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Forum Review Article

Parkinson-Linked Genes and Toxins That Affect Neuronal Cell Death Through the Bcl-2 Family

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Abstract

Parkinson's disease (PD) results from the death of specific neuronal populations in the CNS. Potential causative factors include environmental toxins and gene mutations that can combine to dysregulate the processing and degradation of α-synuclein. Oxidative stress induced by the neurotoxins MPTP, paraquat, maneb, and rotenone causes lipid peroxidation and protein misfolding that affects cell death through members of the Bcl-2 family. Sufficient activation of Bax and Bak facilitates mitochondrial outer-membrane permeabilization, which releases death-inducing factors that cause apoptotic and nonapoptotic programmed cell death. The formation of α-synuclein aggregates is a defining pathologic feature of PD and is induced by these neurotoxins as well as several Parkinson-linked familial mutations. Of the familial mutations identified thus far, two of the loci encode proteins associated with ubiquitin-proteasome degradation of misfolded proteins (Parkin and Uch-L1), and two encode proteins associated with mitochondria and oxidative stress (DJ-1 and PINK1). Both gene and toxin findings indicate that dopaminergic neuron losses in PD are the result of oxidative stress affecting mitochondria function and ubiquitin-proteasome activity. Here we describe how related cell death mechanisms are involved in the pathophysiology of Parkinson's disease. *Antioxid. Redox Signal.* 11, 529–540.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder of the elderly, affecting ~1% of the population older than 60. It was first described by James Parkinson in an 1817 monograph, "Essay on the Shaking Palsy." Patients typically are first seen with resting tremor, slow movements (bradykinesia), limb stiffness (rigidity), and a shuffling gait. Many patients also have autonomic, cognitive, and psychiatric disturbances. The major symptoms of PD result from the loss of catecholaminergic neuron populations in the brainstem, the most profound of which is a depletion of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNpc). In recent years, several new genes and environmental factors have been implicated in PD, and their impact on DA neuronal cell death is coming into focus.

Neuropathology in PD

Nigrostriatal projections from DA neurons in the SNpc innervate regions of the striatum that modulate thalamic activation of motor and somatosensory areas in the neocortex. PD symptoms are not usually noticed until a depletion of >70% of DA neurons in the SNpc has occurred. Surviving neurons in the affected nuclei often contain cytoplasmic eosinophilic inclusions, referred to as Lewy bodies, which contain fibrillar α-synuclein (9). Although it is commonly thought that PD neuropathology is limited to DA neuron loss in the SNpc, synucleinopathy and neurodegeneration extend well beyond these neurons (13, 43). Neurodegeneration and Lewy body formation also occur in noradrenergic (*locus coeruleus*), serotonergic (*raphe*), and cholinergic (*nucleus basalis of Meynert*, dorsal motor nucleus of vagus) systems, as well as in the cerebral cortex (especially cingulate and entorhinal cortices), olfactory bulb, and the autonomic nervous system.

Intrinsic Cell-Death Pathway

The physiologic elimination of superfluous, damaged, or inappropriate cells from the body is mediated by programmed cell death (PCD), the most common of which is

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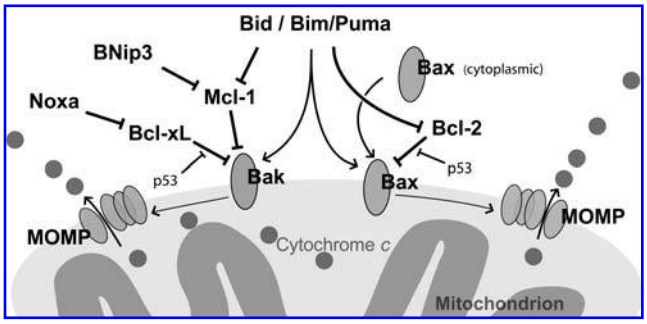

FIG. 1. Regulation of mitochondria outer membrane permeabilization (MOMP) by members of the Bcl-2 family. Antiapoptotic members Bcl-2, Bcl-x_L, and Mcl-1 inhibit apoptosis by direct binding to Bax and Bak. BH3-only members BNip3, Noxa, and Puma bind to antiapoptotic members, freeing Bax and Bak. Bid, Bim, and Puma are BH3 members that may also be able to activate Bax and Bak directly. Activated Bax and Bak form transient membrane pores that permit the release of apoptogenic factors such as cytochrome *c* into the cytoplasm. P53 can disrupt both Bcl-2:Bax and Mcl-1:Bak interactions.

