Lori M. Buhlman *Editor*

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Preface

Our first close-up views of mitochondria, achieved using transmission electron microscopy, revealed their intracellular distribution and organized double-membrane structure. We know now that fixation techniques used to capture these snapshots deceived us into thinking that these "powerhouses of the cell" existed as a static collection of kidney bean-shaped organelles. Modern technology has revealed that, in fact, mitochondria comprise a highly dynamic network in which sections can break off, move rapidly throughout the cell to deliver energy and calcium-buffering capabilities, and then rejoin the network or be digested in lysosomes. We have learned that mitochondria may have evolved from invasive bacteria to become the most important sensors of cell stress and the gatekeepers of apoptosis. Because most cells rely so heavily on mitochondrial ATP production, and because mitochondrial stress can initiate apoptosis, many deadly diseases are known or thought to be caused by aberrant mitochondrial function. Parkinsonism, which is characterized by the presence of particular motor symptoms caused by degeneration of a subset of dopaminergic neurons in the midbrain, can be induced in animals and humans when they are exposed to mitochondrial toxins. Additionally, common Parkinson's disease (PD)-causing mutations implicate poor mitochondrial function in PD pathology. Indeed, aberrations in mitochondrial function and turnover are evident in genetic models and even in patients who inherit these mutations.

Time and time again, hypergeneration of mitochondrial reactive oxygen species (ROS) has been shown to contribute to cellular aging, decreased ATP production, various diseases pathologies, and necrotic and programmed cell death—probably as a result of macromolecule oxidation. Idiopathic PD models are generated by inducing excessive ROS levels either by administration of a reactive dopamine analogue or mitochondrial toxins, and increased ROS levels have been detected in most models of familial PD. Whether ROS are the direct cause of neurodegeneration in PD is unclear, as is whether the culpable source of mitochondrial ROS is dopamine metabolism, effects of mutated mitochondrial DNA, oxidative phosphorylation, or a combination of these. Further, excessive ROS production could be induced by non-mitochondrial sources like inflammation. While there is no treatment that can halt or delay degeneration for PD patients, strides have been and are being made in

the advancement of our understanding of PD pathology. We have learned that manipulation mitochondrial fusion and fission events can improve or even rescue model phenotypes. We also know that PD is less prevalent in the tobacco cigarette-smoking population and that nicotine exposure can prevent cell death in PD animal models. Modulation of peroxisome proliferator-activated receptor (PPAR) family of transcription factors has been shown to improve mitochondrial function in some PD models as well. Still, when we finally understand how altering mitochondrial dynamics, nicotine exposure, or PPAR activity protects cells, delivery of therapeutic molecules to affected mitochondria may be our most daunting obstacle. Yet, with each passing year, nanobiotechnologists create more promising delivery systems.

Here we discuss the products of tireless efforts put forth by many researchers around the world who are optimistic about the idea that PD and other neurodegenerative disorders will one day be treatable. Due to advances in imaging technology and our ability to understand and manipulate gene expression, our understanding of disease mechanisms has expanded exponentially, paving the way for potential therapeutic strategies and delivery methods to be discovered.

Glendale, AZ, USA Lori M. Buhlman

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Chapter 1
Mitochondrial ROS and Apoptosis

Hazem El-Osta and Magdalena L. Circu

1.1 Reactive Oxygen Species and Mitochondrial Sources of Their Generation

The term reactive oxygen species (ROS) comprises O_2-derived free forms that have accepted extra electrons and that can be free radicals like superoxide ($O_2^{\bullet -}$) or hydroxyl radical ($HO^{\bullet}$) as well as O_2-based nonradical species such as hydrogen peroxide (H_2O_2) [1]. Although H_2O_2 is not a free radical, it is considered a ROS because, in the presence of transition metals, it is able to form the $HO^{\bullet}$ via Fenton chemistry. This hydroxyl radical is highly reactive and can damage intracellular macromolecules including proteins, lipids, and DNA [2]. $O_2^{\bullet -}$ is a reactive species that is converted to H_2O_2 by CuZn-SOD (CuZn-superoxide dismutase) in the cytosol and in the mitochondrial intermembrane space and by MnSOD in the mitochondrial matrix. Next, H_2O_2 is converted to oxygen and water by catalase (CAT) and glutathione peroxidase (GPx) (Fig. 1.1). Small amounts of mitochondrial H_2O_2 have been shown to function as a signaling molecule in the cytosol being involved in several signaling pathways associated with the cell cycle, stress response, autophagy, and redox balance [3, 4]. Locally, mitochondria-generated H_2O_2 is a key regulator of mitochondrial ROS level via activation of mitochondrial uncoupling proteins [5]. Although physiological concentrations of ROS can be neutralized by mitochondrial antioxidant redox systems, higher levels can become detrimental and cause irreversible oxidative damages to cellular macromolecules leading to apoptosis or necrosis.

Mitochondria are the major source of ROS in the majority of eukaryotic cells. Mitochondria consume nearly 90 % of total oxygen content in the cell for oxidative

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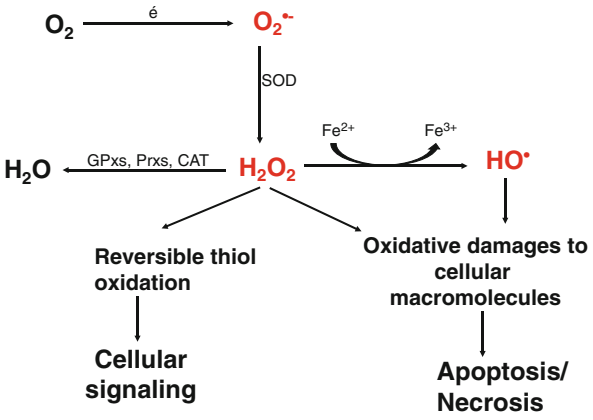

Fig. 1.1 ROS formation and ROS metabolism. Monoelectron reduction of O_2 leads to the formation of superoxide anion radical ($O_2^{\bullet-}$). Cellular superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to H_2O_2, a nonradical ROS. H_2O_2 is reduced to water by several cellular enzymes including glutathione peroxidase (Gpx), peroxiredoxins (Prx), and catalase (CAT). In the presence of transition metals, H_2O_2 can form hydroxyl radical via Fenton chemistry, a toxic species that causes oxidative modifications to cellular macromolecules. Similarly, high amounts of H_2O_2 can damage proteins, lipids, or DNA leading to oxidative stress-induced necrosis or apoptosis. Conversely, small amounts of H_2O_2 can function in cellular signaling via reversible modification of cysteine residues

phosphorylation and cellular ATP production [6]. It was estimated that about 2–5 % of oxygen consumption results in $O_2^{\bullet-}$ formation and that a high amount of superoxide generation is associated with electron flow through the Q cycle in complex III or with reverse electron transport at the level of complex I [7]. However, under physiological conditions, the rate of superoxide generation is lower and has been calculated to be around 0.1–0.2 % of the oxygen consumed by the mitochondria [8].

Complex III of the electron transport chain catalyzes the electron transfer from ubiquinol to cytochrome *c* being known as the ubiquinol-cytochrome *c* oxidoreductase. The monomer of ~240 kDa comprises 11 polypeptides, three hems, and an Fe-S center. The transfer of electrons through complex III occurs via Q cycle in which four protons are translocated across the membrane for every two electrons transferred from ubiquinol to cytochrome *c*. First, a molecule of ubiquinol diffuses to the ubiquinol oxidizing site (Q_o), close to the cytoplasmic face of the mitochondrial membrane and close to the iron-sulfur protein (Rieske protein). Oxidation of ubiquinol at the Q_o site results in the transfer of one electron from UQH_2 to the Rieske protein with the release of two protons to the intermembrane space and leading to the formation of the free radical semi-ubiquinone anion species $UQ^{\bullet-}$ at the Q_o site. The iron-sulfur protein transfers down the electron to cytochrome *c1* and then to cytochrome oxidase. The strongly reducing ubisemiquinone anion passes an electron to cytochrome bL (proximal to the cytosolic site) which then transfers an electron to the other heme bH site (proximal to the matrix site). The reduced bH

transfers the electron to ubiquinone at the ubiquinone-reducing site (Q_i) resulting in the formation of a stable semi-ubiquinone $UQ^{\bullet-}$ which is firmly bound at the Q_i site. To complete the Q cycle, a second molecule of UQH_2 is oxidized at Q_o, another electron passes to cytochrome *c*, and another passes from bL to bH. This electron leads to the reduction of $UQ^{\bullet-}$ to UQH_2 with the uptake of two protons from the matrix. With the formation of UQH_2, the Q cycle can restart again.

For long time complex III has been regarded as a source of $O_2^{\bullet-}$ within mitochondria [9]. At the level of mitochondrial complex III, semi-ubiquinone can yield $O_2^{\bullet-}$ [10]. $UQ^{\bullet-}$ is formed at both the Q_o and Q_i sites. Because the $UQ^{\bullet-}$/UQ pair is highly reducing, $O_2^{\bullet-}$ may be formed via electron transfer in the presence of oxygen at both sites [11]. Due to the location of the Q_o site near the intermembrane space, $O_2^{\bullet-}$ is expelled from the membrane to the cytosolic side [12], but it can also be released into the mitochondrial matrix [13]. Experiments with respiratory inhibitors confirm that $UQ^{\bullet-}$ is the source of the $O_2^{\bullet-}$ at the level of complex III. For example, in the presence of CQH_2 and antimycin, a known inhibitor of the Q_i site, complex III produces large amounts of $O_2^{\bullet-}$ via the Q_o site [7, 10, 11]. In addition, experiments in pigeon heart mitochondria bovine showed a linear relationship between reducible ubiquinone content and H_2O_2 production from $O_2^{\bullet-}$ [14].

Complex I of the electron transport chain is the other place where $O_2^{\bullet-}$ can be formed, but the precise nature of the site remains obscure [15]. Complex I contains 14 subunits and 40 different polypeptides. In this large multi-subunit complex, the electrons are transferred from NADH to FMN, flavin mononucleotide, then to a series of Fe-S clusters which then reduce a membrane-embedded ubiquinone (coenzyme Q, CoQ) to form ubiquinol that is coupled with the transport of four protons across the mitochondrial membrane. It is generally believed that the electron flow in complex I is at near equilibrium with the superoxide formation taking place by both forward electron transfer (FET) and reverse electron transfer (RET) [16]. We can expect that complex I produces $O_2^{\bullet-}$ when the matrix NADH/NAD^+ ratio is high leading to a reduced FMN site or when the forward electron transport slows down, at high mitochondrial membrane potential ($\Delta\psi$), during non-phosphorylating respiration [17]. Production of $O_2^{\bullet-}$ by NADH:ubiquinone reductase was demonstrated by Takeshige and Minakami in bovine heart submitochondrial particles [18]. $O_2^{\bullet-}$ generation was influenced by different inhibitors of the electron transport chain with rotenone, antimycin A, and KCN shown to stimulate the reaction. RET occurs when electron supply reduces the CoQ pool, which, due to a high $\Delta\psi$, forces electrons back from $CoQH_2$ into complex I, thereby reducing NAD^+ to NADH at the FMN site [19]. Cino and Del Maestro found that a succinate-induced high rate of respiration in isolated mitochondria involves RET from succinate to NAD^+, providing reducing equivalents to complex I that served as a site for $O_2^{\bullet-}$ generation [20]. Notably, inhibition of complex I with rotenone enhances $O_2^{\bullet-}$ generation leading to the backing up of the electrons onto FMN which will produce $O_2^{\bullet-}$ [18]. In addition, Lambert and Brand found that $O_2^{\bullet-}$ generation by RET as well as the effect of rotenone and piericidin on $O_2^{\bullet-}$ production resides in the membrane region of ubisemiquinone-binding site of the complex I [21]. Other researchers located the production of $O_2^{\bullet-}$

at the N1a Fe-S center of the 24 kDa subunit on the matrix-protruding arm of the complex I [22]. Genova et al. suggested that the site of $O_2^{\bullet-}$ production was at N2 Fe-S center on the 20 kDa PSST membrane subunit, whereas Liu et al. provided arguments for the involvement of FMN group in $O_2^{\bullet-}$ generation [23, 24]. The high $O_2^{\bullet-}$ production via RET is sensitive to membrane potential and to mild uncoupling [25]. In addition, conversion of pH gradient to membrane potential reduces $O_2^{\bullet-}$ generation by complex I [26].

Mitochondrial glycerol-3-phosphate dehydrogenase (mtGPDH) located on the external site of the mitochondrial inner membrane is a component of mammalian electron transport chain and is involved in glycerol phosphate shuttle between mitochondria and cytosol. Oxidation of α-glycerol-3-phosphate by mtGPDH in brown adipose tissue mitochondria is associated with production of significant amounts of H_2O_2, a process that was linked to RET that occurred at the level of complex I [27]. It is believed that the levels of ROS generated by mtGPDH are similar with those yielded at the level of complex III when inhibited with antimycin A [28]. Several other flavin enzymes including quinone oxidoreductase [29], ETF-Q dehydrogenase [30], and succinate dehydrogenase [31] can contribute to overall ROS production within mitochondria.

Similarly, dihydrolipoamide dehydrogenase (DLD), a component of 2-oxoglutarate dehydrogenase complex, generates $O_2^{\bullet-}$ when inhibited by NAD^+ or activated by Ca^{2+} [32]. In addition, monoamine oxidase, which oxidizes biogenic amines, was described to produce H_2O_2 within mitochondria [33]. Interestingly, the apoptosis-initiating factor (AIF), a proapoptotic protein, was reported to produce $O_2^{\bullet-}$ via NADH oxidase activity [34]. Mitochondrial dihydroorotate dehydrogenase, located on the outer surface of the inner mitochondrial membrane and which catalyzes the oxidation of dihydroorotate to orotate causing the reduction of CQ, is linked to $O_2^{\bullet-}$ generation [35]. In conclusion, mitochondria produce several groups of chemical species, each with its own reactivity and ability to diffuse in different microenvironments, the mitochondrial matrix, the intermembrane space, or the cytosol. When not neutralized by mitochondrial antioxidant systems, mtROS can induce oxidative damages to mitochondrial macromolecules including components of the electron transport chain, lipids, and DNA.

1.2 Mitochondrial Redox Systems

Central to maintaining an optimal redox environment for proper activity of mitochondrial functions and for preserving the redox state of mitochondrial proteins and the integrity of mitochondrial DNA against mitochondria-produced ROS are the glutathione (GSH)- and thioredoxin (Trx)-dependent enzymes. The metabolism of mtROS and the enzymatic detoxification pathways are presented in Fig. 1.2.

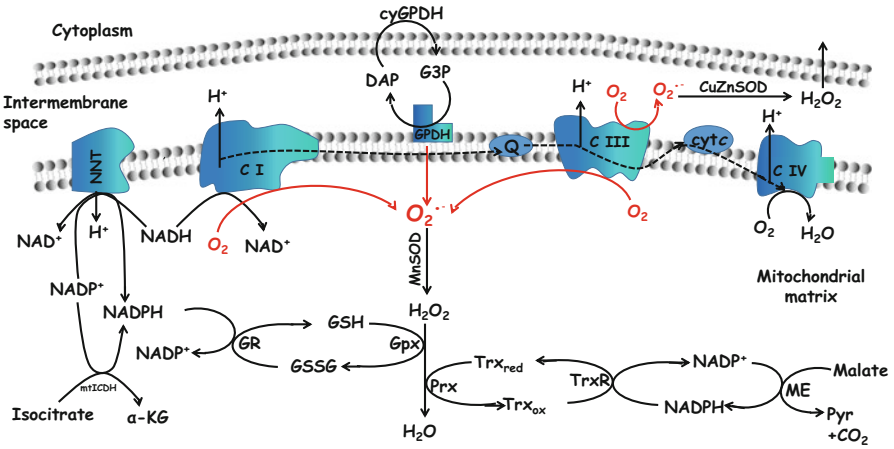

Fig. 1.2 Mitochondrial ROS generation and its metabolism. Superoxide radical anions ($O_2^{\bullet-}$) are generated by monoelectron reduction of O_2 mainly at the level of complexes I and III or by the mitochondrial glycerol-3-phosphate dehydrogenase (GPDH). $O_2^{\bullet-}$ is dismutated to H_2O_2 by CuZn-SOD in the intermembrane space or by MnSOD in the mitochondrial matrix. Next, H_2O_2 can be removed by reduced glutathione (GSH)- or thioredoxin (Trx)-dependent enzymes, glutathione peroxidase (Gpx), or peroxiredoxins (Prx), using GSH or Trx as substrate, respectively. The reduction of oxidized GSSG or Trx_{ox} is catalyzed by glutathione reductase (GR) or thioredoxin reductase (TrxR), using NADPH as reducing equivalent. Mitochondrial sources of NADPH include NAD^+/$NADP^+$ transhydrogenase (NNT), $NADP^+$-isocitrate dehydrogenase, and malic enzyme (ME). *Abbrev*. Dihydroxyacetone phosphate (DAP), glyceraldehyde-3-phosphate (G3P), α-ketoglutarate (α-KG), $NADP^+$-isocitrate dehydrogenase (mtICDH)

1.2.1 The Mitochondrial GSH and Glutathione-Dependent Enzymes

The tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is found in millimolar concentrations in the majority of cells. GSH participates in the antioxidant defense via direct interaction with ROS or as a cofactor of the detoxification enzyme GSH peroxidase (Gpx) [36]. Intracellularly, GSH is predominantly in the thiol-reduced form, GSH, which, under oxidizing conditions, can form GSH disulfide, GSSG, leading to a decrease in the GSH-to-GSSG ratio which is often associated with oxidative stress condition. GSH homeostasis is achieved through de novo synthesis from precursor amino acids [37], regeneration from GSSG [38], or import at the plasma membrane [39]. Intracellular GSH synthesis occurs in the cytosol through two ATP-dependent steps catalyzed by glutamate cysteine ligase (GCL) and GSH synthase [40]. Upon synthesis, GSH is distributed to intracellular compartments including the endoplasmic reticulum (ER), mitochondria, and the nucleus where it resides as separate redox pools [41, 42]. The mitochondrial GSH/GSSG redox pool is distinct from the cytosolic one in terms of GSH and GSSG forms, their

redox potential, and their control of cellular activities. Notably, mitochondrial GSH (mtGSH) achieves similar concentrations as the cytosolic pool (5–10 mM) and represents 10–15 % and 30 % of the total cellular GSH in the liver and kidney, respectively [43, 44]. Cytosol-to-mitochondria GSH distribution occurs through active transport via inner membrane dicarboxylate (DIC) and 2-oxoglutarate (OGC) GSH carriers, two well-characterized transporters in the liver and kidney [45, 46]. Another mtGSH carrier was reported in brain mitochondria [47]. Although believed that GSH in the mitochondrial intermembrane space is at equilibrium with the cytosolic pool, Hu et al. reported a more oxidized environment within mitochondrial intermembrane space than in the cytosol or mitochondrial matrix suggesting that the intermembrane redox pool is regulated independently [48].

