aggregate formation. Another study in DJ-1-deficient mice showed that the complex I inhibitor paraquat decreased proteasome activity concomitant with decreased ATP and regulatory subunit levels [279]. In addition to these effects, the levels of a transcription factor (nuclear factor erythroid 2-related factor 2) that activates cytoprotective genes were reduced. This provided evidence for a role for DJ-1 as a regulator of transcription. The mechanism by which DJ-1 protects against oxidative stress was described by Im et al. [280]. In that study, the authors demonstrated that DJ-1 regulates the MAP3 kinase apoptosis signaling-regulating kinase 1 (ASK1)/thioredoxin 1 (Trx1) complex. ASK1 is a major effector of cell death induced by oxidative stress and is physiologically inhibited by Trx1. Oxidative insults disrupt this complex, and DJ-1 null cells are more susceptible to this dissociation, leading to increased activation of downstream cell death mediators.

A mitochondria–DJ-1 association has also been established. Lev et al. [272] described a cellular redistribution of DJ-1 in cells exposed to neurotoxins. This work was extended by Hayashi et al. [281], who showed DJ-1 binding to NADH dehydrogenase (ubiquinone) 1α-subcomplex 4 and to mitochondrial-encoded NADH dehydrogenase 1, nuclear and mitochondrial DNA-encoding subunits of complex I, respectively, validating the importance of DJ-1 in mitochondrial function. Recently, a direct link between loss of DJ-1, impaired mitochondrial stress response, and reduced clearance of mitochondria by lysosomal degradation was described [282]. An accumulation of fragmented and dysfunctional mitochondria upon reduced basal autophagy contributed to the loss-of-function phenotype in cells from DJ-1 knockout mouse and human carriers of the E64D mutation in the DJ-1 gene [282].

In summary, PD individuals with autosomal recessive inheritance differ generally from those with idiopathic PD, although cases with clinical evolution similar to that of the typical disease have been reported. An increasing body of recent information provides strong support to the idea that mitochondrial dysfunction may be central to the pathophysiology of familial PD, particularly to that of parkinsonian syndromes with autosomal recessive inheritance that directly associate with mitochondria, such as parkin, PINK1, and DJ-1.

Psychostimulant drugs of abuse and PD

Psychostimulant drugs of abuse are psychoactive drugs that induce temporary improvements in mental and/or physical function, such as enhanced alertness, wakefulness, and locomotion. These drugs increase the extracellular concentration of monoamines, such as dopamine, leading to increased dopamine transmission [283]. Dopamine is involved in controlling reward, cognition, and movement, explaining why these drugs of abuse affect locomotion [284,285]. Thus, dopamine and its related oxidative stress are a common link between PD and psychostimulant neurotoxicity.

Some common aspects between psychostimulant abuse and PD may be found, and exposure to amphetamines was suggested to affect the progression of PD, because increased risk of PD was found in individuals hospitalized with conditions related to the use of methamphetamine or other amphetamine-type drugs [286]. Thus, amphetamines may be environmental factors for PD, accounting for a percentage of sporadic PD cases.

Similar to PD, the neurotoxicity observed upon exposure to amphetamines may be due to dopamine-induced oxidative stress. Amphetamines increase extraneuronal dopamine and other monoamine levels, through a nonexocytotic mechanism, by directly interacting with monoaminergic cells [283,287,288]. Because of its structural similarity to dopamine, amphetamine is a substrate for the DAT [289] and, when in low concentrations, it is transported by the DAT to the cytosol. This leads to an increase in the intracellular binding sites of DAT for dopamine, resulting in the exchange of extracellular amphetamine by intracellular dopamine and leading to an increase in extracellular dopamine [290]. When present in higher extracellular concentrations, amphetamine may diffuse into the cell, because of its lipophilicity [291,292]. Amphetamine also interferes with VMAT2 function, impairing the active transport of the monoamines into synaptic vesicles, where they are stored. In addition, amphetamine may enter into the vesicles by diffusion,