type I PCD, also known as apoptosis (14). Apoptosis causes stereotyped morphologic changes that maintain plasma membrane integrity while organelles are disassembled and cellular contents repackaged for easy phagocytosis by neighboring cells, or professional macrophages. This process is the result of caspase activity disrupting critical metabolic and structural elements by proteolytic cleavage at highly specific peptide sequences (*i.e.*, they are not degradative enzymes). Caspases are expressed by all cells as inactive zymogen precursors that become fully active after proteolytic cleavage by other caspases. Therefore, activation of the caspase cascade is tightly regulated and normally triggered by one of two macromolecular complexes. The "extrinsic" cell-death pathway is activated by clustering of ligand-bound transmembrane death receptors, such as Fas or TNFR I, which permits the formation of a death-inducing signaling complex (DISC) on their cytoplasmic tails (81, 117). Alternatively, the "intrinsic" cell-death pathway can be activated by the disruption of biochemical processes that constitutively repress apoptosis (27, 77). Regulation of the intrinsic cell-death pathway centers on the release of death-inducing proteins from the intermembrane space of mitochondria. These factors include cytochrome *c*, SMAC/DIABLO (second mitochondria-derived activator of caspases/direct-IAP binding protein with low pI), AIF (apoptosis-inducing factor), and HtrA2/Omi. Members of the Bcl-2 protein family act as gatekeepers for the release of these factors by regulating permeability of the outer mitochondrial membrane (18). The proapoptotic family members Bax and Bak are able to bind heterologously and homologously to form pores that cause mitochondrial outer membrane permeabilization (MOMP; Fig. 1). Antiapoptotic members such as Bcl-2 and Bcl-x_L bind directly to Bax and Bak and stabilize the outer mitochondrial membrane (OMM). BH3-only members (so named because they contain only the third Bcl-2 homology domain) facilitate MOMP by inhibiting Bcl-2 and Bcl-x_L through direct binding (52). Additionally, further activation of Bax and Bak through direct binding by tBid, Bim, or Puma may be required. MOMP permits the release of cytochrome *c* into the cytoplasm, where it interacts with the adapter protein Apaf-1, which in turn binds to procaspase-9, forming a fully active apoptosome (Fig. 2). Thus, MOMP and cytochrome *c* release depends on ratios of specific proapoptotic to antiapoptotic Bcl-2 family members (89, 120). Factors that shift the ratio toward a higher proportion of proapoptotic members will activate Bax and/or Bak, induce cytochrome *c* release, and allow apoptosis to proceed. UV irradiation, growth factor withdrawal, and a variety of other cellular insults have been shown to trigger apoptosis through the intrinsic cell-death pathway. Interestingly, p53, a protein that is lost in approximately half of all malignant human cancers, has recently been shown to form a p53–Bax–Bcl-2 complex and activate Bax in a manner similar to that of BH3-only members (19). Further, p53 can also activate Bak by disrupting Bak–Mcl-1 binding (59). Apoptotic cell death has been found to underlie DA neuron losses in toxin-based models of PD.

Environmental Toxins and PD

In the early 1980s, severe parkinsonism was observed in several young heroin addicts who had self-administered a designer drug that contained large amounts of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (53). They displayed clinical signs consistent with late-stage PD, specifically an extensive depletion of DA neurons in the SNpc. Over the past 20-year period, studies of MPTP neurotoxicity in animal models have provided important insights into PD pathogenesis and the role of apoptosis in PD (115).

MPTP readily crosses the blood–brain barrier and is metabolized into 1-methyl-4-penylpyridinium ion (MPP^+) by monoamine oxidase (75). Structural similarities with dopamine allow high-affinity binding of MPP^+ by the dopamine transporter (DAT), resulting in the selective uptake and accumulation of this toxin in DA neurons, as they express DAT. Once inside neurons, MPP^+ binds to complex I of the mitochondrial electron-transport chain and inhibits oxidative phosphorylation (Fig. 3). Complex I, also known as NADH coenzyme Q reductase, is the first enzyme complex of the electron-transfer chain. It is located in the inner mitochondrial membrane and protrudes into the matrix. MPP^+ block-

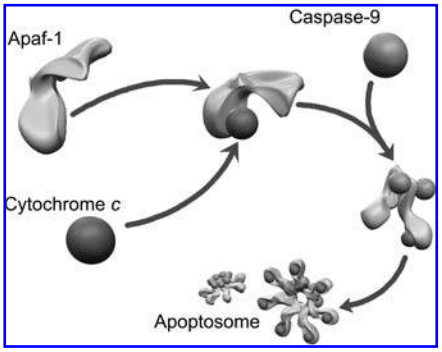

FIG. 2. Apoptosome formation. Cytoplasmic cytochrome *c* interacts with the adapter protein Apaf-1, which then recruits caspase-9. Seven of these triplets form the ~700-kDa apoptosome structure.

ade of complex I rapidly leads to lower ATP levels and increased production of reactive oxygen species (ROS) that react with nucleic acids, proteins, and lipids giving rise to profound oxidative stress (42). Clear evidence of oxidative stress has been found in postmortem PD brain, including high levels of lipid peroxidation and protein nitration in the SNpc and within Lewy bodies (4). Under MPTP intoxication, Bax is strongly upregulated in nigrostriatal DA neurons, whereas Bcl-2 levels are decreased. The key role of Bax in MPTP-induced neurotoxicity was confirmed by the demonstration that Bax-deficient mice are resistant to MPTP toxicity (114). Overexpressing Bcl-2 also protects DA neurons against MPTP-induced neurodegeneration (76, 121). Interestingly, the tumor-suppressor protein p53 regulates Bax expression, and p53 is activated after MPTP intoxication, possibly in response to DNA damage resulting from high levels of ROS (64). Further, p53-null mice are resistant to MPTP-mediated death of DA neurons (108). Activation of the Jun N-terminal kinase (JNK) pathway has also been observed after MPTP administration (88, 119). Altogether, strong evidence indicates that p53 and JNK work in concert to activate Bax, resulting in cell death through the intrinsic pathway.