Intramitochondrial antioxidant defense is mediated by GSH-dependent enzymes, glutathione peroxidase (Gpx), and glutaredoxin (Grx) [49]. Among the Gpx isoenzymes, Gpx1 is found in the mitochondrial matrix, whereas Gpx4 (also known as phospholipid hydroperoxide Gpx) [50] is located in the inner mitochondrial membrane and detoxifies mainly phospholipid hydroperoxides. Mitochondrial Gpx1 is the major H_2O_2 reducing selenoprotein. Formation of mixed disulfides between protein cysteine sulfhydryls and GSH, known as S-glutathionylation, is a protective mechanism against irreversible oxidative modifications that affect the activity of many mitochondrial enzymes including aconitase [51], α-ketoglutarate dehydrogenase [52], isocitrate dehydrogenase [53], succinyl-CoA transferase [54], and aldehyde dehydrogenase [55]. These enzymes are inhibited by S-glutathionylation during oxidative stress condition. Similarly, the activity of respiratory complex I [56], II [57], and V [58] is modulated via oxidative stress-induced S-glutathionylation. Within mitochondria, the de-glutathionylation of protein mixed disulfides is catalyzed by Grx2 through thiol-disulfide exchange mechanism [59]. Oxidized Grx2 is reduced back by either GSH or Trx redox systems [60].

1.2.2 Mitochondrial Thioredoxin and Thioredoxin-Dependent Enzymes

In mammalian cells, the thioredoxin system is composed from reduced nicotinamide dinucleotide phosphate (NADPH), thioredoxin (Trx), and thioredoxin reductase (TrxR). Thioredoxins are small ubiquitous proteins that possess two redox cysteines in the catalytic site [61]. Trxs catalyze the reduction of mixed protein disulfides with concomitant oxidation of the cys in the catalytic site. The regeneration of the Trx active site is catalyzed by Trx reductase (TrxR) and NADPH [61]. The mitochondria-specific Trx is Trx2 which functions in mitochondrial redox homeostasis [62]. In addition, Trx2 is critical for cell survival since homozygous $Trx2^{-/-}$ mouse embryonic fibroblasts (MEFs) were not viable [63]. TrxR, a monodimeric 115 kDa flavoenzyme containing a penultimate C-terminal selenocysteine in its catalytic site, is a ubiquitous enzyme which catalyzes the reduction of TrxSS using NADPH as cofactor [64].

Peroxiredoxins are a group of non-seleno-thiol peroxidases that contribute to the detoxification of organic peroxides and H_2O_2 [65]. All Prxs have a peroxidatic cys within the catalytic site that is oxidized and forms a disulfide bond with a second cys located in the C-terminal subunit. The regeneration of the active cys occurs via Trx/TrxR system. Of the six Prx isoenzymes, Prx2 and Prx5 are mitochondrial. In oxidative stress conditions, the peroxidatic cys is oxidized from sulfenic acid, –S–OH (as in normal catalytic condition) to sulfinic acid (Prx-Cys-SO_2H) resulting in loss of peroxidasic activity [66]. Importantly, to reduce oxidized sulfinic acid of Prx to sulfenic acid, mammalian sulfiredoxin (Srx) translocates from the cytosol to mitochondria [67].

Notably, the redox status of NADPH/$NADP^+$ is tightened to the redox status of GSH and Trx since NADPH is the common electron donor in GSH and Trx reduction (Fig. 1.2). Within mitochondria, there are three enzymatic mechanisms for NADPH generation: (1) $NADP^+$-dependent isocitrate dehydrogenase (mtICDH), (2) $NAD(P)^+$-malic enzyme (mtME), and (3) NAD^+/$NADP^+$ transhydrogenase (NNT). It is believed that the reaction catalyzed by NNT accounts for more than 50 % of mitochondrial NADPH (mtNADPH) pool [68]. NNT, a mitochondrial 110 kDa protein located in the mitochondrial membrane, functions as a proton translocator using the respiration electrochemical gradient to convert $NADP^+$ to NADPH coupled to the oxidation of NADH to NAD^+ [69]. Therefore, the proton gradient stimulates NADPH production, whereas the decrease in the membrane potential lowers the activity of NNT. Since NNT cannot sustain NADPH production, subsequent oxidative damages may occur due to diminished capacity of H_2O_2 reduction [70]. The role of NNT in mitochondrial redox regulation was emphasized in studies in which NNT inhibition impaired GSH redox status and enhanced sensitivity to oxidative stress [71, 72]. In addition to NNT, the activity of $NADP^+$-isocitrate dehydrogenase and mtME contributes to the maintenance of mtNADPH redox pool [73]. Among the three mammalian ME, the mitochondrial ME (ME3) is both NAD^+ and $NADP^+$ specific. ME3 catalyzes the oxidative decarboxylation of malate to pyruvate using $NADP^+$ as a cofactor.

1.3 Overview of Apoptosis Pathways

Apoptosis or programmed cell death is an evolutionary conserved pathway characterized by nuclear changes, DNA fragmentation, chromatin shrinkage, membrane blebbing, and the formation of the apoptotic bodies containing the remains of the dying cell [74]. Depending on the nature of the apoptotic stimuli, the trigger of apoptosis can occur at the plasma membrane through membrane-associated death receptor (extrinsic) signaling or at the mitochondria (intrinsic) signaling (Fig. 1.3). Both pathways lead to the activation of caspases, a family of cysteine protease, and result in specific morphological changes characteristic of apoptotic cell death.

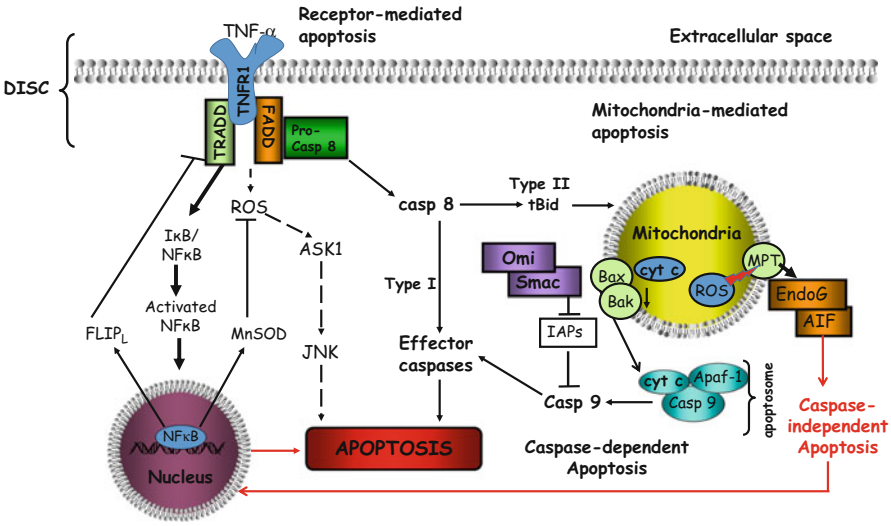

Fig. 1.3 Death receptor and mitochondria-mediated apoptosis. Upon binding of the ligand (e.g., TNF-α) to cognate receptor, TNFR1, formation of death-inducing signaling complex (DISC) activates caspase 8 which in turn activates effector caspases (type I cells) or engages the mitochondria via tBid (type II cells). In addition, activation of the NF-kB pathway enhances transcription of the antiapoptotic proteins like $FLIP_L$ or MnSOD, leading to apoptotic blockade. In parallel, ROS generated at the receptor activate the ASK1/JNK pathway that triggers apoptosis and induces redox changes of the NF-kB promoting its inactivation. Different proapoptotic stimuli like ROS can mediate the release of apoptogenic factors from mitochondria via pore formation by Bax/Bak or via opening of mitochondrial transition pore (MPT). Once in the cytosol, proapoptotic factors can trigger caspase-dependent and caspase-independent apoptotic signaling. In caspase-dependent signaling, cytochrome *c* together with Apaf-1 and dATP forms the apoptosome complex where initiator pro-caspase 9 is recruited and activated. Moreover, apoptogenic proteins like Smac and Omi neutralize caspase inhibitors, enhancing apoptotic signal. In caspase-independent signaling, proteins like AIF and EndoG translocate to the nucleus where induce DNA fragmentation

1.3.1 Receptor-Mediated (Extrinsic) Apoptosis

The death receptor pathway is initiated at the plasma membrane by extrinsic signals such as extracellular hormones or components of the tumor necrosis factor family (TNF) including TNFα, Fas/CD95 ligand, or TRAIL (TNF-related apoptosis-inducing ligand). The death receptors include Fas, TNF receptor 1 (TNFR1), TRAIL receptor 1 (TRAILR1), and TRAIL receptor 2 (TRAILR2). Binding of the ligand to the cognate receptor induces oligomerization of the receptor followed by the recruitment of adaptor proteins containing death domains or death effector domains (Fas-associated death domain [FAAD] or TNF receptor type 1-associated death domain [TRADD]) at the cytosolic death domain of the receptor resulting in the formation of DISC (death-inducing signaling complex). At the DISC initiator caspases, such as caspase 8 or caspase 10, are recruited and activated. Depending on cell type, the

downstream signaling can lead to either activation of executioner caspases or to the engagement of mitochondrial apoptotic signaling. For example, in type I cells high magnitude of initiator caspase activation causes the cleavage and activation of executioner caspases 3 and 7. In type II cells, low initiator caspase activation engages mitochondrial apoptotic signaling via truncated Bid (tBid) which interacts with pro-apoptotic proteins Bax/Bak causing pore formation in the outer mitochondrial membrane [75].

In addition to caspase activation, several other events occur at the death receptor. For example, binding of TNFα to TNFR1 is linked to the production of ROS via plasma membrane NADPH oxidase, Nox1, and mitochondrial and cytosolic sources [76]. It is believed that ROS can modulate two signaling pathways, namely, the NF-kB and JNK (c-Jun N-terminal kinases) pathways. NF-kB, a pro-survival pathway that is activated by death signals, enhances transcription of survival genes and blocks death signals at the level of TNFR1 via $FLIP_L$ (c-FLICE inhibitory proteins) and other antiapoptotic proteins [77]. However, high level of ROS can modulate NF-kB signaling through redox changes, thus mediating its inactivation and promoting apoptotic signaling [76]. In parallel, ASK1 (apoptosis signaling-regulating kinase 1), a MAPK kinase kinase, is inhibited in cytoplasm by Trx1 which contains critical redox-sensitive thiols [78, 79]. The oxidation of these thiols by ROS causes the dissociation of Trx1 and self-activation of ASK1. The activated ASK1 will phosphorylate and activate MKK, a MAPK kinase, which phosphorylates and activates JNK. A feedforward loop has been unveiled in which JNK translocates to the mitochondria where it initiates phosphorylation and inactivation of pyruvate dehydrogenase, thus promoting ROS generation which in turn triggers enhanced JNK activation [80]. Once activated, JNK targets mitochondria causing mitochondrial membrane potential collapse, the release of cytochrome *c* into the cytosol, and phosphorylation and inactivation of the antiapoptotic Bcl-xl protein [81].

1.3.2 Mitochondria-Derived (Intrinsic) Apoptosis

The intrinsic apoptotic signaling starts at the mitochondria when different apoptotic stimuli cause mitochondria-to-cytosol release of proapoptotic proteins (e.g., cytochrome *c*, [cyt *c*], apoptosis-inducing factor [AIF]) located in the mitochondrial intermembrane space. Once in the cytosol, proapoptotic factors trigger caspase-dependent and caspase-independent signaling leading to the execution of cell death [2, 42]. In caspase-dependent signaling, cyt *c* binds to the apoptotic protease-activating factor 1 (Apaf-1) forming the apoptosome which, in the presence dATP, recruits and activates pro-caspase 9 [82]. The activated caspase 9 signals downstream by cleavage/activation of effector caspases 3, 6, and 7, the executioners of apoptosis. Other proapoptotic proteins assist and enhance the apoptotic signal by blocking the inhibitory effects of inhibitory proteins, IAPs, an effect caused by Smac/Diablo (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI). In caspase-independent signaling, AIF and endonuclease G

(endoG) translocate from the mitochondria to the nucleus where promote nuclear chromatin condensation and large-scale DNA fragmentation [83].

How mitochondrial apoptogenic factors are released from mitochondria is less understood. Several mechanisms have been proposed to occur including formation of a megapore, mitochondrial permeability transition (MPT) pore, or pore formation within mitochondrial outer membrane [84, 85]. MPT pore, formed at the level of inner and outer mitochondrial membrane, comprises voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), the translocator protein TSPO (previously called peripheral benzodiazepine receptor), and cyclophilin D (cypD) [84, 86]. Recent evidence found that only cypD is a permanent component of MPT pore, being also its main regulator [87, 88]. In addition, MPT opening can be modulated by mitochondrial membrane potential ($\Delta\psi$), mitochondrial Ca^{2+}, thiol oxidation, or oxidative stress conditions [89]. A second mechanism of mitochondrial apoptogenic factors' release involves the Bcl-2 proteins, a superfamily of proteins consisting of over 30 members, namely, antiapoptotic proteins or proapoptotic proteins and BH3-only proteins. In normal conditions, the antiapoptotic proteins (e.g., Bcl-2, Bcl-xl, Diva, Mcl-1) react with the proapoptotic proteins (e.g., Bid, Bad, Bim, Bax, Noxa, Puma) and prevent the outer mitochondrial membrane permeabilization (MOMP). However, activation/truncation of the cytosolic BH3-only protein Bid or the activation of Bim enhances Bax/Bak (Bcl-2-associated x/Bcl-2 killer) protein conformational change and subsequent oligomerization which causes their insertion in the mitochondrial outer membrane [90, 91]. Upon insertion, Bax and Bak activate mitochondrial metalloproteinase AMA1 which cleaves the dynamin-like GTPase optic atrophy protein 1, OPA1, an event that is key to the remodeling of mitochondrial cristae and to the pore formation [92]. Additionally, fragmentation of mitochondrial network via fusion and fission processes occurs during extrinsic and intrinsic apoptosis and can lead to cyt *c* release. The role of OPA1, an inner membrane-associated protein that controls the morphology of crista junctions (CJ), has been documented. In particular, Zhang et al. found that Ca^{2}-induced ER stress resulted in MPT-mediated mitochondrial remodeling via OPA1 loss and cyt *c* release [93]. Interestingly, OPA1 response to apoptotic stimuli was dependent on tBid which widened the CJ and promoted mitochondria-to-cytosol cyt *c* translocation [94].

1.4 Mitochondrial ROS (mtROS) and Implication for Intrinsic Apoptosis

The mitochondrial electron transport chain is the main cellular process that generates ROS in mammalian cells under physiological conditions [95]. Mitochondrial ROS are a major determinant of cell apoptosis. It is believed that a low level of ROS can initiate apoptosis, while a high level of ROS promotes necrosis. Among ROS,

H_2O_2 has the longer half-life and can be transported outside mitochondria via aquaporins, a family of proteins that act as peroxiporins [96], where it can act as a second messenger in biochemical processes like autophagy, cell cycle, stress response, and redox balance. Importantly, aquaporins can modulate mtROS production. For example, silencing of inner mitochondrial membrane aquaporin 8 in human HepG2 cells enhanced mtROS generation leading to $\Delta\psi$ collapse via MPT pore opening and cell death [97].

Under normal physiological conditions, the inner mitochondrial membrane is key to maintenance of the electrochemical gradient necessary for respiration and energy production. Opening of the MPT pore by Ca^{2+} or oxidant-induced ROS leads to loss of negative electrochemical gradient across the inner mitochondrial membrane that maintains $\Delta\psi$. Dissipation of the $\Delta\psi$ results in depolarization of the membrane and dysfunctional mitochondria that promote increased ROS generation and decreased ATP production, events that finally trigger intrinsic apoptosis [98]. A direct involvement of mtROS generation in the initiation of mitochondria-derived apoptosis was demonstrated in various cell lines [99, 100]. Mechanistically, mtROS interact with proteins of the MPT pore to induce oxidative modifications that promote changes in the anion influx and the release of mitochondrial apoptogenic factors into the cytosol. Notably, treatment of cardiac myocytes with H_2O_2 caused hyperpolarization of mitochondrial membrane mediating mitochondrial translocation/insertion of Bax/Bak and the release of cyt *c* into the cytosol [101]. In another study, the Gli inhibitor, GANT61, triggered mitochondrial superoxide production inducing G1 phase arrest and apoptosis of human MMe cells [102]. In addition, cyclosporin A-dependent mtROS generation induced an increase in the expression of dynamin-related protein 1 (Drp1), a fission protein, and a decrease in the expression of mitofusin 2 (Mfn2) and Opa1, fusion proteins that modified the mitochondrial network and promoted apoptosis [103]. Since the antioxidant vitamin E protected against cyclosporine A effects, this indicated that mtROS were central to the apoptotic signaling [103]. Similarly, glutamate-induced mtROS generation facilitates a concomitant release of Opa1 and cyt *c* into the cytosol followed by mitochondrial fragmentation and intrinsic apoptosis of HT22 cells [104]. In another study, cytotoxic lymphocyte protease granzyme B-induced mtROS formation caused $\Delta\psi$ loss and MOMP. Notably, mtROS promoted cleavage of the subunits of NADH-ubiquinone oxidoreductase complex I leading to the disorganization of the respiratory chain that results in loss of CJ and the release of apoptogenic factors into the cytosol [105].

Another mechanism of mtROS-induced mitochondrial apoptotic signaling was unveiled in neutrophils. In this study, pyocyanin, a membrane-permeable pigment and a toxin released by *Pseudomonas aeruginosa*, enhanced mtROS generation that led to dissipation of $\Delta\psi$ and to the activation of mitochondrial sphingomyelinases. The subsequent formation of ceramide within mitochondrial membranes promoted cyt *c* release and neutrophil apoptosis [106]. Although much is known about the role of mtROS in apoptosis, some novel mechanisms are yet to be uncovered.

1.4.1 Cardiolipin Peroxidation and Initiation of Mitochondrial Apoptotic Signaling

Cardiolipin (CL), an acidic glycerophospholipid found exclusively in mitochondrial membranes, is essential for the eukaryotic energy metabolism and for the maintenance of mitochondrial structure and function [107]. Within the mitochondrial inner membrane, CL enables the formation of supercomplexes with enhanced activity, maintains membrane fluidity, and binds cyt *c* via electrostatic and hydrophobic interactions [107, 108]. Normally, cyt *c*, a heme containing protein bound to the outer leaflet of the mitochondrial inner membrane, shuttles electrons between complexes III and IV of the respiratory chain. In order for cyt *c* to translocate to cytosol via pore formation, it must first be released from its interaction with CL. Notably, the oxidation of CL is a prerequisite step in the release of cyt *c* into the intermembrane mitochondrial space [107]. Indeed, compared to the other phospholipids of the mitochondria, CL is more prone to ROS-induced oxidative modification because of its unsaturated carbon chains [109]. At low mtROS, tightly bound cyt *c* exhibits peroxidase activity, and significant amounts of oxidized CL have been measured after treatment of cells with mtROS-producing compounds [103]. Moreover, low levels of mitochondria-derived H_2O_2 can promote the unfolding of cyt *c* and the enhancement of its peroxidatic activity [110]. Peroxidation of saturated fatty acids of CL subsequently releases cyt *c* from mitochondria [110]. Oxidized CL is next distributed to the outer leaflet of the outer mitochondrial membrane where it functions as a platform for proapoptotic protein, tBid, enabling mitochondrial membrane permeabilization and the release of the cyt *c* into the cytosol [111]. Consequently, cardiolipin-cytochrome *c* can be viewed as a mitochondrial oxidative stress sensor and redox regulator of apoptotic signaling.