because of its weak base properties [293, for review]. Because amphetamine is a weak base, at acidic pH it accepts protons, leading to alkalinization inside the vesicles. A low pH inside the vesicles is essential to maintaining the proton gradient used by VMAT2 for active transport of monoamines into the vesicles. Therefore, amphetamine induces the release of vesicular dopamine into the cytosol and impairs the storage of dopamine in the vesicles. Cytosolic dopamine is then released into the extracellular space via reverse transport by the DAT. Amphetamine also interferes with dopamine metabolism, by inhibiting MAO [294], and with dopamine synthesis, by inhibiting TH [295]. Thus, long-term exposure to amphetamine leads to dopamine depletion [296] and reduction in dopaminergic markers [297].

Amphetamines have been shown to induce common features of PD. A significant association between variations in a highly polymorphic sequence (T10A7) of the α-syn gene and methamphetamine psychosis/dependence was found in female human abusers of methamphetamine [298].

Neurotoxic events occurring in amphetamine consumption and in PD seem to share a number of steps related to the mechanisms of neuronal dysfunction involving the UPS. In particular, amphetamines were previously described to induce neuronal inclusions and affect proteins belonging to the UPS, in the central nervous system [299]; moreover, ultrastructural and molecular changes were similar to those occurring in PD [300]. Amphetamine-induced neuronal inclusions present many features similar to those observed in PD, and the maturation of these inclusions can be reproduced by continuous exposure to parkinsonian neurotoxins such as rotenone [84,301] or MPTP [302]. In mice, the occurrence of neuronal inclusions combined with increased nigral expression of α-syn within dopaminergic neurons was found upon treatment with amphetamine derivatives [303], similar to what occurs after administration of the parkinsonian toxin MPTP [304]. Furthermore, treatment of mice with methamphetamine increased markers of oxidative stress in the striatum and increased the levels of oligomeric α-syn in the SN [305]. In this context, increased α-syn expression after methamphetamine may be a