Rotenone

Another inhibitor of complex I is rotenone (Fig. 3), a natural cytotoxic ketone made by tropical plants that is widely used as an insecticide and fish poison. Rotenone is extremely lipophilic, allowing it to easily cross cell membranes, including the blood–brain barrier. Continuous rotenone infusion in rats reduces complex I activity by 75% and causes the selective degeneration of nigrostriatal DA neurons (7). Rotenone-induced pathology closely matches that seen in sporadic PD, with neurodegeneration beginning in nerve terminals and progressing retrogradely to the cell body. Further, rotenone-induced degeneration is accompanied by synucleinopathy and oxidative protein damage in the same brain regions affected in PD (7, 90). Rotenone is also thought to trigger apoptosis as caspase-3 activity increases in rotenone-treated HL-60 cells in a time-dependent manner (100). Moreover, the expression of antiapoptotic Bcl-2 can inhibit rotenone-induced changes in mitochondrial membrane potential and the formation of DNA ladders, in BJAB cells. Nanomolar concentrations of rotenone have also been shown to induce caspase-3–mediated apoptosis of primary DA neurons using ventral mesencephalic cultures from E15 rats (2). Moreover, the caspase-3 inhibitor, DEVD-*fmk*, can protect tyrosine hydroxylase-immunopositive (TH^+)-neurons from rotenone-induced death, at least temporarily (2).

Paraquat

Soon after the discovery of MPTP neurotoxicity, it was proposed that similar toxins may account for the higher incidence of sporadic PD in agricultural communities. Because of structural similarities with MPP^+, the herbicide paraquat/PQ (1,1′-dimethyl-4,4′-bipyridinium) became a prime candidate (30). PQ is a nonselective contact herbicide with high pulmonary toxicity (24). When administered by intraperitoneal injection, PQ crosses the blood–brain barrier *via* the neutral amino acid transporter (93). Systemic administration of PQ in mice leads to higher α-synuclein expression and synucleinopathy in PD-affected brain regions, as well as the loss of nigrostriatal DA neurons (29, 66, 69). Although PQ and MPP^+ do share some structural features, more recent evidence has shown that PQ does not require a functional dopamine transporter (DAT) for neurotoxicity nor is it a particularly good inhibitor of complex I (85). Instead, PQ reacts with molecular oxygen to generate superoxide anions that can form toxic hydroxyl radicals or react with nitric oxide to form peroxynitrite (124). Divergence is also found in the subcellular localization of oxidized thioredoxin, which is primarily in mitochondria with MPP^+ but mostly cytoplasmic with PQ (86). MPP^+ activates cell death through JNK and p53, which ultimately leads to Bax-mediated release of cytochrome *c*. Although PQ also activates JNK and its downstream target c-jun (80), a recent report has shown that, unlike MPTP, Bax is not required for PQ-induced cell death; instead, PQ triggers BNip3 and Noxa activation of Bak (34).

Bax resides in the cytoplasm and translocates to the OMM during activation, but Bak is constitutively present on the OMM and must be continuously inhibited to prevent MOMP. Bak is most strongly bound by anti-apoptotic Mcl-1 and Bcl-x_L, both of which must be neutralized to provide the double hit necessary for efficient Bak-induced MOMP (35, 118). PQ induces higher BNip3 and Noxa expression, two specific blockers of Bcl-x_L and Mcl-1, respectively. Knockdown of BNip3, Noxa, or Bak makes neuroblastoma cells resistant to PQ-induced apoptosis. Importantly, Bak-deficient mice are resistant to PQ, providing conclusive evidence that Bak is necessary for PQ neurotoxicity, *in vivo* (34). This mechanism relies on high BNip3 and Noxa expression to inhibit Bcl-x_L and Mcl-1, which is consistent with the double-hit model for Bak activation. When levels of uninhibited/activated Bak surpass threshold, then MOMP releases apoptogenic factors, such as cytochrome *c*.

Synergy of paraquat and maneb

Maneb [manganese ethylenebisdithiocarbamate (manganese-EBDTC)] is a dithiocarbamate fungicide that is used for the control of field-crop infections. Several studies have shown that PQ and maneb neurotoxicities are dramatically enhanced when the two are combined (103–106). Maneb acts synergistically with PQ in mice, with treated animals being characterized by sustained decreases in motor activity and

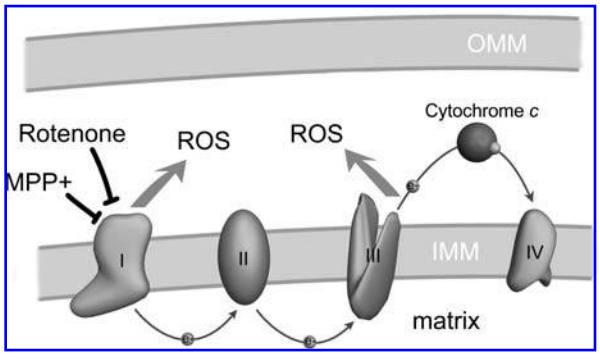

FIG. 3. Complex I blockade. The electron-transport chain passes electrons from complex I to III, and cytochrome *c* carries electrons from complex III to complex IV, as part of oxidative phosphorylation. Rotenone and MPP^+ bind to complex I with high affinity and block its activity, resulting in ROS generation from complexes I and III.