1.5 Role of Mitochondrial GSH in Oxidant-Induced Intrinsic Apoptosis

A known consequence of increased mtROS is the loss of mtGSH. Within mitochondria, GSH is important for maintaining the integrity of mitochondrial proteins and lipids and in controlling mitochondrial ROS generation. Therefore, it is not surprising that mtGSH decrease is associated with cell demise by apoptosis [41, 42], whereas an increase in mtGSH has a protective role against oxidative stress [112]. Mechanistically, ROS-induced mtGSH loss results in $\Delta\psi$ collapse and mitochondria-to-cytosol release of cyt *c* that triggers intrinsic apoptosis [113]. Notably, ethanol-mediated mtGSH depletion increases the sensitivity of rat hepatocytes to TNF-α and promoted apoptosis via CL oxidation and mitochondria-to-cytosol translocation of apoptogenic factors [114]. In addition, chronic models of alcoholism, where mtGSH is low and its transport is deficient, are characterized by mitochondrial damage associated with swelling, disruption, and disorganization of the normal cristae, effects that lead to lower capacity to produce ATP [115].

In acute diabetic rats, mtROS-mediated mtGSH decrease promoted Δψ loss and intrinsic apoptosis via activation of caspases 9 and 3 [116]. In addition, ROS-mediated mtGSH loss in heart mitochondria increased susceptibility to oxidative stress underscoring the importance of mtGSH in diabetes-mediated cardiomyocyte apoptosis [116]. Depletion of mtGSH in neurons leads to a subsequent increase in mitochondrial ROS production that was associated with Δψ collapse and cell death [117]. In colon carcinoma HT29 cells, menadione-induced cellular and mtGSH decrease promoted loss of mitochondrial membrane potential and apoptosis via cyt *c* release and activation of caspases 9 and 3 [113]. Thus, ROS-induced mtGSH loss is a prerequisite step in oxidant-induced mitochondrial apoptotic signaling.

Because of the technical challenge to quantify the mitochondrial GSH pool, the contribution of this redox compartment to the initiation of apoptotic signaling has not been evaluated. A significant advancement in procedures to alter mtGSH uptake was the genetic manipulation of the two transporters, OGC and DIC, located in the mitochondrial inner membrane [46, 118]. Studies in the Lash laboratory found that overexpression of mtGSH carriers, OGC or DIC, in rat renal proximal tubular NRK-52E cells protected against *tert*-buthylhydroperoxide (*t*BH)- or *S*-(1,2-diclorevinyl)-L-cysteine (DCVC)-induced apoptosis in association with higher mtGSH concentrations [119, 120]. In exchange, overexpression of cysteine mutant of OGC, a nonfunctional transporter, abrogated the protection against *t*BH and DCVC due to lower mtGSH levels. We used a similar strategy in colon carcinoma cells and found that overexpression of OGC carrier in HT29 cells protected against menadione-induced mitochondrial respiratory activity and intestinal apoptosis [113].

Notably, a direct link between mtGSH and the apoptotic machinery has been unveiled in which mtGSH interacts with the BH3 groove of the antiapoptotic protein in association with an antioxidant function of Bcl-2. In contrast, proapoptotic proteins Bax and BH3-only proteins displace GSH from its interaction with Bcl-2 and inhibit mitochondrial GSH transport into isolated rat brain mitochondria [121].

1.5.1 Mitochondrial GSH and Mitochondrial DNA Damage-Induced Apoptosis

As compared with nuclear DNA, mitochondrial DNA (mtDNA) is highly susceptible to oxidative damage due to the lack of histone protection and its close proximity to the mitochondrial electron transport chain, the main source of ROS within mitochondria [122]. Since mtDNA encodes several proteins of the respiratory chain, oxidative damage to mtDNA would decrease expression of these proteins and lead to dysfunction of mitochondrial respiration [123]. The subsequent disruption in the electron transport chain increases ROS formation and creates a vicious cycle that finally triggers intrinsic apoptosis [124]. By directly blunting ROS or by participating as a cofactor in the antioxidant reaction, mtGSH contributes to the preservation of the integrity of the mitochondrial genome and cell survival during oxidative stress. In this respect, studies have shown that there is an increase in mtDNA damage when the mtGSH level is decreased [125]. In vivo studies in rat lactating

mammary gland found that there is a direct relationship between low levels of mtGSH and greater mtDNA damage in association with increased superoxide production by mitochondria that induced apoptosis [126]. Similarly, Suliman et al. found that hemin-induced mtGSH loss promotes sensitization of a specific mtDNA region to deletion resulting in mitochondria-derived apoptosis in rat liver in association with increased expression of pro- and anti-apoptotic proteins Bax and Bcl-xl [127]. Our study in intestinal cells indicated that menadione (MQ)-mediated mtGSH loss exhibited a dose-dependent increase in mtDNA damage that was reversed by *N*-acetyl cysteine suggesting that mtDNA oxidative damage is redox dependent. Moreover, blockage of cytosolic GSH synthase exacerbated oxidative mtDNA damage in association with decreased mtGSH. In contrast, overexpression of the mtGSH carrier, OGC, conferred protection of mtDNA against MQ, thus confirming the close relationship between mtGSH and oxidative damage to mtDNA [128]. In conclusion, these results are supportive of a central role of mtGSH redox as a major contributor to mitochondrial genomic integrity and, moreover, to the control of mitochondrial apoptotic initiation during oxidative stress conditions.

1.6 Trx2 and Intrinsic Apoptosis

Within mitochondrial matrix, Trx2 acts in concert with Prx3 and Prx5 to catalyze the elimination of mtROS; therefore, Trx2 plays a role in regulating intrinsic apoptosis. For example, in $Trx2^{+/-}$ mice there is an increase in liver apoptosis compared with wild-type mice upon diquat treatment [129]. Similarly, in Trx2-deficient chicken, DT40, inhibition of the Trx2 gene promotes intracellular ROS generation and mitochondria-to-cytosol translocation of cyt *c* followed by caspase 9 and 3 activation [130]. Notably, transfection of DT40 cells with hTrx2 blocks these events, an effect that might be associated with the fact that Trx2 maintains Bcl-xl protein levels and preserves the redox state of the mitochondrial outer membrane [131]. In HeLa cells disruption of Trx2 redox state by cationic triphenylmethanes promotes the release of cyt *c* and AIF into the cytosol [132].

Trx2 can be involved in the control of apoptosis via its interaction with ASK1 [133]. Namely, the reduced form of Trx2 binds to mitochondria-bound ASK1 and inhibits its protein kinase activity, thus preventing oxidative stress- and cytokine-induced apoptosis. In oxidative stress conditions, the Trx-interacting protein, TXNIP, translocates from the nucleus to the mitochondria where it removes/competes with Trx2 from its binding with ASK1; this results in the induction of mitochondria-dependent apoptotic signaling [134]. Recently, Huang et al. reported that Trx2 deletion in mouse cardiomyocytes, Trx2-cKO, promotes mtROS generation, mitochondrial dysfunction with concomitant reduction in ATP production, and ASK1 initiation of apoptosis via caspase 3 cleavage [135]. In exchange, overexpression of Tx2 blocks ASK1-dependent apoptosis in association with a central role for Trx2 in apoptosis initiation [134].

1.7 MtROS and Neurodegenerative Disorders

Mitochondrial dysfunction and oxidative stress-induced intrinsic apoptotic signaling are central to neurodegenerative disorders including Parkinson's disease (PD) and Alzheimer's disease (AD). Current evidence indicates that, as compared with other tissues, the brain possesses lower tissue GSH levels in association with reduced capacity to import cysteine [136]. In addition, the brain consumes about 20 % of the O_2 supply of the body, and the mitochondrial electron transport chain is a major contributor to ROS generation [137]. Consequently, neurons are highly susceptible to oxidative stress. Notably, mitochondrial parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an inhibitor of complex I, enhances cellular and mtROS production and oxidative damage to lipids, DNA, and proteins and results in activation of mitochondria-dependent apoptotic molecular pathways [138]. MPTP treatment in mice causes the release of cyt *c* that is followed by the activation of caspases 9 and 3. Similar to MPTP, mitochondrial parkinsonian toxin rotenone, an inhibitor of complex I, promotes cyt *c* translocation, caspase 3 activation, and apoptotic signaling in SH-SY5Y cells [139] and in ventral mesencephalic dopaminergic neurons [140]. In MPTP-treated mice, apoptotic cell death has been shown to be dependent of proapoptotic protein Bax as the release of cyt *c* and the activation of caspase 3 are accompanied by upregulation of Bax and by its translocation to the mitochondria [138]. It is believed that mtROS-induced oxidative damage secondary to complex I inhibition, such as peroxidation of CL, increases the soluble pool of cyt *c* in the mitochondrial intermembrane space that can be subsequently released by activated Bax [138]. Based on these obseervation, a model of PD-related dopaminergic neurodegeneration was proposed in which MPTP-induced inhibition of complex I promotes mitochondrial ROS generation. Next, mtROS increases the soluble pool of cyt *c* via CL peroxidation, whereas ROS outside of mitochondria induces oxidative damage to lipids, protein, and DNA which triggers the activation of p53 and JNK. The p53 induces upregulation of Bax, whereas JNK induces transcriptional activation of BH3-only protein Bim which participates in Bax mitochondrial translocation.

AD, an age-related neurodegenerative disorder characterized by progressive deterioration of memory and intellectual function, is associated with oxidative stress-induced apoptotic signaling. The accumulation of misfolded proteins like Aβ (β-amyloid) kills neurons via oxidative stress [141]. For example, Aβ (25–35) and Aβ (1–40) peptides can induce ROS formation, lipid peroxidation, and GSH loss that collectively impair mitochondrial respiratory function and decrease ATP production [142]. Notably, exposure of teratocarcinoma ρ^0 cells, cells lacking mtDNA with nonfunctional mitochondria, to Aβ (25–35) did not elicit oxidative stress providing evidence that mitochondria are the source of ROS in β-amyloid-induced neurotoxicity [143]. Mechanistically, Aβ-induced mtROS formation triggers the activation of JNK and p38 [144]. Next, JNK downregulates antiapoptotic protein Bcl-xl and Bcl-w expression, and suppression of Bcl-w potentiates the release of apoptogenic factor Smac into the cytosol where it promotes caspase 9 activation [145].

Taken together, these results indicate that oxidative stress and JNK activation accompanied Aβ-induced neuronal apoptosis. In another study, generation of ROS in neurons cultured from transgenic mice with targeted overexpression of a mutant form of amyloid precursor was associated with cyt *c* release from mitochondria, caspase 3 activation, DNA fragmentation, and neuronal mitochondrial apoptosis [146]. In addition, non-amyloid-beta component of AD has been shown to enhance mtROS generation and apoptosis in cortical neurons of rat brain [147]. Collectively, these results suggest that mtROS-induced oxidative stress is a common denominator in neuronal apoptosis in PD and AD.

1.8 Summary and Perspective

Current understanding of the redox mechanisms involved in regulation of apoptotic signaling is incomplete despite intense research in the field. High levels of mtROS and oxidative damages to mitochondrial macromolecules are import factors associated with the initiation of mitochondrial apoptotic signaling. Also, changes in mtGSH/GSSG redox status, in conjunction with the other intramitochondrial redox systems, are central to coordination and control of mtROS-induced oxidative damage and in redox regulation of intrinsic apoptosis. In addition, CL, the phospholipid characteristic to mitochondria, is prone to ROS-induced oxidative damage. This phospholipid has dual roles, namely, to bind cyt *c* and to function as a platform for proapoptotic proteins when oxidized. Less understood is the contribution of oxidative damage to mtDNA. Apart from encoding polypeptides of the mitochondrial electron transport chain, oxidative damage to mtDNA induces a decrease in respiratory function enhancing ROS generation and ultimately triggers apoptosis. At present, the precise role of ROS-induced mtDNA damage in regulation of intrinsic apoptosis is unclear and should provide and challenge new research.

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Chapter 2
Dopamine Metabolism and Reactive Oxygen Species Production

Sylvie Delcambre, Yannic Nonnenmacher, and Karsten Hiller

Abbreviations

AADC	Aromatic amino acid decarboxylase
ALDH	Aldehyde dehydrogenase
ANLSH	Astrocyte–neuron lactate shuttle hypothesis
ApN	Aminopeptidase N
BBB	Blood–brain barrier
BH4	Tetrahydrobiopterin
CA	Catecholamine
COMT	Catechol-*O*-methyltransferase
DA	Dopamine
DAB	Dopamine-beta-hydroxylase
DAT	Dopamine transporter
DHPR	Dihydropteridine reductase
DOPAC	3,4-Dihydroxyphenylacetic acid
DOPAL	3,4-Dihydroxyphenylaldehyde
DOPET	3,4-Dihydroxyphenylethanol
DT Diaphorase	NAD(P)H:quinone oxidoreductase
E	Epinephrine
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione

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HVA	Homovanillic acid
L-DOPA	L-3,4-Dihydroxyphenylalanine
MAO	Monoamine oxidase
MCT	Monocarboxylate transporter
NE	Norepinephrine
NM	Neuromelanin
NO	Nitric oxide
NOS	Nitric oxide synthase
PD	Parkinson's disease
PNMT	Phenylethanolamine-*N*-methyltransferase
PST	Phenylsulfotransferase
PTP	Permeability transition pore
ROS	Reactive oxygen species
SN	Substantia nigra
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
TH	Tyrosine hydroxylase
UGT	Uridine diphosphoglucuronosyltransferases
VMAT2	Vesicular monoamine transporter 2
γ-GT	Gamma-glutamyl transpeptidase

2.1 Dopamine Metabolism

2.1.1 Dopamine Biosynthesis

Dopamine (DA) is a hormone and neurotransmitter and belongs to the group of catecholamines (CAs). It can be found in both the central and the peripheral nervous system. After its discovery at the beginning of the twentieth century, DA was primarily believed to be the precursor of norepinephrine. It was not until 1959 that Carlsson suggested a more important role for DA itself after finding that the distribution pattern of DA in the brain is different from that of norepinephrine [1]. Based on current knowledge, DA can be synthesized by cells via three different pathways (Fig. 2.1). The first and main pathway, which is carried out in neuronal cells in vivo, was first described in 1939 and starts from the amino acid tyrosine [2]. In a first step, L-3,4-dihydroxyphenylalanine (L-DOPA) is produced by hydroxylation of tyrosine at the *ortho*-position of the phenol ring. This reaction is catalyzed by the enzyme tyrosine hydroxylase (TH) that belongs to the family of biopterin-dependent aromatic amino acid hydroxylases. During the second step, L-DOPA is decarboxylated by the enzyme aromatic amino acid decarboxylase (AADC) to form DA. The hydroxylation by TH represents the regulatory switch of DA-synthesis: (1) TH activity is dependent on the availability of its cofactor tetrahydrobiopterin (BH_4), and (2) TH is strongly regulated on both the transcriptional and posttranscriptional level, including allosteric control, covalent modifications, and protein–protein interactions [3]. A second option to produce DA is given through the activity of the

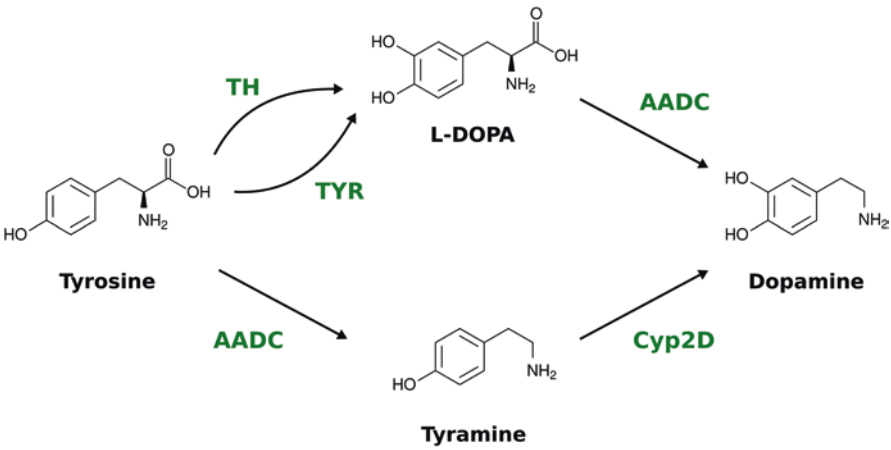

Fig. 2.1 Dopamine synthesis. DA can be synthesized via three different pathways. The classical pathway consists of hydroxylation of tyrosine by TH followed by AADC-catalyzed decarboxylation. Under TH-deficient conditions, TYR has been shown to produce L-DOPA instead of dopaquinone in order to ensure sufficient DA production. In a third pathway, tyrosine is first decarboxylated to form tyramine which is subsequently hydroxylated by the P450 enzyme Cyp2D

metalloenzyme tyrosinase. Like TH, this enzyme catalyzes the hydroxylation of tyrosine to L-DOPA, but in a second reaction further oxidizes L-DOPA to form dopaquinone. In melanocytes (pigment-producing cells present in the skin), dopaquinone is used as a precursor to form the black polymer–pigment eumelanin [4–6]. However, it has also been shown that tyrosinase activity can lead to DA production in TH-deficient mice [7]. In this case the reaction stops at L-DOPA, which is subsequently decarboxylated to DA. A third DA-producing pathway was identified in rats in vivo [8]. For this pathway, the series of reactions is reversed compared to the other two pathways: tyrosine is first decarboxylated by AADC to yield tyramine and then hydroxylated by Cyp2D enzymes belonging to the cytochrome-P450 family to form DA.