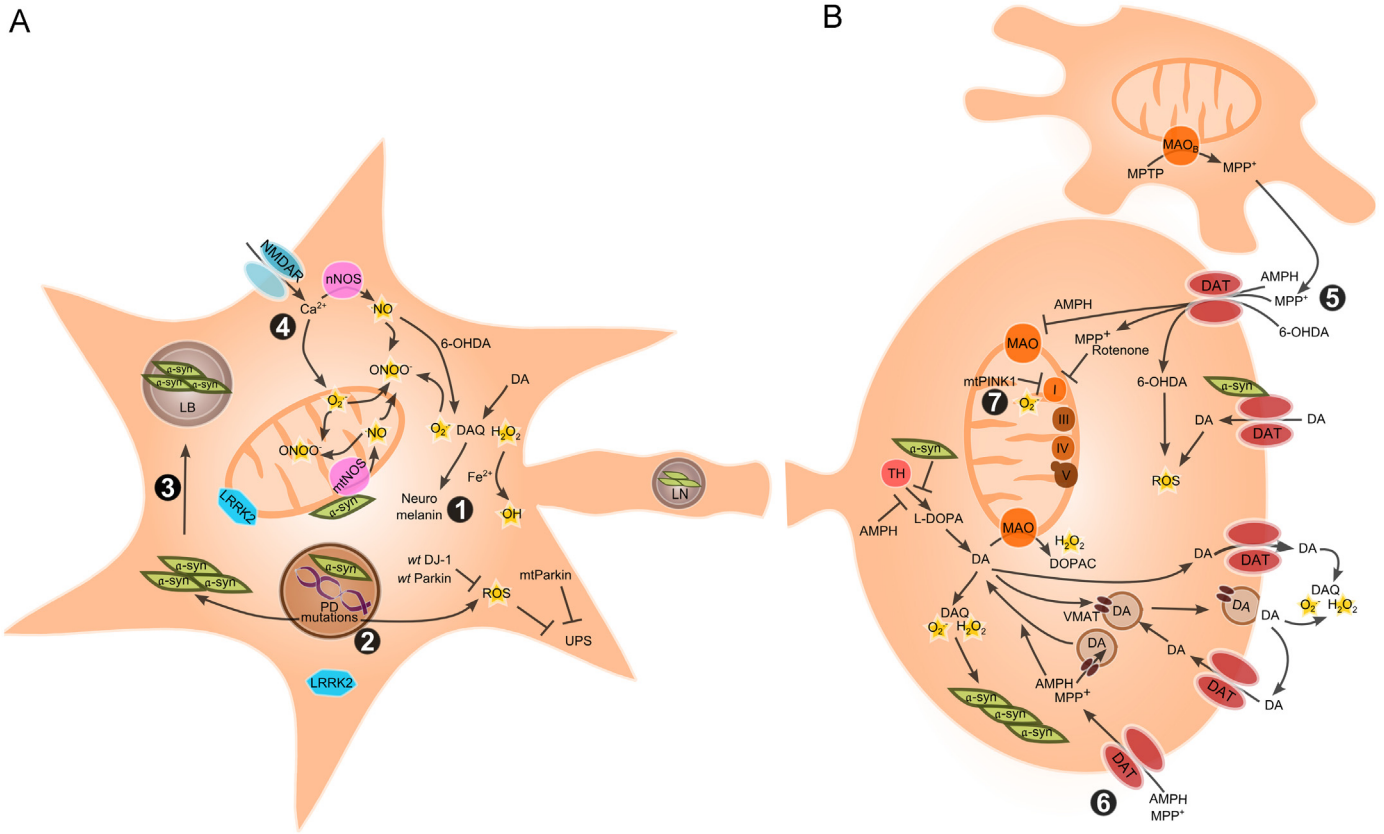

Fig. 3. Mechanisms involved in oxidative stress in familial and sporadic Parkinson disease (PD). (A) (1) Dopaminergic neurons in the substantia nigra are selectively affected in PD. These neurons contain Fe^{2+} and neuromelanin, which result from dopamine (DA) oxidation, in a process that generates dopamine quinone (DAQ) and reactive oxygen species (ROS). Neuromelanin may also be protective, because it binds Fe^{2+}, known to promote the formation of the highly toxic hydroxyl ($^{\bullet}OH$) radical by the Fenton reaction. (2) Familial PD may be caused by duplication/triplication of α-synuclein (α-syn) or by mutations in this and other proteins, such as LRRK2, DJ-1, parkin, and PINK1, which may induce oxidative stress by gain or loss of function. Wild-type DJ-1 and parkin have antioxidant effects that are lost with the pathogenic mutations. (3) A common characteristic of various forms of PD is the occurrence of α-syn aggregation and the presence of α-syn-containing Lewy bodies (LB) and Lewy neurites (LN). (4) Impairment of energy metabolism in PD, resulting from mitochondrial dysfunction, may increase glutamate release, exacerbating *N*-methyl-D-aspartate (NMDA) receptor activation. This leads to an increase in intracellular Ca^{2+}, which activates neuronal nitric oxide ($^{\bullet}NO$) synthase (nNOS), leading to the formation of $^{\bullet}NO$, which readily reacts with the superoxide anion ($O_2^{\bullet -}$), forming the highly toxic peroxynitrite ($ONOO^-$). (B) (5) PD phenotype may be re-created by a number of mitochondrial toxins. MPTP is converted into MPP^+ by glial monoamine oxidases (MAO-B) and this metabolite may be transported by the dopamine transporter (DAT) into the DA nerve terminals, where it inhibits mitochondrial electron transport chain (ETC) complex I; 6-„hydroxydopamine (6-OHDA) is also taken up by DAT and may be oxidized in the cytosol, leading to DAQ and ROS formation; rotenone is a pesticide that induces a PD-like phenotype by inhibiting mitochondrial complex I. (6) Amphetamine drugs directly affect DA-ergic nerve terminals, reproducing most of the PD molecular hallmarks. Amphetamine (AMPH) is taken up by the DAT and impairs the active transport of DA into synaptic vesicles, leading to an increase in cytosolic DA, which is metabolized by MAO, leading to H_2O_2 formation, or by autoxidation, with the generation of DAQ, $O_2^{\bullet -}$, and H_2O_2. DAQ may react with α-syn, stabilizing α-syn protofibrils and promoting α-syn aggregation. This may prevent DAQ from producing greater oxidative damage in DA-ergic neurons. Cytosolic DA may also be released to the extracellular space via reverse transport by the DAT. AMPH also interferes with DA metabolism, by inhibiting MAO, and with DA synthesis, by inhibiting tyrosine hydroxylase (TH), which can lead to dopamine depletion after prolonged exposure to this drug. AMPH also induces mitochondrial dysfunction involving a loss of mitochondrial potential. (7) Mutant PINK1 may also inhibit mitochondrial complex I.