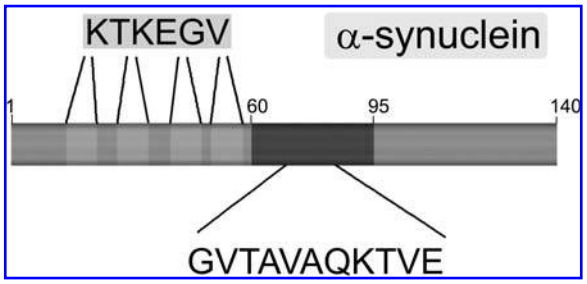

FIG. 4. Structure of α-synuclein. The amino terminus (amino acids 1–60) contains four near-perfect repeats and help to form an α-helical structure when the protein associates with lipid vesicles. The central region (amino acids 61–95) contains the non–amyloid-β component (NAC) sequence (GVTAVAQKTVE). The carboxyl terminus (amino acids 96–140) contains many acidic amino acids and prolines, as well as three conserved tyrosines.

increased damage of both striatal terminals and nigral cell bodies. In a study of mice at different ages, PQ+maneb had its strongest effects on locomotor activity, dopamine metabolites, and dopamine turnover in older mice (18 months old). Those studies established that aging dopamine pathways have enhanced sensitivities to the synergistic effects of PQ and maneb (105).

PQ and maneb activate Bak in neuroblastoma SK-N-SH cells when used individually, but together they trigger Bax-dependent cell death (33). PQ+maneb increase the expression of three strong Bak inhibitors, Bfl-1, Bcl-x_L, and Mcl-1, while simultaneously inducing Bax activators that include Bik and Bim. This combination favors Bax-dependent MOMP and apoptosis. SiRNA knockdown of Bax and Bak confirmed that individually PQ and maneb induce Bak-dependent cell death, but together, they block the Bak pathway and activate apoptosis through Bax. Interestingly, high levels of Bax have also been observed in the midbrains of mice given intraperitoneal injections of PQ+maneb (104).

α-Synuclein and the Ubiquitin-Proteasome Pathway

Abnormal deposition of protein aggregates, such as α-synuclein, is a common feature in several age-related neurodegenerative diseases. α-Synuclein is the predominant component of cytoplasmic Lewy bodies within the few surviving neurons in the SNpc of PD patients. Although α-synuclein is an abundant synaptic protein, its function is still unknown, but overexpression triggers neuronal apoptosis *in vitro* and parkinsonian features in mice. A30P and A53T substitutions in α-synuclein have been linked with early-onset autosomal dominant PD (84). α-Synuclein is a typical natively unfolded protein that is extended under physiologic conditions, but can adopt many different conformations, depending on environmental influences that can include pH, temperature, metals and other cations, interactions with lipid membranes, and up to 50 protein-binding partners (111). Residues 1 to 60 contain four near-perfect repeats of a conserved KTKEGV motif (Fig. 4) that facilitates interactions with lipid layers (15), resulting in a curved alpha helix conformation required for α-synuclein insertion into small vesicles and seems to protect against aggregation (128). High levels of α-synuclein in presynaptic terminals and its influence on vesicle dynamics are consistent with a functional role in neurotransmitter vesicle trafficking (23, 73). Residues 61 to 95 contain an aggregation-prone GVTAVAQKTVE motif called the non–amyloid-β component (NAC), which was originally isolated from β-amyloid plaques in Alzheimer brain (41, 110), although subsequent studies were not able to detect NAC in amyloid plaques (6). Carboxyl-terminal residues (amino acids 96 to 140) of α-synuclein contain a high number of acidic and proline amino acids and three conserved tyrosines.

The transformation of this natively unfolded protein into synucleinopathic aggregates, such as Lewy bodies and Lewy neurites, has been intensively investigated since the discovery of α-synuclein (Fig. 5). In addition to the membrane-bound form of α-synuclein in vesicles, it can also enter a molten globular state in response to high temperatures, low pH, metal and nonmetal ions, organic solvents, and PQ, among others (111). This molten state seems to be an entry point for aggregation, which leads to dimers, and then oligomeric fibrils that can be particles or spheroids. Eventually spheroids collapse into rings and crescents, which coalesce into amorphous aggregates, such as Lewy bodies and Lewy neurites.

Cells respond to misfolded proteins with heat-shock protein (Hsp) chaperones and the ubiquitin–proteasome pathway (UPP). The poly-ubiquitylation of proteins targets them for degradation *via* the 26S proteasome (Fig. 6). Free ubiquitin is phosphorylated by E1 ubiquitin-activating enzymes, which is then used by E2 ubiquitin-conjugating enzymes. Although E2 can ubiquitylate some proteins directly, they usually do so with the assistance of E3 ubiquitin ligases that provide more specificity. Some E3 ligases work in multiprotein complexes to recruit substrates. Mono-ubiquitylation can affect signal transduction in cellular processes such as DNA repair and transcriptional regulation; whereas poly-ubiquitin chains are recognized by the 26S proteasome, which then