2.1.2 Dopamine Degradation

The controlled and enzymatically catalyzed degradation of cytoplasmic DA represents a crucial part of its metabolism in the brain (Fig. 2.2). If DA is not actively degraded by the cell, it easily auto-oxidizes, leading to the formation of highly reactive intermediates as well as reactive oxygen species (ROS). DA degradation is carried out both in glial cells and DAergic neurons. Glial cells take up DA released by neurons into the synaptic cleft during signal transmission, but transporter-mediated DA reuptake is also observed in neurons [9]. There, it is recycled by being repackaged into synaptic vesicles via the vesicular monoamine transporter 2

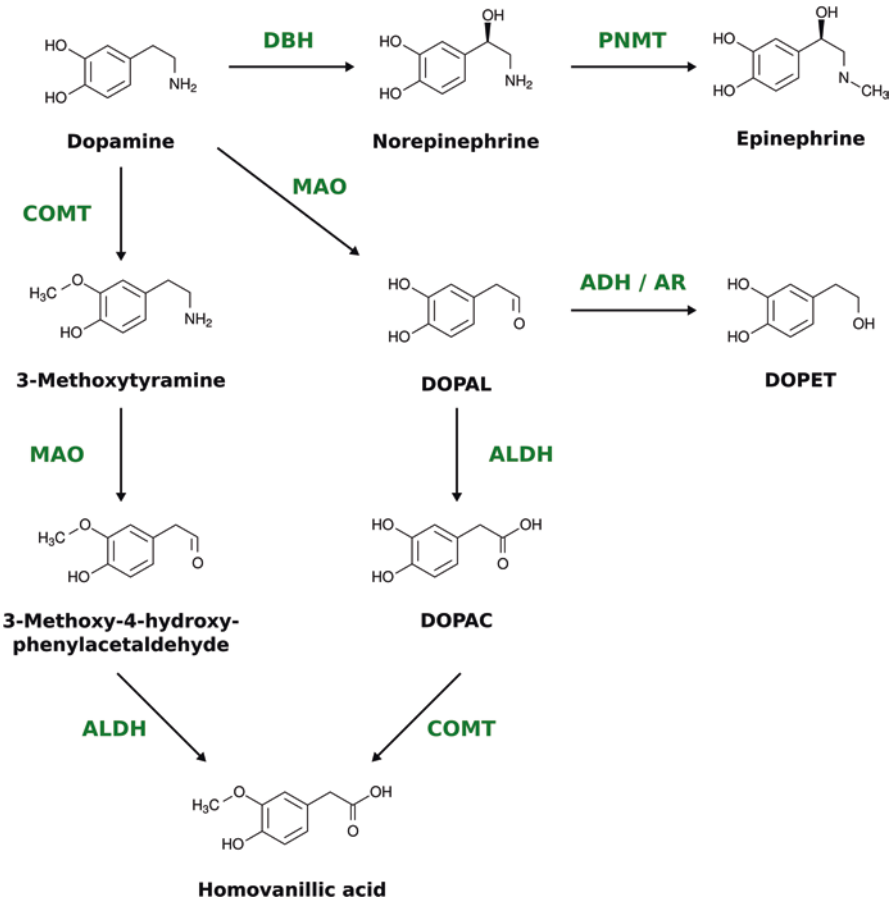

Fig. 2.2 Enzymatic dopamine degradation. DA can be hydroxylated to form the neurotransmitter norepinephrine. Further methylation at the amino group results in epinephrine. The DA degradation product homovanillic acid can be produced by two pathways, both using the enzymes MAO, ALDH, and COMT in different order. DOPET is derived from the degradation intermediate DOPAL via ADH- or AR-catalyzed reduction of the aldehyde moiety

(VMAT2). In neurons, DA degradation only takes place when DA leaks into the cytosol. Both cell types are able to oxidize DA via the mitochondria-associated enzyme monoamine oxidase (MAO) to form 3,4-dihydroxyphenylacetaldehyde (DOPAL). Hydrogen peroxide is formed as a by-product of this reaction. DOPAL can afterwards either be oxidized to its corresponding acid (3,4-dihydroxyphenylacetic acid, DOPAC) or reduced to the corresponding alcohol (3,4-dihydroxyphenylethanol, DOPET). DOPAC can undergo a further methylation step of the 3′-hydroxyl group of the catechol ring, resulting in homovanillic acid (HVA). This step is catalyzed by the catechol-*O*-methyltransferase (COMT). COMT can also first act on DA to form 3-methoxytyramine, which is then oxidized by MAO. The resulting intermediate

3-methoxy-4-hydroxyphenylacetaldehyde is subsequently oxidized to HVA by aldehyde dehydrogenase (ALDH). In addition to its own role as a neurotransmitter, DA is also the direct precursor of norepinephrine, which is formed by the DA-β-hydroxylase (DAB) reaction. Subsequently, norepinephrine can be methylated at the amino group to form epinephrine. This reversible reaction is carried out by the enzyme phenylethanolamine-*N*-methyltransferase (PNMT).

Besides these processes, which are mainly based on oxidation and reduction, there is also the possibility of DA degradation by sulfate and glucuronide conjugation. Such processes are referred to as *phase II* reactions and are often performed by hepatic and renal enzymes, but can also be found in the central nervous system [10]. The aim of these reactions is to covalently attach polar groups to target molecules and increase their water solubility. The modified molecules can then easily be eliminated via the renal system. Sulfurylation and glucuronidation are not only performed on DA itself but also on its degradation products [11]. The responsible enzymes for this mechanism are phenylsulfotransferases (PSTs) and uridine diphosphoglucuronosyltransferases (UGTs), which transfer the respective groups from either 3′-phosphoadenosine-5′-phosphosulfate or UDP glucuronic acid to the target molecule. Conjugation always takes place at one of the hydroxyl groups of the catechol moiety, resulting in a mix of 3- and 4-sulfates and glucuronides [12, 13]. The extent to which either of these reactions is performed strongly differs between species and even tissues.

2.2 The Role of Neuromelanin in Dopaminergic Neurons

Under physiological conditions, intracellular dopamine (DA) is prone to spontaneous and iron-catalyzed oxidation [14]. The oxidation products emerging from this process are highly reactive and can produce ROS which in turn cause elevated oxidative stress levels to DA-producing cells. There are several mechanisms available to a cell to prevent the accumulation of excessive amounts of DA: (1) transport of DA into synaptic vesicles and their subsequent secretion [15], (2) enzymatic degradation of DA [3], and (3) polymerization of DA and its metabolites to form neuromelanin (NM) [16].

Since the first two of these processes are associated with strongly regulated physiological functions of DAergic cells, their ability to decrease cytoplasmic DA levels is limited. The lesser regulated polymerization of DA and its oxidation products to form NM however seems to represent a more effective sink for these highly reactive compounds. NM is a polymer pigment mostly found in catecholaminergic neurons of the substantia nigra (SN) and the locus coeruleus of the human brain [17–19], but was also shown to be present in other brain areas in lower amounts [20–24]. NM consists of a melanic structure with proteins and lipids covalently attached to it (Fig. 2.3). Comparison with synthetic melanins has suggested that the melanic part of NM is a mixed-type pigment comprising both eu- and pheomelanin [25]. The eumelanin part mainly consists of polymerized oxidation products of DA and

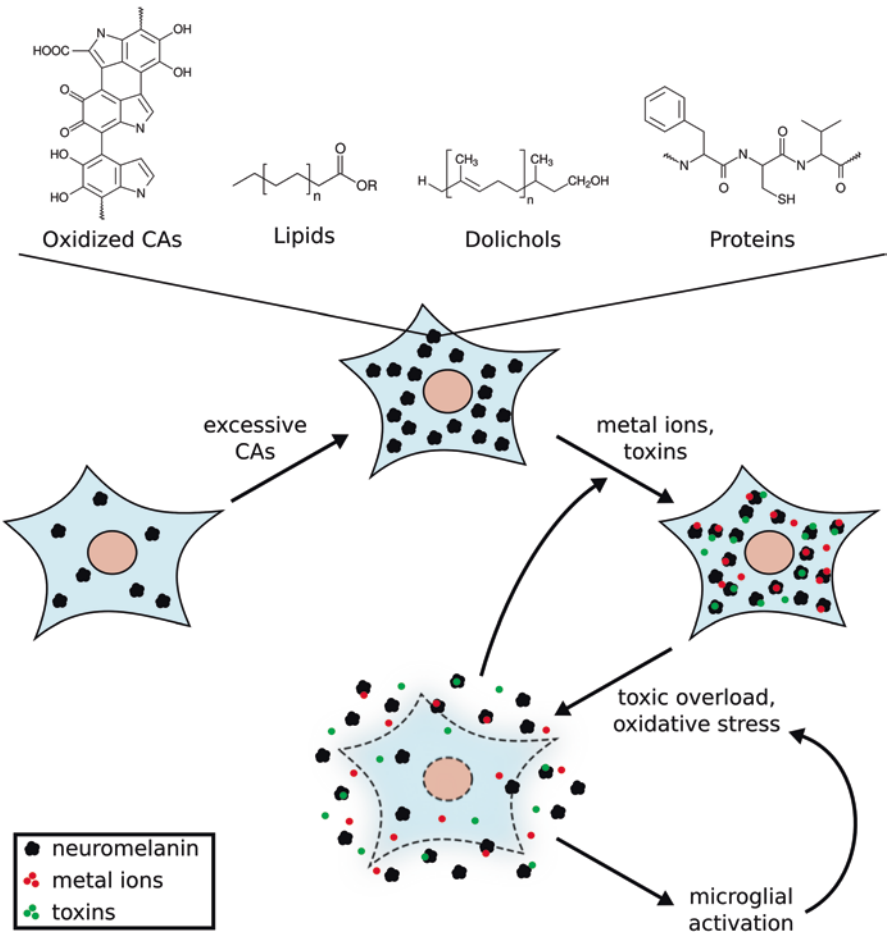

Fig. 2.3 NM biosynthesis in SN neurons is triggered by excessive cytoplasmic levels of CAs. NM can bind substantial amounts of metal ions (mostly iron) and organic toxins to prevent cell damage. These molecules are released upon cell death and can cause damage to surrounding cells. NM release further induces an inflammatory response by microglial activation which leads to oxidative stress

L-DOPA, namely, 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid [26]. For the pheomelanin part, oxidized cysteine is additionally integrated into the polymer. Studies investigating the composition and macromolecular structure of NM have found that lipids (mainly dolichols) account for around 18 % of pigment mass, while another 12–15 % are composed of peptides [27]. NM granules undergo autophagy and are enclosed by a lipid bilayer. The produced organelles can later fuse with lysosomes during their maturation process.

The synthesis process of NM has not yet been completely elucidated; however, several findings point toward a mostly auto-oxidative buildup of the pigment.

The metalloenzyme tyrosinase plays a vital role during the synthesis of peripheral melanins [4–6], but NM levels in tyrosinase-deficient albinos remain unchanged [18], thus suggesting a tyrosinase-independent synthesis process. In addition, no other enzymes are known to play a role during NM synthesis. Sulzer and colleagues have shown that treatment of PC-12 cells with the dopamine precursor L-DOPA leads to an increased production of NM granules [28]. Overexpression of the synaptic vesicle CA transporter VMAT2 inhibited NM biosynthesis by decreasing cytosolic DA levels. These results show that NM synthesis strongly depends on the cystolic availability of DA. By removing excessive amounts of potentially harmful CA intermediates from the cytosol, NM might thus exert a neuroprotective function.

Besides its ability to trap CAs, NM is also able to bind large quantities of iron and other metal ions (e.g., copper, zinc, and lead) as well as neuronal toxins (e.g., paraquat and MPP^+) [29–32]. This does not only play a pivotal role in iron homeostasis but also in the prevention of iron-catalyzed oxidation of CAs and metal ion- or toxin-mediated neurotoxicity. The iron contained in NM is mostly in the ferric state (Fe^{3+}) and can be bound to the melanic portion at two sites, differing in their binding strength [33, 34]. Under normal conditions, most of the iron is tightly bound to high affinity sites. In these sites, the iron is coordinated by catechol groups and remains in an inactive form, thus preventing oxidative reactions. When an excess of iron is present in the cytosol, as reported during Parkinson's disease (PD) [35, 36], most of these sites are occupied. Iron is then also bound to low affinity sites. As the binding to these sites is not as effective as to high affinity sites, the iron remains redox-active, thus promoting oxidative reactions.

Following neuronal cell death, NM is released into the extracellular space, where it can trigger a series of detrimental events. Released NM has been shown to induce an immune response by microglial activation in PD patients [37–39]. As a part of this process, several pro-inflammatory proteins and molecules such as tumor necrosis factor alpha, interleukin-6, and nitric oxide are released from the microglial cells [40]. The initial response is readily turned into a chronic inflammatory process [41]. This might be explained by the fact that NM is not soluble and, due to its complex composition, only slowly degraded. The high density of microglial cells in the SN additionally increases the intensity of this response [42]. Metal ions and toxins which were previously bound to the pigment can be released into the extracellular space. This in turn represents another source for ROS production, eventually leading to the death of more neurons and glial cells. The result of this series of processes is a vicious cycle where oxidative stress leads to neuronal cell death and vice versa (Fig. 2.3). It is conceivable that this series of events is one of the factors which are driving the progression of PD in vivo.

In summary, NM can play two opposing roles, depending on the cellular context [43]. Under normal conditions, NM is able to serve as a sink for excessive amounts of CAs, which helps in preventing the accumulation of ROS. It also plays a vital role in iron homeostasis and can bind other metal ions and organic compounds, thus decreasing their toxicity. This beneficial role is however easily reversed under stressed conditions as occurring during PD. Iron overload leads to its binding to low

affinity sites, where it remains redox-active. NM released from dead cells is only slowly degraded and induces an immune response by microglial activation. When present in the extracellular space, NM releases previously bound metals and toxins, which represent an additional burden to the already stressed cells.

2.3 ROS Production During Dopamine Metabolism

ROS are produced during the metabolism of DA either enzymatically or nonenzymatically. In the first case, the most important enzyme involved in ROS production is MAO. This process is believed to occur under low concentrations of intracellular DA. With increasing intracellular DA concentrations, an increase in ROS production caused by the autoxidation of degradation products results in an inhibition of mitochondrial respiration [44].

2.3.1 MAO-Linked ROS Production

MAO is located in the outer membrane of mitochondria [45] and catalyzes the degradation of DA to DOPAL (Fig. 2.4) via the following reaction (2.1):

$$\mathrm{DA} + \mathrm{O_2} + \mathrm{H_2O} \rightarrow \mathrm{DOPAL} + \mathrm{H_2O_2} + \mathrm{NH_3}. \tag{2.1}$$

DOPAL has been shown to induce mitochondrial permeability transition pore (PTP) opening, which can trigger cell death [46]. PTP opening will be discussed in more detail later on.

Hydrogen peroxide (H_2O_2) is a natural by-product of respiration, and about 1–3 % of the O_2 consumed by a cell is converted to H_2O_2 [47]. However, during MAO activity, the amount of H_2O_2 produced can increase 100-fold [48]. Due to the iron-rich environment in the substantia nigra pars compacta [49], H_2O_2 can react with iron via the Fenton reaction and generate hydroxyl radicals, which are highly reactive and damaging, causing DNA mutations, lipid peroxidation, and modification of certain amino acids [50].

Thus, given the risks generated by high concentration of H_2O_2, it has to be detoxified. Because mitochondria do not contain the catalase enzyme [45], the enzyme catalyzing this reaction is glutathione peroxidase (GPx). This enzyme is located in the mitochondrial matrix and at the contact points between the inner and outer mitochondrial membrane [51]. It catalyzes the following reaction (2.2):

$$\mathrm{H_2O_2} + 2\mathrm{GSH} \rightarrow \mathrm{GSSG} + 2\mathrm{H_2O}. \tag{2.2}$$

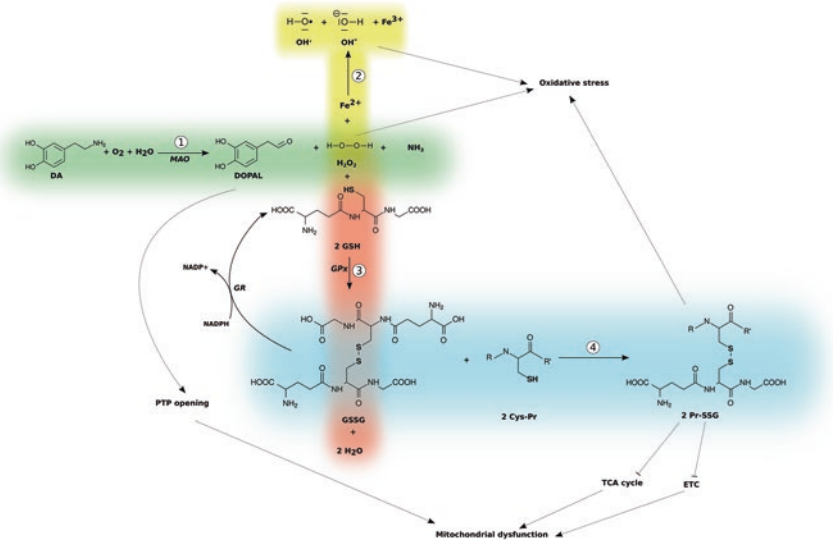

Fig. 2.4 Degradation of dopamine via MAO and induced toxicity. (*1*) The monoamine oxidase (MAO) catalyzes the oxidation of DA to DOPAL and produces H_2O_2 as by-product. High concentrations of DOPAL induce PTP opening and lead to mitochondrial dysfunction. (*2*) H_2O_2 can react with Fe^{2+} via the Fenton reaction and lead to the production of hydroxide ion and hydroxyl radicals which in turn increase oxidative stress levels within the cell. (*3*) H_2O_2 can be detoxified via the glutathione system. 2GSH will react with H_2O_2 to produce $2H_2O$ and GSSG via the glutathione peroxidase (GPx). GSSG is recycled into GSH by the action of GSH reductase (GR) using NADPH as a cofactor. (*4*) GSSG can react with free cysteinyl residues of proteins to form disulfide bonds. If this reaction occurs in the catalytic site of an enzyme, it can induce its inactivation. Proteins sensitive to this reaction include enzymes involved in the tricarboxylic acid (TCA) cycle or the electron transport chain (ETC), leading to the inactivation of these two pathways and ultimately to mitochondrial dysfunction. R and R′ represent the peptidic chain of the protein

Although at this stage H_2O_2 has been detoxified, the oxidized glutathione (GSSG) can spontaneously react with the thiol group of cysteinyl residues of proteins to form disulfide bonds. If a cysteinyl residue is located in the catalytic site of an enzyme, disulfide bonds formation leads to its inactivation. Enzymes shown to be inactivated this way include succinate dehydrogenase, NADH dehydrogenase, ATPase, isocitrate dehydrogenase, pyruvate dehydrogenase, alpha ketoglutarate dehydrogenase, and aconitase [45]. The inactivation of these enzymes induces an inhibition of the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain. Sulfhydryl modifications have also been shown to induce PTP opening and regulation [52].

PD is also associated with a higher dopamine turnover due to the lower number of dopaminergic neurons [53]. This induces a higher activity of MAO and thus an increase in MAO-associated toxicity. The increased turnover also promotes an increase in the autoxidation of dopamine and its metabolites which is described in the following section.

2.3.2 *Autoxidation-Linked ROS Production*

High intracellular concentrations of dopamine can lead to the formation of intermediate quinone products [44] (Fig. 2.5). These quinones can be produced either enzymatically or by autoxidation. The enzymatic formation of superoxide radicals or hydrogen peroxide increases the rate of CA oxidation [54]. Enzymes involved in the process include prostaglandin H synthase, lipoxygenase, and xanthine oxidase [55]. CA autoxidation can also occur in the presence of reactive nitrogen species that are derived from nitric oxide (NO) [56]. NO is synthesized in the central nervous system by the neuronal nitric oxide synthase (nNOS) as a mediator of the response to excitatory amino acids [57].