protective mechanism, because α-syn fibrils sequester the toxic species formed upon intracellular dopamine oxidation, preventing further oxidative damage [128].

Exposure to amphetamine or amphetamine derivatives has been shown to induce oxidative stress in the nervous system [306,307]. Methamphetamine and D-amphetamine have been shown to increase ROS levels upon in vitro [308,309] or in vivo [310,311] exposure and the toxic effects of these drugs may be prevented by antioxidants. Moreover, in human methamphetamine abusers the activity of antioxidant enzymes was affected in several regions of the brain [312]. In animal models, methamphetamine [313] and D-amphetamine [314,315] also affected the activity of antioxidant enzymes. In addition, evidence of oxidative damage has been reported in the brains of human methamphetamine abusers [316] and animals exposed to methamphetamine [313] or D-amphetamine [310].

In PC12 catecholaminergic cell cultures, we have previously shown that prolonged exposure to low (nontoxic) concentrations of D-amphetamine induces a partial protection against H_2O_2-induced toxicity, which was suggested to be associated with adaptation to oxidative stress [317]. Other authors showed that a short-term exposure to subtoxic concentrations of methamphetamine can protect dopaminergic cells against a larger oxidative stress injury, through upregulation of Bcl-2 [318].

Thus, amphetamines affect the major mechanisms associated with sporadic and genetic PD, including dopamine depletion, α-syn oligomerization, UPS, and mitochondrial dysfunctions and oxidative stress.

General conclusions

Cardinal cellular features of idiopathic PD are classically linked to mitochondrial dysfunction and oxidative stress, which has a strong basis in both mitochondrial complex I inhibition and dopamine autoxidation. Interestingly, α-syn, a protein present in LBs and whose expression and mutations cause one of the familial autosomal dominant forms of PD, promotes ROS formation and interacts with dopamine quinones. On the other hand, LRRK2 mutations have been linked to late-onset cases of PD (resembling sporadic forms), leading to altered mitochondrial function and α-syn aggregation. Evidence for mitochondrial dysfunction and oxidative stress is also very clear today in some autosomal recessive familial forms of PD caused by mutations in parkin, PINK1, and DJ-1, which directly associate with and regulate mitochondrial function, dynamics, and degradation.

Interestingly, abuse of psychostimulants, namely amphetamines, has been largely described as affecting dopaminergic transmission and function and inducing dopamine depletion. Although these drugs may not directly evoke PD, they may predispose the central nervous system for PD-like syndromes in long-term exposure, as evidenced by neurotoxic events linked to dopamine-induced oxidative stress and decreased protein quality control. Taking into account the described commonalities between PD pathogenesis and amphetamine addiction, defining effective strategies for protection of dopaminergic neurons in PD patients could also benefit drug abusers. Fig. 3 summarizes the sources of oxidative stress in familial and sporadic PD, as well as the mechanisms elicited by amphetamine drugs of abuse, as described in this review.

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