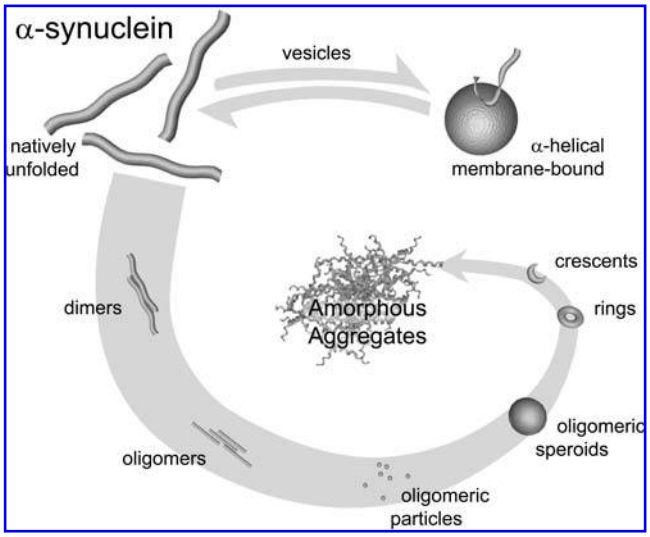

FIG. 5. Aggregation of α-synuclein. Native unfolded α-synuclein shifts to a molten state in response to a variety of factors. Molten α-synuclein transitions into oligomeric forms that are particles, then spheroids, and then rings or crescents before finally contributing to the formation of larger amorphous aggregates, such as Lewy bodies and Lewy neurites.

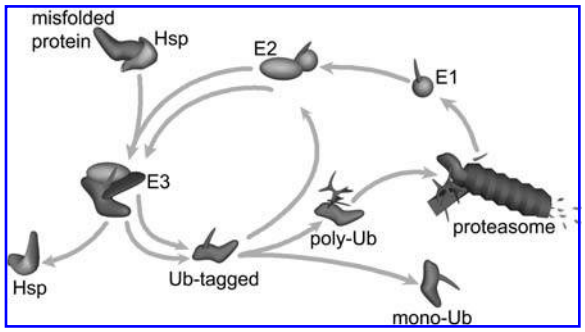

FIG. 6. The ubiquitin-proteasome pathway. Misfolded proteins are recognized by heat-shock proteins (Hsp), which refold them or target them for degradation by recruiting E2 and E3 ligases, which will connect ubiquitin side chains to the misfolded protein. Mono-ubiquitylation is used for signaling in some pathways; however, proteins with polyubiquitin side chains are recognized by the 26S proteasome and degraded. Ubiquitin fragments are not degraded, but clipped off, and recycled with the help of E1. Once ubiquitin is recharged by E1, it is handed off to E2 and reused to tag more proteins for degradation.

degrades the ubiquitin-tagged protein. An important function of the UPP in apoptosis is in maintaining high turnover of the tumor-suppressor p53 through Mdm2 ubiquitylation and subsequent proteasomal degradation. Mdm2 directs this ubiquitylation with a RING-finger E3 ligase domain that can be inhibited by its interaction with Mdmx and p19ARF (11). Mdm2 itself is also susceptible to ubiquitylation and degradation by SUMO-1. The critical role played by p53 in apoptosis and its sensitivity to ubiquitylation suggest that its regulation may be altered by mutations in *parkin*, *uch-L1,* or *α-synuclein* loci (Table 1).

Mutations in PARK2/*parkin* are linked with early-onset juvenile PD (50). The gene product, Parkin, is a 465-amino acid/55-kDa protein with ubiquitin homology at its N-terminus and two RING domains at its C-terminus, the latter of which interacts with E2 enzymes. The ubiquitylating activity of Parkin associates with hSel-10 and Cullin-1 in the SCF ubiquitin ligase multiprotein complex (97). Substrates for Parkin-mediated ubiquitylation include glycosylated α-synuclein (91, 92), Pael-R, synphilin-1, CDCrel-1 and cyclinE. Therefore, Parkin may play a critical role in the identification and elimination of potentially toxic proteins. A recent report shows that Parkin facilitates the elimination of Ataxin-3 mutants containing expanded polyglutamine repeats (109). The chaperone Hsp70 increases Parkin-mediated ubiquitylation of Ataxia-3polyQ, indicating that Hsp70 may help recognize and recruit misfolded proteins for Parkin's E3 ligase activity. Others showed that Parkin overexpression can suppress cyclinE accumulation and apoptosis in cerebellar granule neurons treated with the neurotoxin kainite (97). High cyclin activities have been described in PD, Alzheimer's disease, and other neurodegenerative disorders (44). Moreover, DA neurons in the SNpc of Parkin-deficient mice show elevated cyclinE expression.

Mutations in Parkin, Uch-L1, and α-synuclein could alter UPP activity in DA neurons and may affect the regulation of proteins that trigger apoptosis. Parkin has been reported to protect catecholaminergic neurons from mutant α-synuclein–associated toxicity (83). Further, Parkin is selectively S-nitrosylated in the brains of MPTP-treated mice and in patients with sporadic PD (20). Overexpression of the Parkin substrate PaeL-R in *Drosophila* led to DA neuron death, which could be rescued by Parkin overexpression (122). Further, Parkin overexpression in *Drosophila* can also overcome neurotoxicity resulting from α-synuclein overexpression. Several clues suggest that Parkin may be directly involved in the removal of certain forms of α-synuclein and so could attenuate its aggregation and/or toxicity (37). Observations supporting functional interactions between α-synuclein and Parkin that involve the proteasome include (a) α-synuclein is usually degraded by the proteasome (36, 95), (b) α-synuclein overexpression inhibits proteasome activity (98), and (c) PD-linked α-synuclein mutations increase the sensitivity of cells to proteasome inhibition (83, 102). Taken together, these findings suggest that high levels of misfolded α-synuclein will impair proteasome activity, and that, in turn, will further exacerbate α-synuclein accumulation.