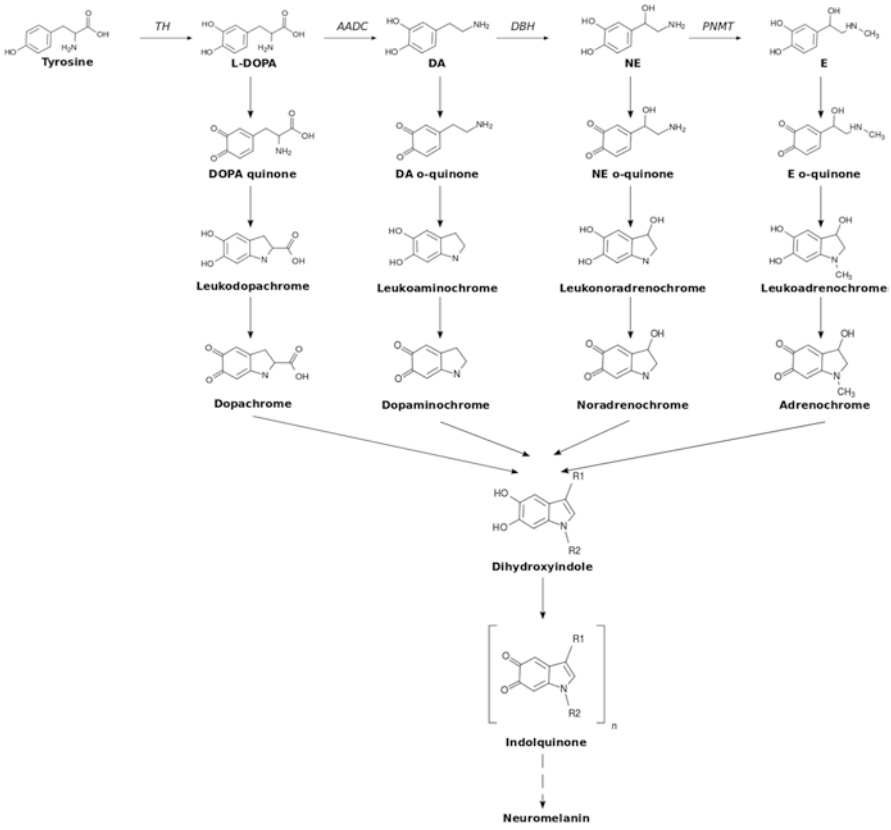

Fig. 2.5 Autoxidation of CAs. The synthesis of epinephrine from tyrosine occurs enzymatically as described in Fig. 2.1. Each of the metabolites of the CA synthesis pathway can auto-oxidize and form the respective quinones. Quinones then cyclize into leuko chrome intermediates, which further oxidize into chrome intermediates. The next step in the pathway is the formation of dihydroxyindole which oxidizes to form indolequinone which can polymerize to form neuromelanin. Depending on the precursor, R1 represents either H or OH and R2 represents H or CH_3

All products of the CA synthesis pathways are prone to oxidation, including epinephrine and norepinephrine. Products of dopamine oxidation include dopamine-*o*-quinone that cyclizes irreversibly to form leukoaminochrome and dopaminochrome. The oxidation pathways converge to dihydroxyindole and then to indole quinones that can polymerize to form neuromelanin [58].

Oxidation reactions producing CA quinones lead to the activation of proteases and lipases, irreversible inactivation of COMT, and an increase in cytosolic calcium concentration [56]. Quinones also impair mitochondrial function [55]. They are electron-deficient and react with nucleophiles such as cysteines, reduced glutathione (GSH), and cysteinyl residues of proteins to form 5-cysteinyl-dopamine [55].

The formation of quinone intermediates is reversible and can be prevented by antioxidants such as the GSH/GPx-system or simply cysteine due to its thiol group or by ascorbate [55]. However, once quinones have been formed, they can either be used as substrates by the NAD(P)H:quinone oxidoreductase (DT diaphorase) (two-electron reduction) or by the NADPH cytochrome P450 reductase (one-electron reduction) [54, 59] (Fig. 2.6). The action of the DT diaphorase leads to the production of leukochromes that are more redox stable and can subsequently be secreted [60]. This enzyme thus bypasses the semiquinone intermediate produced by the NADPH cytochrome P450 reductase. Semiquinone intermediates rapidly oxidize in the presence of oxygen to form back the quinone and superoxide anion radicals [59]. Other enzymes involved in quinone detoxification include sulfotransferases, COMT, or superoxide dismutase (SOD) [56]. Another important class of enzymes involved in quinone detoxification is metallothioneins. Their primary function is to maintain metal homeostasis by binding to heavy metals. As the name suggests, these proteins are containing a big amount of cysteinyl residues that can make up to 40 % of the total protein content [55].

Many enzymes contain cysteinyl residues playing a critical role in their function. The formation of 5-cysteinyl-dopamine has been shown to interfere with the activity of enzymes of the dopamine synthesis and transport pathways such as TH, dopamine transporter (DAT) [55], or dihydropteridine reductase (DHPR) [61]. These enzymes are crucial since TH is the rate-limiting step in dopamine synthesis and DAT regulates the duration and magnitude of dopamine signaling at the extracellular level and the dopamine concentration at the intracellular level [62]. DHPR is the enzyme responsible for BH_4 recycling, which is an important cofactor for TH, but also for phenylalanine hydroxylase (converting phenylalanine to tyrosine) as well as the tryptophan hydroxylase (first enzyme of the serotonin synthesis pathway) [52]. The inhibition of these enzymes under oxidative stress conditions contributes to cell protection by decreasing the intracellular amounts of dopamine and thus limiting its toxicity.

A protein that has been shown to be associated with PD is also affected: α-synuclein. This protein is initially produced in a soluble form. In order to form fibrils, it is aggregated to an intermediate state, the protofibrils. These protofibrils are toxic when not processed and accumulate to form Lewy bodies in PD patients. Although this protein does not contain cysteinyl residues, quinones are thought to affect its tyrosine and lysine residues. This formation of quinone–protein complexes

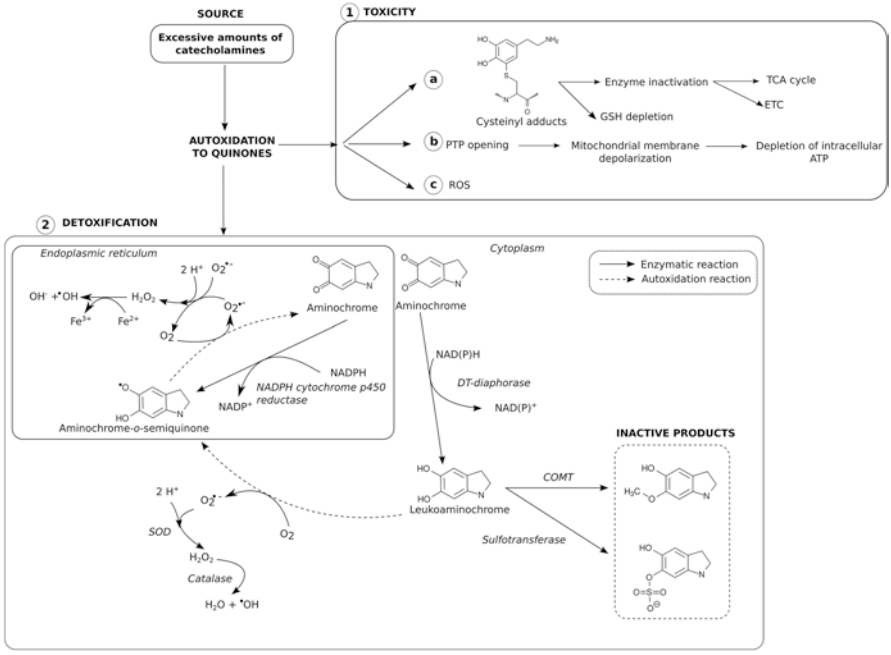

Fig. 2.6 Cytotoxicity and degradation of quinones. Excess of dopamine not transported into vesicles can auto-oxidize in the cytosol to form quinones (Fig. 2.5). (*1*) Toxicity. (*a*) Dopamine-*o*-quinone can react with cysteinyl residues of proteins. If these residues are located in the catalytic site of an enzyme, it leads to its inactivation. Affected enzymes include succinate dehydrogenase, NADH dehydrogenase, ATPase, isocitrate dehydrogenase, pyruvate dehydrogenase, alpha ketoglutarate dehydrogenase, and aconitase. Dopamine-*o*-quinone can also react with the cysteinyl residue of GSH, which subsequently cannot be used as antioxidant anymore leading to GSH depletion. (*b*) Oxidation of quinones also induces the opening of the permeability transition pore (PTP) thus increasing the permeability of the inner mitochondrial membrane. This causes a proton leak across the membrane which leads to an uncoupling of ATP synthesis and ultimately to ATP depletion. (*c*) The autoxidation process consumes oxygen and produces superoxide radicals which contribute to the increase in intracellular reactive oxygen species (ROS). (*2*) Detoxification. Enzymes involved in the detoxification are the NADPH cytochrome p450 and the DT diaphorase. The former is located in the endoplasmic reticulum and uses NADPH to catalyze the one-electron reduction of aminochrome to aminochrome-*o*-semiquinone. The aminochrome-*o*-quinone can auto-oxidize back to aminochrome and generate superoxide radicals. Superoxide radicals and hydrogen peroxide induce an increase in the autoxidation rate. In the cytoplasm, the DT diaphorase uses the same substrate, but converts it to leukoaminochrome via a two-electron reduction reaction. This enzyme can use both NADH and NADPH. Leukoaminochrome can auto-oxidize to aminochrome-*o*-quinone, but in the cytosol this can be prevented by the superoxide dismutase (SOD) and catalase which remove superoxide radicals and hydrogen peroxide, thus decreasing the rate of autoxidation. Finally, catechol-*o*-methyltransferase and sulfotransferase can inactivate leukoaminochrome by covalent modification which can then be secreted

prevents the conversion of the protofibrils to fibrils, thus leading to an accumulation of the toxic protofibrils in the cytoplasm [55].

On the other hand, another protein associated with PD has a protective effect over quinone toxicity: Parkin is an E3 ubiquitin ligase. Its function is to recognize sub-

strates and promote their ubiquitination, thus targeting them for degradation by the ubiquitin–proteasome pathway [63]. Knockdown of the enzyme induced accumulation of dopaminochrome and apoptotic death in a neuronal cell model [55].

As discussed in the previous section, many enzymes of the TCA cycle and the mitochondrial electron transport chain are affected by modification of cysteinyl residues [45]. Furthermore, 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBT-1), an oxidation product of 5-cysteinyl-dopamine, can also affect mitochondrial complex I [55].

Exposure to dopamine quinones has also been reported to cause a proton leak across the inner mitochondrial membrane, thus uncoupling ATP synthesis. This leads to respiration without ATP production and in turn depletion of intracellular ATP levels [52].

Oxidation products of dopamine do not only affect neurons but also activate microglia and induce expression of neurotoxic genes such as inflammation markers, cytokines, prostaglandins, and chemokines. On the other hand, quinones also induce a downregulation of the neuroprotective genes P2X and of the sestrin 1 gene which is responsible for balancing antioxidant levels, thus contributing to an increase in oxidative stress [64].

Several drugs have been identified to protect against quinone-mediated toxicity. One class includes DT diaphorase activators, such as 3-*tert*-butyl-4-hydroxyanisole (BHA), *tert*-butylhydroquinone, or dimethyl fumarate which also activates the GSH S-transferase and GSH reductase. Another class of compounds protecting against quinone-mediated toxicity is the nonsteroidal anti-inflammatory drugs (NSAIDs). Their main role is to protect against inflammation, but they also have been shown to inhibit the activity of prostaglandin H synthase, thus preventing quinone formation [55].

2.3.3 Permeability Transition Pores (PTPs)

One of the consequences of dopamine degradation, either via the activity of MAO or via autoxidation, is the induction of PTP opening. This is highly relevant since this opening can induce a loss of mitochondrial function and will thus be further discussed in this section.

PTPs are calcium-dependent protein channels that allow solutes smaller than 1500 kDa to cross the impermeable inner mitochondrial membrane. The perturbation of permeability induces the depolarization of the membrane potential, followed by the release of small solutes and finally proteins. The consequences are a swelling of the mitochondria and a loss of oxidative phosphorylation [52].

PTP opening is induced by oxidants and toxic quinones. In this case, the inner membrane permeability is increased, which leads to a proton leak across the membrane and thus, an uncoupling of ATP synthesis. This can be experimentally circumvented by the addition of cyclosporin A, which prevents PTP opening, highlighting the effect of quinones on PTPs [52].

Cysteinyl residues located in the active site of PTPs have also been implicated in their regulation, rendering these proteins sensitive to sulfhydryl modifications, such

as disulfide bond formation between quinones and cysteinyl residues of proteins [52]. Another regulator of PTP opening is the redox status of pyridine nucleotides. Oxidation of NADH and NADPH to NAD^+ and $NADP^+$ can induce pore opening. This shift can be achieved through the DT diaphorase enzyme, which detoxifies quinones via a two-electron reduction while oxidizing either NADH or NADPH [52].

Finally, it has been shown that PTP opening is also dependent on the activity of mitochondrial complex I [46]. If the activity of complex I is compromised as, for example, by the action of environmental toxins, PTPs will be more sensitive to DOPAL toxicity or the modification of cysteinyl residues.

2.3.4 L-DOPA Toxicity

L-DOPA is the direct precursor of dopamine. It was reported as such in 1938, although it had first been isolated in 1913 by Guggenheim who suggested that it was biologically inactive [65].

It was not before 1960 that dopamine deficiency was identified as the cause of the motor symptoms in PD and that subsequent intravenous injection of L-DOPA could transiently sooth the symptoms. The US Food and Drug Administration approved L-DOPA for PD treatment in 1970, and nowadays, it is still the most commonly used drug to cope with the symptoms. However, L-DOPA is not curing the disease, and 40–75 % of the patients under treatment will eventually redevelop motor symptoms within 4–6 years of L-DOPA therapy [65].

The efficacy of L-DOPA treatment versus direct dopamine administration is due to the fact that L-DOPA, in contrast to DA, can be transported across the blood–brain barrier (BBB) via the large neutral amino acid transport system. In order to increase the half-life of L-DOPA in the blood of the patients, it is commonly given in combination with AADC inhibitors such as carbidopa (which cannot cross the BBB), MAO inhibitors, or DA agonists [65].

The toxicity induced by L-DOPA treatment is similar to that induced by oxidation of DA: L-DOPA can also auto-oxidize and form reactive quinones that will contribute to an increase in oxidative stress [58]. Chronic L-DOPA therapy in rats reduces the activity of mitochondrial complex I. This is also the case when rats are treated with DA, but not with DOPAC or HVA, two products further down in CA metabolism [66].

Treatment with L-DOPA increases neuronal levels of DA but also of its downstream metabolites, including ROS formation. Since PD patients are treated with L-DOPA over long periods of time (years to decades), the permanent exposure might also affect other cell types in the body. Studies have shown that patients diagnosed with PD show a lower risk of developing most types of cancer [67]. However, an increase in cases of melanoma, a tumor of pigment-producing cells (melanocytes) in the skin, has been reported in patients with PD [68, 69]. One possible explanation for this correlation might be that melanocytes oxidize the administered L-DOPA and use it as a building block for eumelanin synthesis. The ROS produced

during these processes might over time lead to cancer development [70, 71]. There are indeed some studies which have indicated that the higher prevalence of melanoma in PD patients could be caused by long-term L-DOPA treatment [72, 73]. However, more recent cohort studies could not confirm this hypothesis [74, 75].

2.4 Astrocyte–Neuron Interactions

In the brain, dopaminergic neurons only account for 1–2 % of the total cell population [76]. While microglia are responsible for the immune surveillance in the brain, astrocytes are supporting other cell types by providing them with nutrients or protecting them against ROS-induced neurotoxicity [77]. Astrocytes and neurons interact in several ways (Fig. 2.7).

One of the examples of astrocyte–neuron cross talk is the astrocyte–neuron lactate shuttle hypothesis (ANLSH). This hypothesis states that during periods of high activity, such as hypoglycemia or hypoxia [78], astrocytes use anaerobic glycolysis to produce energy in the form of ATP and export lactate. Neurons can take up extracellular lactate via the monocarboxylate transporters (MCT) and oxidize it to pyruvate to fuel the TCA cycle. This shuttle is mediated by the glutamate release of neurons [79]. Glutamate is then taken up by astrocytes and induces aerobic glycolysis. This glutamate is converted to glutamine by the glutamine synthetase, an ATP-dependent enzyme absent in neurons, and then exported back for further neuronal use [77].

Astrocytes can also protect neurons from oxidative stress via a mechanism linked to their gap junction network [80], in particular via maintenance of calcium homeostasis. They have the highest antioxidant levels in the brain and can provide precursors for the GSH system to neurons [77]. GSH is a tripeptide formed from glutamate, cysteine, and glycine. It is known that astrocytes contain more GSH than neurons both in vitro and in vivo [81]. It is also interesting to note that most of the GSH produced by astrocytes is released [81]. Astrocytes can use several substrates for GSH synthesis such as exogenous amino acids, sulfur-containing compounds, or peptides. However, GSH can neither cross the blood–brain barrier nor the membrane of neurons. This is circumvented by the extracellular astrocytic expression of γ-glutamyl transpeptidase (γ-GT) that converts GSH into cysteinylglycine (CysGly). CysGly can then be used as substrate by the neuronal ectopeptidase aminopeptidase N (ApN) which breaks it into cysteine and glycine that can be imported into the neurons for subsequent GSH resynthesis. The needed glutamate can then be provided by the astrocytes in the form of glutamine recycled from uptaken neuron-released glutamate [81].

A last interesting point in the neuron–astrocyte interaction in the context of dopaminergic neurons is the ability of astrocytes to degrade dopamine. Upon neuronal depolarization, astrocytes can take up dopamine via DAT and subsequently degrade it via MAO and COMT. Moreover, astrocytes express high levels of GPx allowing them to cope with the toxicity of intracellular dopamine metabolism [77].

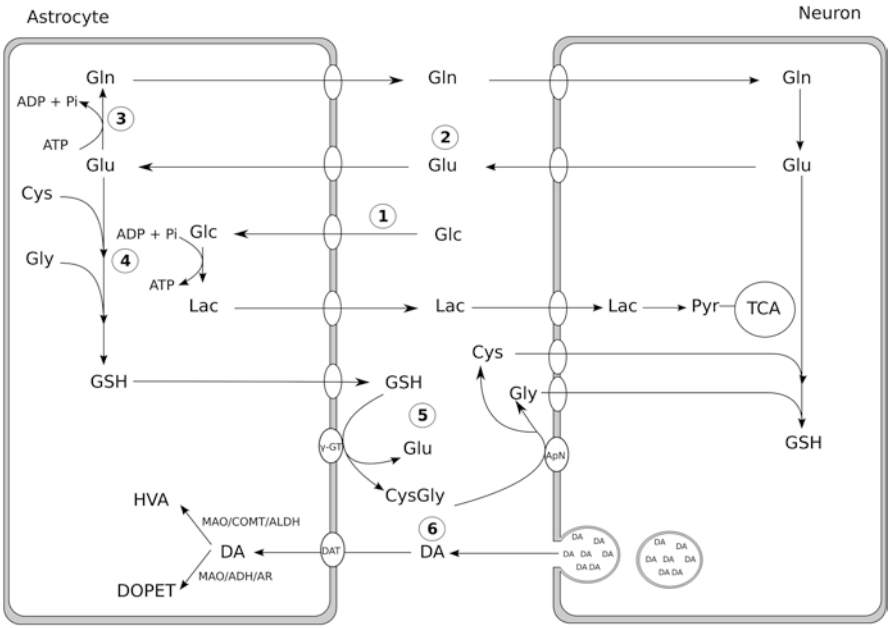

Fig. 2.7 Astrocyte neuron interactions. The metabolic interaction between neurons and astrocytes plays a pivotal role for neuronal survival. (*1*) The first interaction displayed is the astrocyte–neuron lactate shuttle hypothesis (ANLSH) which suggests that astrocytes take up glucose from the blood stream and use glycolysis to produce lactate and ATP. Lactate is then exported out of the cells where it is available for uptake by neurons. The neurons then take up lactate and oxidize it back to pyruvate which can enter the tricarboxylic acid (TCA) cycle to produce energy and activate the electron transport chain. (*2*) A second interaction between neurons and astrocytes is the glutamine–glutamate cycle. Neurons import glutamine and convert it to glutamate via glutaminase. Glutamate is an excitatory neurotransmitter or can be used as precursor for GSH synthesis. After release, glutamate is taken up by astrocytes, (*3*) converted back to glutamine via the glutamine synthase and exported for neuronal use or (*4*) used for GSH synthesis. In astrocytes, most of the GSH produced is exported for neuronal use. Neurons cannot import GSH as such, thus the gamma-glutamyl transpeptidase (γ-GT) cleaves it into glutamate and cysteinylglycine (CysGly) which can be further cleaved into cysteine and glycine via the neuronal aminopeptidase N (ApN) and finally imported into the neuron. (*6*) Another interaction between neurons and astrocytes is the recycling of dopamine (DA) after its release to the synaptic cleft. Astrocytes express COMT and MAO giving them the ability to degrade DA

2.5 Genetic and Environmental Factors Affecting DA Metabolism

This section highlights some of the genetic variation and environmental factors that affect specifically dopaminergic neurons. They can either cause the death of those neurons or be closely linked to the development of PD.

2.5.1 Genetic Factors

2.5.1.1 TH Deficiency

TH is the rate-limiting step in DA synthesis. TH knockout mice are not viable highlighting the importance of TH in prenatal development and postnatal survival [82].

TH impairment in vivo can be either due to a defect in the synthesis of its cofactor BH_4 or to a mutation in the TH gene itself. TH deficiency due to a mutation causes a neurometabolic disorder in which extracerebral production of CAs is not impaired. The symptoms can be improved by L-DOPA therapy, but the patients never reach adulthood [82]. A defect in BH_4 synthesis can arise from a deficiency in any of the enzymes involved in its synthesis pathway. BH_4 is a cofactor for TH, and thus its absence will impair CA production [83] and induce a developmental delay, hypokinesia, and progressive neurological deterioration [84].

2.5.1.2 DAT Genetic Variants

DAT is heavily involved in the duration and magnitude of extracellular DA signaling as well as for the maintenance of intracellular DA levels. Although it does not seem that genetic variations affect DAT expression, epidemiological studies have shown that a combination of certain variants with exposure to exogenous compounds such as fertilizers can increase the risk of developing PD three- to fourfold [85].

2.5.2 Environmental Factors

There are multiple chemicals that can target dopaminergic neurons and cause their degeneration. Here, we will discuss two compounds well known to induce PD in vivo: MPP^+ and rotenone.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of the opioid analgesic 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP). When ingested, it causes irreversible damage to dopaminergic neurons of the SN and to a lesser extent to other catecholaminergic neurons [86], leading to a parkinsonism syndrome which includes the motor symptoms of PD. MPTP, however, is not the active form of the toxin: when it reaches the brain, it is taken up by astrocytes and metabolized via MAO B to 1-methyl-4-phenylpyridinium (MPP^+) [87]. MPP^+ can then enter dopaminergic neurons via DAT where it leads to cell death, most probably by complex I inhibition. A similar mode of action is accounted to the widely used pesticide rotenone. Although it is highly hydrophobic and thus able to cross the cell membrane of almost any cell, it seems to mainly affect dopaminergic neurons [88].

MPP^+ and rotenone are selective and potent inhibitors of mitochondrial complex I, the NADH dehydrogenase. Their toxicity leads to the expression and aggregation of

α-synuclein to Lewy bodies, a hallmark of PD. Both compounds also induce PTP opening and the consequent loss of membrane potential followed by impaired oxidative phosphorylation in combination with increased ROS production [89]. However, the production of ROS following complex I inhibition does not seem to be sufficient to cause the selective death of dopaminergic neurons [90]. Rotenone and MPP^+ also induce microtubule depolymerization [91]. The depolymerization of the microtubules leads to accumulation of vesicles in the soma that cannot be transported through the axon to the synapse. This does not seem to be affecting GABAergic or glutamatergic neurons, but it promotes selective degeneration of dopaminergic neurons [92]. Although all cell types enclose their neurotransmitters into vesicles which are transported across the cell via the microtubule network [92], GABA and glutamate are not readily converted into toxic intermediates. Moreover, it was shown that rotenone can induce a redistribution of DA from vesicles to the cytosol, thus increasing the concentrations of cytosolic DA as well as its autoxidation and MAO-induced degradation [93].

The fungicide benomyl has been associated with risks of developing PD and, like rotenone or MPP^+, can also induce microtubule disassembly. A more important effect in the scope of dopamine metabolism is its capacity to inhibit ALDH, an enzyme important in oxidizing toxic aldehydes including DOPAL. It has been shown that DOPAL is 400-fold more toxic than DA or DOPAC, due to its high potential to form quinones. In postmortem brains of PD patients, the concentration of DOPAL measured was four times higher than the concentration of DA [94].

2.6 Summary

Due to their chemical nature, dopamine and its metabolites are easily oxidized—a process often accompanied by the production of reactive oxygen species. While the MAO-mediated degradation of dopamine leads to the formation of hydrogen peroxide, the autoxidation of many intermediates of dopamine metabolism produces highly reactive quinones. These quinones can bind to cysteinyl residues of reduced GSH or proteins, leading to their inactivation if this residue is located in the active site of a protein. One way to overcome this problem is to increase production or uptake of antioxidants such as GSH or ascorbate. Interaction with astrocytes is an important fact for dopaminergic neuron survival: astrocytes provide the neurons with the GSH-precursor glutamine and are able to degrade excessive dopamine released by neurons. In dopaminergic neurons, excessive amounts of catecholamines can also be inactivated by their polymerization to neuromelanin. This polymer pigment itself can, however, have both a protective or deleterious effect, depending on the cellular context. It should be noted that although L-DOPA still represents the state-of-the-art treatment for Parkinson's disease, it also represents a catecholamine that can contribute to oxidative stress in already damaged dopaminergic neurons. Oxidative damage outside the brain induced by longtime L-DOPA treatment (e.g., in melanocytes) has often been discussed; however, there is little clinical evidence for that theory.

Finally, dopamine metabolism can be altered by genetic factors such as tyrosine hydroxylase deficiency or genetic variants of the dopamine transporter, as well as environmental factors such as pesticides or drugs.

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Chapter 3
The Consequences of Damaged Mitochondrial DNA

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3.1 Introduction of Mitochondrial DNA

Mitochondria are well known for the hereditary independence by possessing unique DNA (mtDNA) and protein synthesis system [1, 2]. Paradoxically, most of the proteins located in mitochondria are encoded by nuclear DNA (nDNA) [1]. The proteins translated from nDNA are transported into mitochondria and, along with mtDNA-encoded proteins, participate in the assembly of mitochondrial complex [3]. Human mtDNA is mainly maternally inherited despite the fact that the sperm contributes around 100 mitochondria during fertilization. The uniparental inheritance is clarified by Sutovsky et al. who suggested that paternal mitochondria are completely degraded by embryo's proteasomes and lysosomes via selective ubiquitination [4]. Although most mtDNA in human cells are homogeneous, various levels of heteroplasmy have been found to distribute in different tissues [5]. The heteroplasmy of mtDNA indicates that a cell is prone to acquire both normal and mutated mtDNA either through inheritance at birth or somatic mutation during lifetime. Increased levels of mutated mtDNA and the subsequent mitochondrial dysfunction are frequently observed in the development of multiple mtDNA-related diseases [6], as addressed later in the chapter. Indeed, high mtDNA mutation rate contributes to the genetic polymorphism in the population, and it is likely linked to both human evolution and migration [6, 7]. Mutations in the mtDNA may lead to significant

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biological defects, compromising essential respiratory chain and impairing ATP bioenergetics [8]. Both endogenous factors such as reactive oxygen species (ROS) and environmental exposures can potentially induce mtDNA damage, resulting in the manifestations of mitochondrial diseases when the damage is beyond repair.

3.1.1 MtDNA Structure

MtDNA differs significantly from nDNA in many aspects, including structure, gene regulation, and damage resistance [9–11]. The structure of mtDNA is characterized by double strands that covalently form a closed circle in nearly all Metazoa [1, 9]. Despite the absence of histones in its structure, it has been found that mtDNA acquires a nucleoid structure via the formation of mtDNA–protein complex with mitochondrial transcription factor A (TFAM) [12]. TFAM, which abounds in mitochondria, plays an important role in the modulation of mtDNA expression [13]. An average of 2.6 mtDNA molecules per mitochondrion exist in mammalian cells [14], while this number is much higher (~4.6) in human cells [15]. Genome size of each mtDNA molecule ranges from 14 to 42 kilobase pair (kbp) among different species. Human mtDNA molecule is composed of 16,569 bp in total [1]. The two strands of mtDNA are termed heavy (H) strand and light (L) strand based on their unique nucleotide distributions. H strand was found to be 2.2 times richer in guanine and 1.2 times richer in thymine as compared to L strand [16].

3.1.2 MtDNA Gene Composition

Mitochondrial genome determines 13 subunit proteins, two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). The genes that code for 12 out of the 13 subunits, two rRNAs, and 14 tRNAs are mainly located on the H strand of mtDNA. On the other hand, the remaining one subunit and 8 tRNAs are encoded from the L strand [9]. The gene content of mtDNA is highly conserved among most vertebrates [17]. This is consistent with our understating on mitochondrion as an ancient organelle that may be derived from the endosymbiont invasion over 1.5 billion years ago [18]. The 13 subunits encoded by mtDNA constitute parts of the crucial respiratory complexes such as NADH dehydrogenase and ATP synthase [1]. Apart from those functional subunits, mtDNA encompasses genes that encode two rRNAs (12S rRNA and 16S rRNA) as well as 22 tRNAs [1]. The 12S rRNA and 16S rRNA are essential components of mitochondrial ribosomes. In addition, the 22 tRNAs utilize a unique system to translate all the codons during protein synthesis [2]. Displacement (D) loop is the only primary noncoding region in the mtDNA, which is a feature of all Metazoa, and plays a major regulatory role in the replication and expression of mitochondrial genes [19]. In particular, the initial transcription sites of both mtDNA strands are located exactly in the D loop region [20].

3.2 Mutations in mtDNA

The genetic aspect of mitochondria is quite complicated partially due to its continuous degradation and replenishment [21]. Within the same cell, mtDNA experienced a faster evolutionary change than nDNA because of its high mutation rate [22, 23]. The respiratory chain in mitochondria generates large amounts of ROS, which exert frequent oxidative attack on DNA [11, 24, 25]. The nonchromatinized structure and the absence of histones contribute to extensive susceptibility of mtDNA to any potential genetic mutation [11]. For example, a practical indicator of oxidative DNA damage, 8′-hydroxy-2′-deoxyguanosine (Oh^8dG), has been shown to exist at a higher level (one per 8000 bases) in mtDNA as compared to nDNA (one per 130,000 bases) [23]. Moreover, the insufficient repair mechanism of mtDNA increases the possibility of irreversible mtDNA mutation [22]. The exact mechanism of mtDNA mutation is not fully elucidated, yet the frequent occurrences of mtDNA deletion have been suggested to be correlated with processes such as slipped mispairing during replication [26].

3.2.1 Oxidative-Induced mtDNA Mutation

Oxidative-induced DNA alteration accounts for the majority of DNA damage, which is a profound phenomenon in mitochondria. The endogenous ROS can attack mtDNA, resulting in the generation of DNA adducts [27]. Yakes and Van Houten utilized PCR techniques to quantitatively monitor hydrogen peroxide (H_2O_2)-induced mutations in both mtDNA and nDNA fragments. They observed a threefold increase in the damage of mtDNA as compared to nuclear genome after H_2O_2 treatment. Following a 60-min incubation in fresh conditioned medium, the oxidative damage in nDNA was significantly alleviated due to recovery. However, no repair was observed in mtDNA [10]. Yakes and Van Houten proposed that this persistent mtDNA damage may be associated with the formation of secondary ROS, including the byproducts of lipid peroxidation [10]. Research has further discussed the role of ROS-related factors such as tumor necrosis factor-α (TNF-α) in the mechanism of mtDNA impairment [28]. It has been suggested that TNF-α can induce mtDNA damage via the production of excessive ROS at the mitochondrial inner membrane, which subsequently contributes to the declined complex III activity. Such damage in mtDNA was effectively prevented by the administration of antioxidants [28]. Due to the remarkable correlation between mtDNA mutation and oxidative stress, mtDNA damage has been implicated as a potential biomarker for ROS-associated diseases [10]. Accordingly, oxidative stress that is elicited by defective mitochondria is proposed to participate in the natural aging process. The accumulation of mutated mtDNA and ROS throughout the lifespan can be regarded as the mechanisms for aging and age-related neurodegenerative diseases [29, 30].

3.2.2 Other Factors in mtDNA Mutation

The high proportion of mutations in mtDNA is commonly detected in aging, tumor cells, and mitochondria-related diseases [21]. The mitochondrial dysfunction due to aging can cause excessive ROS formation, accompanied by a reduced antioxidant activity, fostering the oxidative-induced mtDNA mutation [26]. In addition, Kujoth et al. found that mtDNA mutation was correlated with an overexpression of apoptotic markers in aged mice [31]. Persistent single-strand breaks in mtDNA have been shown to promote ROS formation, triggering the initial apoptosis process [11]. In tumor cells, seemingly neutral mutations may contribute to the neoplasm via clonal expansion, while mitochondrial-related diseases are usually characterized by high proportion of mutated mtDNA [21]. Additionally, environmental factors such as ultraviolet irradiation, smoking, and alcohol can potentially induce mtDNA mutations [26, 32]. The mtDNA adducts generated upon environmental exposures interfere mitochondrial transcription and replication. The gene expression of mitochondria is also altered due to environmentally induced ROS and the chemical modifications of mtDNA [32].

3.3 MtDNA Maintenance and Repair

Recent research has provided new updates on the mtDNA repair mechanisms [33]. The first crucial step to maintaining the mitochondrial genome is to keep the amount of mtDNA stable in the cell. As mentioned previously, TFAM is a transcription factor of mtDNA that is mainly responsible for the regulation of mtDNA copies. An increased number of mtDNA was found in the TFAM-expressed mice [12]. Since the ratio of TFAM to mtDNA was detected as high as 900 in human placental mitochondria, mtDNA is likely to be packed with TFAM to acquire stability [34]. Accordingly, TFAM-suppressed mice largely exhibited mtDNA depletion and failed oxidative phosphorylation in the experiment performed by Larsson et al. [13]. TFAM can also interact actively with p53, possibly delaying the onset of the p53-dependent apoptosis [35].

3.3.1 MtDNA Repair Mechanism-Base Excision Repair (BER)

Several important repair pathways in the mitochondria have been summarized [35]. BER is the primary pathway employed by nDNA and mtDNA to correct small DNA modifications, such as oxidation and alkylation [36]. The basic mechanism of BER involves the recognition and removal of any mutated bp, the recruitment of new nucleotides into the gap, and the ultimate strand ligation [36]. There are two types of BER: short-patch BER and long-patch BER. Short-patch BER is characterized by

single nucleotide substitution, while long-patch BER encompasses the addition of several nucleotides into the abasic gap [36]. Mitochondria were previously known to be only able to carry out short-patch BER. However, recent findings have revealed the crucial participation of long-patch BER in mtDNA repair mechanisms [33, 35, 37].

Each step of BER requires the involvement of specific enzymes [35, 36]. In the first step of BER, DNA glycosylases facilitate the removal of damaged bp and the formation of abasic site by promoting *N*-glycosidic bond hydrolysis between the sugar element and the mutated base [35, 36]. Multiple glycosylases have been found to perform this function in mitochondria. The 8-oxoguanine DNA glycosylase (OGG1) is identified in the mitochondrial matrix, and its lack of activity has been correlated with oxidative damage in mtDNA [35]. Another well-known mitochondrial enzyme is uracil DNA glycosylase (UNG), which is the first repair protein recognized in mitochondria. UNG is responsible for the removal of uracil [35]. Other repair enzymes involved in the abasic site generation include MutY homolog DNA glycosylase and thymine glycol DNA glycosylase [35]. Through the utilization of DNA polymerase (pol) γ, the only DNA pol found in animal mitochondria, the abasic gap is filled with substituted nucleotides [36, 38]. ROS can induce the dysfunction of pol γ thereby affecting mtDNA stability [39]. The final step of BER pathway involves the ligation of the nick in mtDNA via DNA ligases [35]. DNA ligase III (LIG3) is the primary functional enzyme responsible for this repair activity in the mitochondria [35]. Notably, Lakshmipathy and Campbell have shown that antisense LIG3-expressed cells possess lower number of mtDNA as compared with controls [40].