An I93M mutation in PARK5/ubiquitin C-terminal hydrolase (*uch-L1*) has been linked to autosomal dominant PD in two German siblings, although it is not completely penetrant (56). Mice with a naturally occurring mutation in *uch-L1* do not show PD symptoms or pathology but instead develop gracile axonal dystrophy (GAD) (72). Scanning of the *uch-L1* locus identified another polymorphism in humans at S18Y that is linked with decreased susceptibility to PD and age of onset for Huntington's disease.

Current evidence also suggests an intriguing connection between α–synuclein and mitochondria. Complex-I inhibi-

TABLE 1. PARKINSON-LINKED FAMILIAL MUTATIONS

Acronym	*Inheritance*	*Locus*	*Protein*	*Remarks*
PARK1/4	Dominant	4q21-23	α-Synuclein	Major component of synucleinopathies, such as Lewy bodies. Associated with vesicle function
PARK2	Recessive	6q25.2-q27	Parkin	E3 ubiquitin ligase, neuroprotective
PARK3	Dominant	2p13	unknown	
PARK5	Dominant	4p14	Uch-L1	Ubiquitin recycling
PARK6	Recessive	1p35-p36	PINK1	A mitochondrial protein kinase, neuroprotection
PARK7	Recessive	1p36	DJ-1	Antioxidative functino, may work as a chaperone
PARK8	Dominant	12q12	LRRK2	Tyrosine kinase–like, GTPase, WD-40 repeat scaffold protein. Ankyrin-rich repeat domain
PARK9	Recessive	1p36	ATP13A2	Lysosomal ATPase
PARK13	Unknown	2p12	Omi/HtrA2	Mitochondria-dependent cell death (nonapoptotic)

tion both *in vitro* and *in vivo* leads to the accumulation of LB-like α-synuclein–positive inclusions, indicating that synucleinopathy is a downstream consequence of mitochondrial dysfunction (7, 66). Furthermore, α-synuclein knockout mice are resistant to the neurotoxic effects of MPTP, whereas α-synuclein–overexpressing mice show increased sensitivity to MPTP toxicity (25, 96), suggesting that α-synuclein is required for mediating the downstream effects of complex I inhibition.

Mitochondria and Oxidative Stress

Mitochondria play an essential role in the process of apoptotic commitment, and recent experiments have demonstrated that mitochondrial dysfunction may be associated with the pathogenesis of neurodegenerative diseases (55, 116). Although direct links between MPP^+ blockade of complex I and cell-death mechanisms remain unresolved, MPTP does upregulate p53, perhaps as a response to ROS damage of DNA (65). Another target of ROS may be the electron-transport chain itself, leading to mitochondrial dysfunction and amplification of ROS production (22). DA neurons are particularly sensitive to oxidative stress, as dopamine metabolism produces hydrogen peroxide and superoxide radicals. As an environmental agent, PQ is far more prevalent than MPTP, but it produces ROS through redox cycling with molecular oxygen more than with complex I inhibition (Fig. 3). Further evidence for the importance of mitochondria in PD pathophysiology comes from two autosomal-recessive PD-linked loci that encode the mitochondria-associated proteins, PARK6/PINK1 and PARK7/DJ-1.

DJ-1 was found to be deleted or have a substitution mutation in two separate populations in the Netherlands and Italy (10). Further analysis revealed a L166P substitution that may result in the loss of function. A yeast homologue of DJ-1 (YDR533C) has been reported to be elevated in response to oxidative stress (26), and DJ-1 is hydroperoxidase sensitive in both human and mouse cells (71). The exact function of DJ-1 is unclear, but data from studies in cell-culture and animal models suggest that it contributes to oxidative-stress responses in neuronal cells (49, 60, 62, 123). Under oxidative-stress conditions, DJ-1 shifts its isolelectric point from 6.2 to 5.8 (71). The neuroprotective effect of DJ-1 against oxidative stress correlates with an increase in cellular glutathione and an upregulation of γ-glutamylcysteine ligase (GCL), the rate-limiting enzyme in glutathione biosynthesis (126). Moreover, DJ-1 overexpression has been shown to protect cells against mitochondrial complex I inhibitors and oxidative stress induced by hydrogen peroxide. This protective effect is abrogated by PD-linked DJ-1 mutations or by DJ-1 knockdown with siRNA (17, 101). The cytoprotection effect of DJ-1 against apoptosis induced by hydrogen peroxide has been suggested to result from downregulation of Bax in a p53-dependent manner (31). Interestingly, DJ-1–deficient mice have a heightened sensitivity to MPTP neurotoxicity (49).