3.3.2 Other Mechanisms Involved in mtDNA Repair

Mismatch repair (MMR) activity was previously detected only in nDNA repair pathways [35]. However, preliminary evidence has revealed a novel MMR mechanism involved in mtDNA, which is independent from nDNA repair system. Distinct proteins such as MSH1 and Y-box-binding protein (YB-1) are implicated in the mitochondrial MMR activity [33, 41]. Partial inhibition of MSH1-dependent MMR pathway markedly increases the oxidative lesions in mtDNA [41]. Therefore, MMR pathway has been regarded as an essential alternative for BER in mtDNA repair [41]. Homologous recombination is also an important mtDNA repair mechanism, which has recently been confirmed in mammalian mitochondria in the fixation process of double-strand breaks [33]. However, extensive mitochondrial strand breaks can overwhelm the homologous recombination repair system, resulting in the degradation of mtDNA [33]. Indeed, mtDNA degradation is a crucial step of maintenance when damage is beyond repair [33]. Persistent mtDNA damage can disrupt the respiratory chain, leading to excess ROS production and ultimately cell apoptosis [42]. Tann et al. have found that the overproduction of single-strand breaks in mtDNA could elevate ROS levels, further triggering the apoptotic pathway [11].

3.4 Damaged mtDNA Contributes to Multiple Cellular Alterations

Currently, over 300 mtDNA mutations have been recognized since the discovery of the first pathogenic mtDNA mutation in 1988 [8]. When the sequence of a gene encoding for respiratory subunits is disturbed, it can potentially result in the dysfunction of mitochondria. However, the manifestation of heteroplasmic mtDNA defects depends primarily on threshold effect [8]. In order for the clinical expression of tissue dysfunctions to become apparent, a threshold in which sufficient proportions of mutated mtDNA is presented in the mitochondria must be met. Such threshold value indicates that the remaining wild-type mtDNA is overwhelmed by mutated mtDNA. The value varies among tissues, mostly ranging from 60 to 90 % of mutant mtDNA [7, 43]. In addition, the transmission of mutated mtDNA is variable due to genetic bottleneck effect. In the presence of mitochondrial bottleneck, the mutational load of mtDNA varies between generations, and the transmission of deleterious mutations can be largely limited, resulting in a reestablishment of homoplasy (wild-type mtDNA) in the genetic pool of a population [8, 44, 45]. MtDNA mutations may also be lost during random mitotic segregation as the levels of mutant mtDNA differ in the daughter cells. However, differentiated (or postmitotic) cells such as neurons and muscles are incapable of undergoing mitosis, presumably suggesting their involvement in multiple mitochondrial diseases [7, 8, 46].

3.4.1 Impaired Protein Synthesis and Function

It is suggested that fragmented mtDNA can gradually transfer to the nucleus and accumulate in nDNA, leading to the alternation of nuclear information content [47]. Vice versa, nDNA mutations also influence mtDNA [48]. A great amount of nuclear-encoded proteins are involved in the mtDNA maintenance and mitochondrial metabolism. Indeed, mutations in nDNA may mimic the pathological features induced by mtDNA defects [44, 49]. Age-associated mtDNA damage has been shown to impair the function of respiratory complexes I–IV [47, 50]. In addition, mutations in the genes encoded for ribosomal or mitochondrial elongation factors, such as *GFM1* and *TSFM*, respectively, also contribute to the deleterious effects of mtDNA translation [49].

The impact of tRNA point mutations has been demonstrated in various mitochondrial encephalomyopathies, including MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) with $tRNA^{Leu(UUR)}$ A3243G transition and MERRF (myoclonic epilepsy and ragged-red fibers) with $tRNA^{Lys}$ A8344G transition [51]. Indeed, point mutations at the mt-tRNA genes are largely disease-related since the overall mitochondrial translation can be impaired when functional mt-tRNA level is reduced [7]. Point mutations in tRNA may lead to unprocessed or partially processed RNA transcripts, which are likely associated

with oxidative phosphorylation capacity. Moreover, the stability of tRNA is reduced due to mutation-induced conformational changes, affecting the effectiveness of tRNA aminoacylation [51]. The prevalence of mt-tRNA mutations such as m.3243A>G in *MTTL1* gene is approximately 1 in every 400 people [52].

3.4.2 Decreased ATP Bioenergetics

Considering the essential role of mitochondria in cellular respiration, pathogenic mtDNA mutations may result in inefficient ATP synthesis and excess ROS production, compromising cellular respiratory functions [7]. Notably, hundreds of mitochondria reside in the neurons to generate sufficient energy to meet the high demands. Mitochondria travel within the neurons and reach to the areas where high ATP usage is required. The interruption of this movement can induce protein aggregation due to lack of ATP supply [48]. Therefore, the accumulation of ROS and reduced ATP production in the mitochondria can significantly damage neurons, which possibly contribute to the development of neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis [48, 53]. Intriguingly, reduced mitochondrial motility has been observed in mature neurons. Neurons are unable to utilize glycolysis for ATP supply even when the oxidative phosphorylation is limited [48]. Approximately 1–2 % of the consumed oxygen is converted to ROS during cellular respiration. In the study performed by Yakes and Van Houten, H_2O_2 clearly influenced the normal functioning of mitochondria by diminishing succinate dehydrogenase activity, thereby producing excess ROS [10]. Therefore, mitochondria are prompted to generate ROS when the respiratory chain is disrupted.

3.4.3 Nuclear Transcriptional Responses to Mitochondrial Defects

The activation of nuclear transcriptional response has been associated with mtDNA mutations and, to some extent, determines the outcomes of pathogenic mutations. Cortopassi et al. examined the transcriptional profiles of five mitochondrial diseases in nine cell types [54]. Distinct mutations are implicated in different mitochondrial diseases. Through the analysis of microarray, a large amount of transcriptional alterations (e.g., the downregulation of COUP-tf2) are identified in all five mitochondrial diseases, indicating the activation of similar pathophysiological pathways among these diseases [54]. Unfolded protein response (UPR) serves as a quality control of mitochondria to maintain protein homeostasis [55, 56]. It is proposed that UPR is triggered by endoplasmic reticulum (ER) stress induced by mitochondrial dysfunction and subsequently inhibits vesicular secretion. During UPR, the activated PKR-like ER-localized elf2α kinase (PERK) phosphorylates activating transcription factor 4 (ATF4), further preventing neural vesicular secretion and protein

synthesis [54]. Due to the proximity between mitochondria and ER, defects in the mitochondria can significantly influence the function of ER. For instance, bioenergetic disruption affects calcium homeostasis and cross-talk between mitochondria and ER [54, 55]. The downregulation of transcripts involved in oligodendrogenesis, synaptogenesis, and neural migration is essential in the development of multiple neurological features in mitochondrial diseases [54].

3.5 MtDNA-Related (Mitochondrial) Diseases

MtDNA diseases are most characterized by the presence of various neurological features [43]. Clinical syndromes of the disease can be, not exclusively, attributed to specific types of mtDNA mutation [43]. However, the diagnosis of mitochondrial diseases does not solely depend on neurological presentations including neuropathy and movement disorder. Physicians also take into account non-neurological symptoms (e.g., deafness) during evaluation [43]. The symptoms expressed by the patients differ largely, some even remain asymptomatic. Therefore, mitochondrial diseases may be difficult to characterize, and the prevalence of mtDNA diseases has yet to be determined [44, 48]. If mtDNA diseases are associated with common clinical features such as hypertension and diabetes, the diagnosis can be further challenged. In fact, a number of patients with mtDNA mutations are possibly undiagnosed or never recognized [44].

3.5.1 Common Mitochondrial Diseases

Mutations at the mitochondrial tRNA gene can induce diseases such as myoclonic epilepsy with ragged-red fibers (MERRF), a severe neuromuscular disorder with high variability in the mtDNA mutation levels [43, 48]. The mtDNA mutations of MERRF reside at nucleotide 8344 in the $tRNA^{Lys}$-encoded MT-TK gene. The symptoms mostly include myoclonic epilepsy, myopathy, dementia, and ataxia, yet they vary between individuals [48]. Leigh syndrome (LS), also known as subacute necrotizing encephalomyelopathy, has been associated with oxidative metabolism failure that results in the deterioration of basal ganglia or brainstem [43, 48]. It is a neurodegenerative condition that occurs in infancy and childhood [43]. Generally correlated to nDNA mutations of oxidative phosphorylation genes, LS is also attributed to mtDNA point mutations in the MT-ATPase6 gene. Point mutation at the nucleotide 8993 may induce a thymine-to-guanine (m.8993T>G) or thymine-to-cysteine (m.8993T>C) transition, resulting in the substitution of leucine to arginine or proline, respectively. It is suggested that T8993G mutation is related to mitochondrial energy deficiency, which leads to the development of early-onset LS, whereas T8993C mutation is associated with increased ROS production [48]. MtDNA point mutation is also implicated in Leber's hereditary optic neuropathy (LHON). LHON causes a loss of vision. In over 90 % of families, one of three mutations (m.11778G>A, m.3460G>A, or m.14484T>C) is presented in the genes encoded for the NADH ubiquinone–oxidoreductase of

complex I [43]. LHON patients with homoplasmic mtDNA mutations inherently transmit the mutations to all maternal offspring. However, not all offspring develop this disease, suggesting a possible involvement of nuclear genetic factors in the mtDNA-related disease expression [44]. Despite the general perception of LHON development in younger age, cases with late-onset LHON (age 50 or above) have been identified [57]. In addition, important factors such as smoking and alcohol intake may increase the disease penetrance among LHON carriers, which serves as an essential risk aspect for both typical and late-onset LHON [57, 58]. Chronic progressive external ophthalmoplegia (CPEO) is commonly caused by a single deletion at 4977 bp of mitochondrial genome. The mutated mtDNA can cause eye muscle paresis and weakness [43]. Depletion syndromes present in the organs with mtDNA depletion, affecting the functions of muscle, brain, and/or liver [7].

3.5.2 Neurodegeneration and mtDNA Mutations

The gradual accumulation of mtDNA mutations over time has been implicated in contributing to normal aging and related neurodegeneration [59]. Considerable interests have been shown in the research investigating the possibility of mtDNA variants in the predisposition of common diseases such as Alzheimer's and Parkinson's diseases [44, 60]. Neuronal loss due to somatic mtDNA deletions has been shown in the substantial nigra of Parkinson patients [61]. Simon et al. observed higher cumulative mtDNA mutations in the frontal cortex of elderly subjects compared to younger controls, further supporting the positive correlation between somatic mtDNA mutations and age [59]. In Parkinson's disease as well as other age-related neurodegenerative diseases, the declined activity of mitochondrial complex I in the substantia nigra is likely linked to mtDNA mutations [59, 62]. Moreover, a deficient level of cytochrome *c* oxidase (complex IV) has been detected in the hippocampal pyramidal neurons, suggesting its implication in the pathogenesis of Alzheimer's disease [63].

3.6 Potential Treatment Options

There remains a great challenge for treating mtDNA diseases with neurological features. However, several treatments have been proposed and investigated for the proper management of mtDNA diseases. For instance, endurance exercise has demonstrated beneficial effects in both patients and animal models with mitochondrial myopathies [64]. In a 12-week aerobic training study, an improved oxidative capacity and approximately twofold increase in mitochondrial enzyme activity were observed in the myopathic subjects. However, mtDNA levels in the muscle remained unchanged, suggesting an exercise-induced upregulation of mtDNA transcription or translation [65]. Intriguingly, Safdar et al. reported systemic mitochondrial biogenesis in the mice that underwent 5-month endurance training [66]. Other

adaptations associated with exercise including increased antioxidant enzyme activity and muscle strength have also been recognized [64]. Although further studies are essential to develop an optimal exercising plan, exercise is regarded as a safe approach for mtDNA diseases as no deleterious effects have been reported in most studies [64, 65].

Mitochondria-targeted antioxidant therapy is another feasible approach. It is suggested that antioxidants with specificity for the mitochondria are necessary for the successful application in clinical trials. For instance, MitoQ and SS31 are taken up directly by the inner mitochondrial membrane and have been shown to reduce Aβ toxicity in Alzheimer's disease [55, 67]. Moreover, various gene therapies may mitigate ROS-induced mitochondrial dysfunction [55]. In the study performed by Elliott et al., 3168 neonatal-cord-blood and maternal blood samples were analyzed to estimate the rate of *de novo* mutations. Approximately 0.00107 % of individuals obtained an mtDNA mutation that was absent from the maternal samples. The finding emphasizes the essential development of technique for the transmission prevention of mtDNA diseases [68].

3.7 Conclusion

MtDNA is susceptible to mutation due to its proximity to the oxidative phosphorylation enzymes and ineffective mtDNA repair mechanisms. Although sporadic mutations are essential for genetic variation, excess mtDNA mutations that exceed the threshold may lead to significant cellular alterations and possible disease developments. Defects in the mtDNA are often associated with diseases with neurological features. However, the variable phenotypes or expressions of mtDNA mutations make the diagnosis of mitochondrial disorders rather challenging. The management of these diseases also remains difficult. Currently, several treatment approaches, such as exercise and antioxidants, are undergoing continuous investigation, and it is essential that the idea of preventing maternal transmission of mtDNA mutations be established.

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Chapter 4
The Role of Chronic Inflammation in the Etiology of Parkinson's Disease

Yuval Nash and Dan Frenkel

4.1 Introduction

Inflammation is a natural process within tissues in which immune cells, such as macrophages and dendritic cells in the periphery and microglia in the brain, mount a response against external infection or injury to the tissue [1]. This response is characterized by vasodilation (expansion of blood vessels), increased capillary permeability, and migration of phagocytes into the tissue [2]. Such an inflammatory response can be either acute, i.e., short and powerful around the time of insult, or chronic, which lasts over a long period of time. As neurons appear to be more vulnerable to inflammatory responses compared to other tissues, chronic inflammation in the brain could be harmful to the tissue [3].

Inflammation might play a double-edged sword in neurodegenerative diseases. It might be linked to neuronal stress and death signals through cytokines such as interleukin 1 beta (IL-1β) [4], tumor necrosis factor alpha (TNFα) [5], and interferon gamma (IFNγ) [6], increased secretion of reactive oxygen species (ROS) [7], and by activation of the complement system [8]. However, inflammation may also be linked to increased secretion of antiapoptotic cytokines such as IL-10 [9] and IL-4 or neurotrophic factors such as brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), or insulin-like growth factor 1 (IGF-1) [10–12]. It has been suggested that within the central nervous system, astrocytes might play a role as sensors and modulate the inflammation in the microenvironment [13]. In many neurodegenerative diseases, evidence for such inflammatory processes is seen both at the site of the degeneration, as well as in the peripheral system [14–16].

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4.1.1 Inflammation and PD

Parkinson's disease (PD) is characterized by progressive degeneration of neuromelanin-containing dopaminergic neurons throughout the substantia nigra pars compacta (SNc) within the basal nuclei [17]. This degeneration is accompanied by the formation of intracellular inclusion bodies, termed Lewy bodies, within neurons [18]. These inclusion bodies are comprised of several proteins, the most studied of which are ubiquitin and α-synuclein [19].

It has been reported that the inflammatory processes may play a role in pathogenesis of the disease. In PD patients' brains, there is evidence of chronic inflammation, seen in the elevated levels of proinflammatory cytokines such as IL-1_{β} and IL-6 in the cerebrospinal fluid (CSF) and TNFα in the substantia nigra of patients [20]. This reaction is attributed, at least in part, to the activation of microglia and infiltrated monocyte cells, marked by [^{11}C](*R*)-PK11195 reactivity in the midbrain of patients [21] and an increase in EBM11 (CD68)-positive cells in the substantia nigra, in postmortem [22]. In addition to proinflammatory cytokines, activated microglia and monocytes also exhibit a higher expression of inducible nitric-oxide synthase (iNOS) and cyclooxygenases 1 and 2 (COX1 and COX2) which can exert cytotoxic effects through oxidative stress [23]. Since these findings were observed in patients at different disease stages and disease durations, it is possible that the increase in the presence of microglia is linked to chronic inflammation, rather than an ad hoc activation.

In contrast to microglia, the role of astrocytes in chronic inflammation in PD is not clear. In some PD patients' brains, there is no morphological evidence for reactive astrocytes in the substantia nigra, compared to control samples [24]. In comparison, others have found astrocytic activation in the substantia nigra accompanied with increased expression of intercellular adhesion molecule-1 (ICAM-1) [25].

The complement system also appears to play a role in the pathogenesis of PD, as early-stage complement proteins such as iC3b, as well as late-stage complement proteins such as C9, surround Lewy bodies in PD patients' brains [26]. Of note, iC3b was also observed around melanized neurons in the SN, i.e., dopaminergic neurons still containing neuromelanin. This suggests that complement activation could play a role in triggering the death of dopaminergic neurons in the SN [26].

Immune cells in the periphery also show abnormalities in PD patients: PD patients exhibit lower levels of circulating $CD4^+$ T-helper 1 lymphocytes and lower levels of B cells, concomitant with increased levels of natural killer (NK) cells [27]. Similarly, PD patients show lower levels of induced secretion of IL-2 [28]. In line with these findings, PD patients' monocyte-derived macrophages also showed impairment in inducible expression of CD200R, the ligand for the T-cell-expressed CD200 protein [29]. As the CD200-CD200R signaling is thought to exert an inhibitory effect on myeloid-lineage cells, such as macrophages and microglia [30], impairment in this signaling could lead to excessive activation of proinflammatory functions among these cells. Finally, PD patients also exhibit increased levels of infiltrating $CD4^+$ and $CD8^+$ T cells within the substantia nigra, suggesting that peripheral lymphocytes play a role in degenerative processes in PD [31].

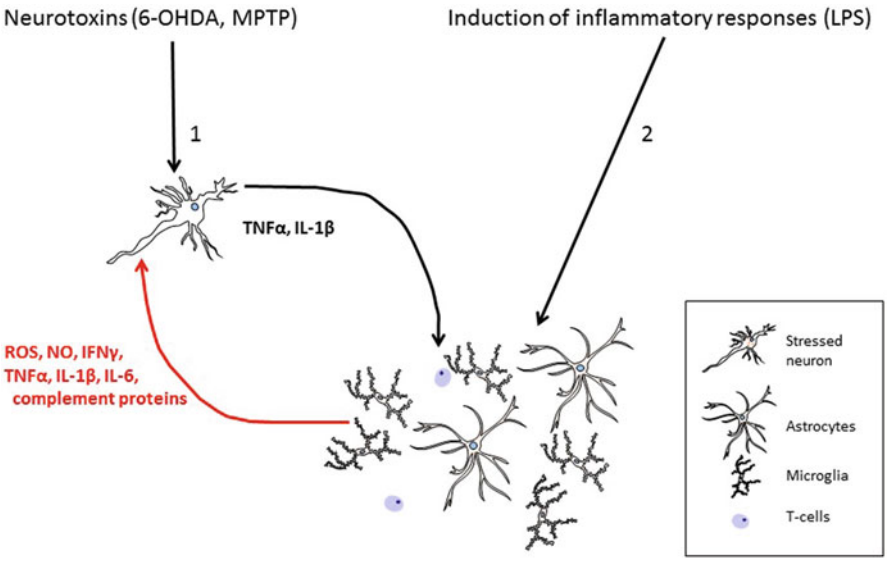

Fig. 4.1 The role of inflammation in triggering and exacerbating dopaminergic neurons death in PD. Inflammation can cause neuronal death through two potential mechanisms: (*1*) As a result from stress signals of apoptotic dopaminergic neurons. Toxin-based models can induce mitochondrial dysfunction and inhibition of dopamine synthesis in dopaminergic neurons, leading to secretion of stress signals that induce neurotoxic inflammatory responses. (*2*) As an initiation stage by which inflammatory activation of brain-resident cells and infiltrating cells may trigger dopaminergic neuronal stress. Inflammation-based models (lipopolysaccharide, or LPS) induce inflammatory activation of brain-resident cells and infiltrating cells, which in turn induce toxicity in dopaminergic neurons by Inflammatory signals such as nitric oxide (NO), ROS, IFNγ, TNFα, IL-1β, and complement proteins. Abbreviations: 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

Together, these findings point to an abnormality in lymphocyte cells and their interaction with macrophages in the peripheral system of PD patients, which has possible implications on processes within the central nervous system (see Fig. 4.1).