PINK1 encodes a putative serine/threonine kinase with a mitochondrial targeting sequence. PINK1 can partially protect against mitochondrial dysfunction induced by oxidative stress and proteasome inhibition (113), which is lost by the PD-linked mutations that also cause mitochondria to develop fragmented cristae. The relation of PINK1 with the PI3kinase/PTEN pathway and its mitochondria localization suggests an involvement in regulating the intrinsic cell-death pathway (113). It has recently been shown that PINK1 overexpression in SH-SY5Y cells reduces cytochrome *c* release and caspase-3 activation in response to staurosporine (82). Notably, PINK1-knockout flies have a higher sensitivity to oxidative stressors, including PQ and rotenone (21, 79). PINK1- and Parkin-knockout flies have a nearly identical phenotype that is not enhanced in double-knockout flies. Further, Parkin overexpression can rescue the PINK1-knockout phenotype, indicating that they are in a linear pathway with Parkin downstream of PINK1.

LRRK2

Autosomal-dominant PD inheritance has recently been shown for amino acid substitutions in leucine-rich repeat kinase 2 (LRRK2) (8, 48, 68); these account for ~7% of familial and a significant fraction of sporadic PD cases (32, 67, 125). LRRK2 is a massive protein (2,527 amino acids) comprising six distinct domains, including an ankyrin-rich repeat domain (ANK), a leucine-rich repeat domain (LRR), a Roc GTPase domain and associated COR domain, a tyrosine kinase–like (TKL) domain, and a C-terminal WD-40 domain. Multiple substitutions in each domain have been linked with PD, suggesting that LRRK2 plays a central role in PD pathophysiology. Investigations of protein interactors are complicated by the potential of LRRK2 to form an extremely large complex. The WD-40 domain forms a seven-bladed β-sheet propeller structure (1) that is a common protein-interaction motif and may trigger a higher-order complex. For example, six- and seven-blade propellers in the WD-40 domain of Apaf-1 bind to cytochrome c, which allows the hub domain to interact with other Apaf-1 proteins and form the seven-spoked apoptosome of >700 kDa (Fig. 2). That is not to suggest that the WD-40 repeat in LRRK2 necessarily binds to cytochrome *c*, but significant cellular events may activate a higher-order LRRK2 complex.

LRRK2 contains a conserved GTPase domain that is homologous with the Roco family (12), in which the Roc domain is always found in tandem with a COR domain. Although the COR domain's function is unclear, this Roc-COR module has been highly conserved throughout evolution. GTPase family members nearest to Roc are the Rab family, including Rab3 and Rab5, which are critical for vesicular trafficking in the presynaptic terminal. Localization of α-synuclein to presynaptic terminals makes this connection particularly intriguing. The COR domain of LRRK2 can interact with Parkin (94), although it is unknown whether LRRK2 affects α-synuclein processing by the UPP.

The kinase domain of LRRK2 is similar to other TLK serine-threonine kinases, with the LRRK2 domain most closely resembling receptor-interacting protein kinases (RIPKs). RIPKs integrate cell-stress responses (70) by activating NF-κB and MAP kinases, including Erk1/2, JNKs, and p38 (45, 46). The most prevalent substitution in LRRK2 is within the kinase domain at G2019S and is responsible for ~40% of familial cases and sporadic PD cases in samples from North African Arabs (57, 58), ~30% of Ashkenazi Jews (78), and significant fractions of European and North American cases (28, 32, 38, 47, 74). G2019 causes late-onset disease that is indistinguishable from sporadic PD, including Lewy body pathology (87). Mg^{2+} positioning within the catalytic domain

of most protein kinases depends on phosphorylation of an activation domain that is bordered by a DF/YG sequence; G2019S and I2020T lie at the N-terminal boundary of this activation domain, where they could significantly affect kinase activity. Some investigators have suggested that G2019S and I2020T impair kinase activity by interfering with Mg^{2+} positioning in the catalytic cleft (3, 63), whereas others argue that these substitutions cause a gain of function (47, 107). As LRRK2 function is largely unknown, it is unclear what effects oxidative stress and ROS may have on Roc-COR interactions or function. However, LRRK2 is such a large protein, it is highly probable that ROS-inducing toxins, such as MPP^+, PQ, and maneb, will affect TLK activity, GTPase activation, or at least some of its protein–protein interactions. Gene-toxicant interactions between LRRK2 isoforms and PD-linked neurotoxins are currently unknown. LRRK2 toxicity in cortical neurons and neuroblastoma cells, and its tendency to aggregate when overexpressed in those cells, has been reported (40). LRRK2 induces features of apoptotic death, including cytoplasmic and nuclear shrinkage and TUNEL labeling (40, 94), but more details on the cell-death mechanism engaged are still emerging.

Molecular Chaperones

Heat-shock proteins and other molecular chaperones provide a first line of defense against misfolded, aggregation-prone proteins and thus assist in the maintenance of cellular integrity and viability. These enzymes transiently stabilize and mediate the folding or assembly of unfolded protein substrates, or identify them to the UPP for degradation. Many reports have shown that the induction of Hsp protects cells from apoptotic death and may even clear intracellular inclusions. The overexpression of Hsp70 in an α-synuclein/synphilin cell model markedly decreased the number of cells that contained inclusion bodies (51). Hsp70 also caused a decrease in detergent-insoluble, high-molecular-mass α-synuclein species, as well as a decrease in total α-synuclein protein, indicating that Hsp70 might enhance refolding or promote degradation of α-synuclein. Furthermore, Hsp70 overexpression decreased toxicity induced by α-synuclein overexpression. In *Drosophila*, the overexpression of molecular chaperones also rescues flies from pathologic features induced by transgenic overexpression of normal or mutant α-synuclein (5), which further suggests that Hsp70 might have a protective role in PD. DJ-1 is structurally similar to the chaperone Hsp31 and has been shown to possess chaperone activity *in vitro* (54). DJ-1 interacts with α-synuclein and inhibits its aggregation *in vitro* and in cellular models (127). Finally, DJ-1 has been shown to upregulate Hsp70 in a dopaminergic cell line (126) and primary dopaminergic neurons (62), and this upregulation correlates with DJ-1–dependent suppression of α-synuclein toxicity.

Conclusions

Over the past 20-year period, genetic and toxin-based studies have significantly advanced our understanding of PD pathogenesis. Studies of MPTP, PQ, rotenone, maneb, and other neurotoxins have clearly identified roles for oxidative stress and mitochondrial dysfunction. The identification of PD-linked familial mutations provided key insights into those oxidative/mitochondria responses, and also showed the importance of the UPP in this disease (Fig. 7). Ultimately, these PD-linked toxins and PD-linked mutations converge with the loss of specific populations of neurons through PCD, but it is unclear which pathways are paramount in this process. Recent studies on MPTP and PQ neurotoxicity have shown that these two toxins result in the activation of Bax and Bak, respectively; although they both trigger MOMP and the release of apoptogenic factors into the cytoplasm, it is accomplished through divergent mechanisms. This same theme may also hold true for other PD-relevant mechanisms wherein PD can result from a wide variety of gene-toxicant interactions that may show why PD patients have different combinations of symptoms. Ulti-

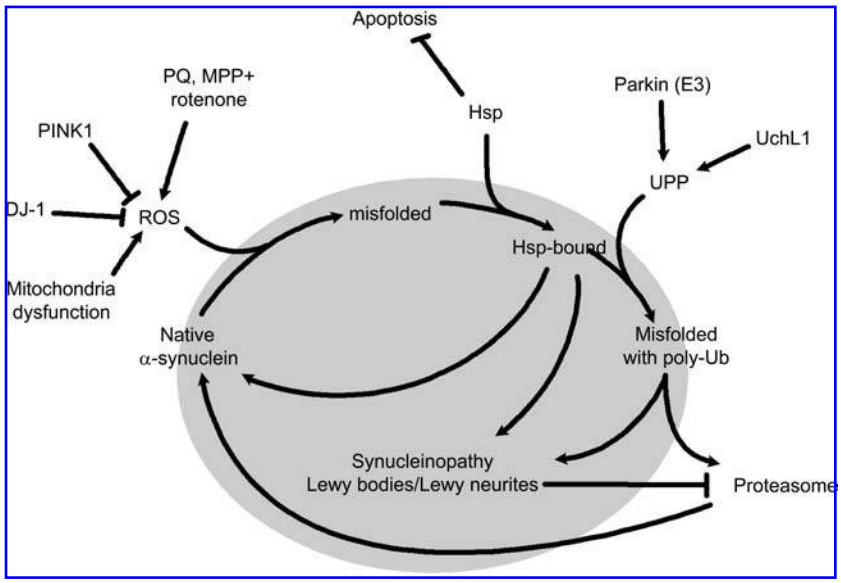

FIG. 7. Schematic diagram showing possible connections between key genes and toxins linked with PD through the formation of synucleinopathies. Mitochondrial dysfunction, PQ, MPP+, rotenone, or loss of DJ-1 or PINK1 increases oxidative stress and ROS production. High ROS levels oxidize amino acids and cause proteins to become misfolded, including α-synuclein. Misfolded α-synuclein is recognized by Hsp, which may refold it back into the native form or recruit UPP components to ubiquitylate the protein. Parkin and UchL1 contribute to this UPP process, and PD-linked loss-of-function mutations would reduce the efficacy of this process. Once misfolded, α-synuclein is polyubiquitylated; it may be degraded by the 26S proteasome or coalesce on synucleinopathic aggregates, such as Lewy bodies or Lewy neurites. α-Synuclein aggregates inhibit proteasome function, which in turn increases cellular concentrations of α-synuclein. High concentrations of misfolded α-synuclein, in soluble or aggregated form, reduce the availability of Hsp to suppress apoptosis, resulting in cell death.

mately, neuronal death is the final convergence point for PD, so it will be interesting to see how many ways there are to arrive at this same end point.

Abbreviations

AIF, apoptosis-inducing factor; DA, dopaminergic; DAT, dopamine transporter; DISC, death-inducing signaling complex; HSP, heat-shock protein; JNK, c-jun-N-terminal kinase; LRRK2, leucine-rich repeat kinase 2; maneb, manganese ethylenebisdithiocarbamate; MOMP, mitochondrial outer membrane permeabilization; MPP^+, 1-methyl-4-penylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OMM, outer mitochondrial membrane; PCD, programmed cell death; PD, Parkinson's Disease; PINK1, PTEN-induced putative kinase 1; PQ, 1,1′-dimethyl-4,4′ bipyridium dichloride; aRIPK, receptor-interacting protein kinase; ROS, reactive oxygen species; SMAC/DIABLO, second mitochondria-derived activator of caspases/direct-IAP binding protein with low PI; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; UCHL1, ubiquitin carboxyl-terminal hydrolase L1; UPP, ubiquitin-proteasome pathway.

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