4.2 Parkinson Genes and Inflammation

Several genes have been linked to familial forms of PD, which can present either autosomal-dominant or autosomal-recessive inheritance, and often cause early onset of the disease [32]. Furthermore, recent studies regarding PD biomarkers in patients reveal an association between PD cases and variation in the *human leukocyte antigen* (*HLA*) gene, which is linked to regulation of immune functions, suggesting that variations in immune functions affect the risk of developing PD [33].

It has been reported that some of the genes which cause familial forms of PD can also directly modulate a proinflammatory response or their expressed proteins might trigger inflammation.

α-Synuclein: Alpha-synuclein is a protein predominantly expressed in the central nervous system and found in presynaptic terminals of neurons [34], as well as in astrocytes, microglia, and oligodendrocytes [35]. Mutations in the *α-synuclein* gene are linked to dominant inheritance of PD [36], although duplication mutations of the gene are also implicated in the disease [32].

Several α-synuclein mouse models for PD exist, with either overexpression of wild-type α-synuclein or expression of mutant forms of α-synuclein, through different promoters [37]. While such models exhibit only some dopaminergic degeneration in older ages, they exhibit chronic microglial activation in the SN, accompanied by increased expression of inflammatory markers such as ICAM-1, IL-1β, IL-6, TNF-α, and iNOS [38, 39].

Interestingly, α-synuclein has been shown to be a negative regulator of cellular degradation processes such as autophagy in T cells [40]. As autophagy has been shown to degrade aggregated α-synuclein in these cells, this could represent a positive-feedback mechanism, in which α-synuclein accumulation prevents its own degradation, furthering the accumulation and cellular burden [40]. Likewise, α-synuclein has been shown to play a role in mediating B-cell-dependent immune responses, as *α-synuclein*$^{-/-}$ animals have lower levels of B cells, and reduced production of IgG antibodies in response to immune challenges [41].

PINK-1: Phosphotensin-induced kinase 1 (PINK1) is a serine/threonine kinase which is located both in the cytosol and in the mitochondrial membrane [42, 43] and is important for various mitochondrial functions [44]. As PD-related mutations are thought to act through a loss of function mechanism [44], most of the research on PINK1 pathology is carried through knockout or knockdown models.

PINK1$^{-/-}$ mice do not exhibit major abnormalities compared to WT mice, except for some mitochondrial impairments [37]. In contrast, organotypic cortical slices of *PINK1*$^{-/-}$ mice show higher expression of proinflammatory genes such as TNF-α, IL-6, and IL-1β [45], and these mice produce larger amounts of IL-1β, IL-12, and IL-10 in response to a peripheral injection of lipopolysaccharides (LPS) [46].

DJ-1: DJ-1 is almost ubiquitously expressed in human tissues [47] and is located both in the cells' cytosols and around mitochondria [48]. DJ-1 acts as an oxidative-stress response protein which protects neurons from various oxidative-stress conditions [49]. Several mutations observed in human PD patients have been found to generate unstable proteins, generating an "effective knockout" or knockdown of DJ-1 [50], suggesting that DJ-1-related pathology is due to DJ-1 loss of function.

DJ-1$^{-/-}$ mice do not show prominent Parkinsonian symptoms or marked neurodegeneration [37]. In vitro models of DJ-1 deficiency, however, reveal impairments in immune responses to various challenges: primary astrocytes derived from *DJ-1*$^{-/-}$ mice exhibited stronger proinflammatory and neurotoxic effects in response to an LPS challenge, compared to astrocytes from WT animal [51], and the same phenomena are observed in microglia [52]. Of note, glial cells cultured from *DJ-1* KO mice exhibit increased phosphorylation of the inflammatory signaling molecule signal transducers and activators of transcription 1 (STAT1), resulting in enhanced inflammatory responses following INF-γ stimulation [53]. These findings suggest that DJ-1 exerts anti-inflammatory effects and that loss of DJ-1 function can lead to exacerbation of inflammatory processes [52].

4.3 Inflammation and Toxin Animal Model of PD

Dopaminergic death in PD has been found to be accompanied by an increase in inflammatory markers. However, it is hard to define whether one is a trigger for the other. Indeed, experiments in animal models of PD suggest two potential roles of inflammation in PD: (1) inflammation exacerbates neuronal death following stress signals from neurons, and (2) inflammation triggers dopaminergic neuronal stress and death (see Fig. 4.1).

4.3.1 Toxin-Mediated Animal Model

Parkinson's disease research employs several toxins which cause dopaminergic degeneration and induce PD-like symptoms in animal models, enabling diverse in vitro and in vivo experimental models.

6-OHDA animal model: One of the most studied toxins is 6-hydroxydopamine (6-OHDA), a hydroxylated derivative of dopamine. 6-OHDA enters catecholaminergic neurons via natural reuptake mechanisms [54], where it exerts its toxic effects by two main processes: the first involves the generation of reactive oxygen species through activation of NADPH oxidase [55], and the second involves impairment in mitochondrial activity through inhibition of mitochondrial complex I and complex IV [56].

In addition to its direct effects on dopaminergic neurons, research in recent years has shown that the deleterious effects of 6-OHDA involve significant inflammatory activation, both in vitro and in vivo.

Administration of 6-OHDA to neuronal cells induces rapid translocation of the inflammatory nuclear factor-kB (NF-kB) to the nucleus, where it binds to the DNA [57]. Moreover, striatal injection of 6-OHDA causes astrogliosis, marked by increased numbers of astrocytes and increased expression of GFAP within the astrocytes [58].

Microglia cells show a robust reaction following striatal 6-OHDA injection, around dopaminergic neurons, and these cells also have been shown to participate in phagocytosis of dopaminergic neurons [59]. Microglia-induced degeneration of dopaminergic neurons is attenuated in a mice harboring knock-in of DNAX adaptor protein 12 (DAP-12) [59]. Conversely, enhancement of microglia activity, by blocking the inhibitory CD200-CD200R signaling in microglia, results in increased neurodegeneration after 6-OHDA administration, accompanied by increased secretion of the proinflammatory cytokines IL-6 and TNFα [60].

Intrastriatal injection of 6-OHDA also has been shown to induce disruption of the blood–brain barrier (BBB), leading to SN blood leakage, which co-localizes with degeneration of dopaminergic neurons [61]. Moreover, increased levels of MHC-II-reactive microglia were also observed in the same areas [61]. These findings suggest that 6-OHDA administration induces a secondary reaction by glial cells that contributes to the progressive neurodegeneration.

MTPT animal model: MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is another frequently used substance in PD research. It is rapidly converted into MPP+ (1-methyl-4-phenylpyridinium) by the MAO-B enzyme within astrocytes [62], and in this ionized form, it is readily taken by dopaminergic neurons' dopamine transporter (DAT) [63]. Once inside the cells, MPP^+ inhibits the generation of dopamine by nitration of tyrosine hydroxylase, directly contributing to dopamine depletion in the brain [64]. Moreover, MPP^+ inhibits the activity of the mitochondrial complex I and causes a depletion of ATP levels and the generation of ROS [65].

Aside from its direct effects on dopaminergic neurons, MPTP exerts marked inflammatory responses in the brain: shortly after the exposure to MPTP, there is activation of both microglia and astrocytes in the SN, accompanied with infiltration of CD4+ and CD8+ T cells [66]. Moreover, several days after the administration of the toxin, increased expression of MHC-II and ICAM-1 on microglia cells is observed in the mouse brain [66], and increased expression of astrocytic ICAM-1 and microglia leukocyte function antigen 1 (LFA-1) is observed in the monkey brain [25].

Mice deficient of iNOS show a similar glial response to MPTP, compared to WT mice, but the neurodegeneration is almost completely abolished in the $iNOS^{-/-}$ mice. Interestingly, dopamine levels are still decreased in this phenotype [67]. These findings suggest an active role for inflammatory responses in the MPTP model that occur simultaneously with the direct effects of this toxin on dopaminergic neurons.

Rotenone animal model: Rotenone is an inhibitor of the mitochondrial complex I, and it exerts toxic effects through disruption of cell respiration and ATP synthesis, as well as enhanced production of ROS by the mitochondria [68].

Rotenone induces dopaminergic neurodegeneration in the SN after chronic administration, accompanied by microglial activation [69]. Interestingly, however, in vitro experiments on primary microglia cultures show that microglia do not exhibit inflammatory responses to rotenone [70], suggesting that the activation of microglia cells is not a direct effect of rotenone, but rather a secondary effect, perhaps by signals from damaged neurons.

Paraquat animal model: Paraquat is an inhibitor of mitochondrial complex I, which causes a reduction in cell respiration and increased free radical formation [71].

When administered to lab animals, prolonged exposure to paraquat induces dopaminergic degeneration, simultaneously with oxidative damage [72]. The oxidative damage appears to be a causative neurotoxic factor, as transgenic mice which are more resistant to oxidative damage show no susceptibility to this toxin [72]. Finally, similarly to rotenone, paraquat does not elicit inflammatory reactions in microglia cells [70].

4.3.2 Inflammatory Mediated Animal Model

As inflammation appears to play a role in the pathology of PD, some experimental models utilize inflammatory agents to initiate dopaminergic neuronal pathological processes. These models commonly involve the use of lipopolysaccharide (LPS), a

macromolecule found on the outer membrane of gram-negative bacteria, which elicits inflammatory responses in mammalian cells through Toll-like receptor 4 (TLR4) signaling [73].

A single intraperitoneal injection of LPS can elicit rapid proinflammatory responses in microglia at the substantia nigra and cause a significant reduction in the numbers of dopaminergic neurons 7–10 months after the injection [74]. Furthermore, a single injection of LPS into the substantia nigra can cause selective dopaminergic degeneration and dopamine depletion, starting 4 days after the injection and persisting for 12 months. This effect is preceded by microgliosis, but not astrocyte proliferation in the injection site, starting only 2 days after the injection [75]. These findings suggest that the neurodegeneration is not a primary result of the inflammatory toxin, but rather a secondary process to microglia activation and neurotoxic inflammatory responses.

Of note, several reports have suggested that inflammation and α-synuclein aggregation create a positive-feedback loop, whereas oxidative stress causes α-synuclein aggregation, and α-synuclein aggregation causes inflammation and oxidative stress [20]. Since α-synuclein deposition in PD patients appears to begin in the vagus nerve and in the anterior olfactory nucleus [76], it is possible that inflammatory processes in the gastric epithelium and olfactory epithelium, which are exposed to the external environment and external pathogens, are a key factor in the pathogenesis of PD [20]. Together, various lines of evidence link inflammatory processes to neurotoxic responses from microglia, aggregation of α-synuclein, and the possible spreading of α-synuclein between neighboring cells.

4.4 Anti-inflammatory Treatment and PD

The link between inflammation and PD pathology suggests a potential for an anti-inflammatory approach for treating PD. Indeed, this approach is supported by results in a PD animal model, where attenuation of inflammatory processes in MPTP experimental models appears to mitigate the deleterious effects of this toxin: Inhibition of the signaling of peptide angiotensin II, an inducer of inflammatory responses, reduces microglia activation and mitigates MPTP-induced neurotoxicity [77]. Similarly, administration of the anti-inflammatory drug dexamethasone reduces MPTP-induced upregulation of MHC-II and ICAM-1, reduces microglia reactivity, and significantly abolishes T-cell infiltration into the SN; these processes coincide with reduced neurotoxicity [66]. These findings suggest an active role for inflammatory responses in the MPTP model, which occurs simultaneously with direct effects of this toxin on dopaminergic neurons and significantly contributes to the neurotoxic effects of this substance.

Epidemiological studies among regular users of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) suggest a reduction in the risk for developing PD [78]. Nevertheless, a clinical trial with aspirin and other NAISDs, except ibuprofen [79], did not show significant effects in reducing the risk for PD [80]. Of note, the

positive results with ibuprofen might be mediated through its effect on other genes such as APOE4 [81] and not through its effect on inflammation. Interestingly, some therapeutic approaches in animal models suggest induction of immune responses specifically against α-synuclein may also result in neuroprotection [82].

4.5 Conclusion

Inflammation plays a major role in processes in which the body preserves homeostasis and protects itself from various insults. Those insults may be linked to abnormalities that prompt the development of neurodegenerative diseases such as PD. Further understanding the role of each inflammatory biomarker in the etiology and the progression of PD may elucidate the role of inflammation in the disease and may lead to the rational development of immunomodulation approaches in PD.

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Chapter 5
Ion-Catalyzed Reactive Oxygen Species in Sporadic Models of Parkinson's Disease

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5.1 Introduction

Parkinson's disease (PD), which affects 4.9–19 per 100,000 people worldwide, is the second most common neurodegenerative disorder after Alzheimer's disease (AD). With an age-dependent prevalence, about 1 % of PD cases are diagnosed at age 65 and 4–5 % at age 85. Although the average age of onset for sporadic PD is ~70, about 4 % of PD patients present early-onset symptoms under the age of 50 [1]. As a chronic movement disorder, PD is characterized by four cardinal symptoms: uncontrollable movement at rest (dyskinesia), slowness of movement (bradykinesia), postural imbalance, and rigidity or stiffness. Evidence suggests that non-motor symptoms such as olfactory dysfunction, sleep abnormalities, memory difficulties, and depression can precede the onset of motor symptoms in PD patients [2, 3].

The selective degeneration of the DAergic neurons in the substantia nigra pars compacta (SNpc) within the midbrain is a major contributing factor to dopamine (DA) deficiency in the nigrostriatal pathway involved in the coordination of movement. In addition, affected neurons are characterized by the formation of

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intracytoplasmic proteinaceous inclusions or LBs [4]. LBs are primarily composed of aggregates of the presynaptic protein alpha-synuclein (α-Syn), proteasome subunits, ubiquitin, and heat shock proteins [5–7]. The elevated expression of α-Syn in sporadic and familial PD cases is associated with LB formation and neurotoxicity in the SNpc. Carriers with α-Syn gene (*SNCA*) mutations present early-onset PD, faster progression of motor symptoms, and high prevalence of dementia and non-motor disturbances [8, 9]. This phenomenon is also observed in a transgenic mouse model (*SNCA*-OVX) overexpressing wild-type human α-Syn at twice the endogenous level, exhibiting a PD-like phenotype with specific disturbances in the dorsal striatum accompanied by movement deficits [10].

5.1.1 Sporadic vs. Familial PD

PD was initially considered a sporadic neurological disease caused by synergistic environmental factors. However, a growing number of genetic linkages with inherited mutations and monogenic PD cases have implicated key genetic risk factors with autosomal dominant or recessive inheritance in familial PD, which constitute about ~10–15 % of cases [11–14]. The most extensively studied genes linked to heritable, monogenic PD are *SNCA* (encoding α-Syn), *PARK2* (encoding cytosolic E3 ubiquitin-ligase *Parkin*), and *PARK6* (encoding mitochondrial kinase PINK1).

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Genetic screening of early-onset PD cases has led to the discovery of missense mutations (A30P, A53T, E46K, A53E, H50Q, and G51D) in the *SNCA* gene, directly implicating α-Syn in familial PD [15–20]. Furthermore, early-onset autosomal recessive cases of parkinsonism have been linked to genetic variance in the *PARK2* and *PARK6* genes. Interestingly, some of the clinical and neuropathological characteristics vary between patients bearing these mutations and those with sporadic PD. Indeed, PINK1-related young-onset PD is clinically indistinguishable from idiopathic PD, as it presents similar movement disorder symptoms accompanied with significant formation of LBs and neuronal loss in the SNpc [21]. Conversely, a detailed clinicopathological study suggests that *Parkin*-related PD differs from sporadic PD. *Parkin*-related PD cases are characterized by severe SNpc neurodegeneration, similar to that observed in sporadic PD. However, they present slower symptomatic progression, few or no LBs, and reduced neuronal death in cognitive function regions, which are otherwise highly affected in sporadic PD [22]. Thus, many genetic changes, pathological hallmarks, and symptoms overlap in sporadic and familial PD cases. Henceforth, the identification of unique differences between the sporadic and genetic forms of PD is critical to address why symptoms progress differently and why patients respond differently to levodopa therapy between the two PD groups.

5.1.2 Etiology of PD

PD is characterized by a complex etiology, involving a multifarious interplay between α-Syn pathology, defective protein degradation/clearance, mitochondrial dysfunction, metal toxicity, and OS. Postmortem analysis of brain tissue from PD patients, transgenic mice, and in vitro models collectively suggests that soluble oligomeric forms of α-Syn, which precedes α-Syn's self-assembly into aggregates, are more aggressive toxic species and play a critical role in triggering neuronal degeneration in PD [10, 23, 24]. Furthermore, the deficient disposal of misfolded/aggregated α-Syn indicates dysfunctional lysosomal autophagy and ubiquitin–proteasome systems [25]. Mitochondrial dysfunction comprising high levels of mitochondrial DNA (mtDNA) deletions, decreased activity of oxidative phosphorylation (OXPHOS) complexes, depolarized mitochondrial membrane potential, and altered mitochondrial membrane proteins has been implicated in PD pathogenesis [26–28]. Epidemiological studies as well as postmortem analysis of PD brains strongly suggest the etiological involvement of prooxidant and heavy metal toxicity in PD [29, 30]. Furthermore, high levels of ionic forms of iron (Fe), copper (Cu), and manganese (Mn) in the brain can catalyze oxidative reactions leading to the formation of ROS, thereby enhancing OS burden in the brain. This is consistent with enhanced OS in the PD brain, involving increased levels of oxidized lipids, α-Syn aggregates, and DNA damage [31–33]. These etiologies are commonly observed in both familial and sporadic PD patients and induced PD model organisms; however, the mechanistic cascade leading to cellular death is not completely elucidated.

5.1.3 Current Treatments

Current available treatments for PD only offer temporary symptomatic improvement, but does not slow or reverse the disease progression. These include carbidopa/levodopa, monoamine oxidase (MAO) B inhibitors, anticholinergic agents, and amantadine, which target motor symptoms in early PD, but they progressively cause drug-induced dyskinesia and motor fluctuations, often limiting the drugs' long-term benefits [34]. In late-stage PD patients who are severely affected, various kinds of surgical interventions have reportedly been effective in reducing symptoms. Among these, deep brain stimulation (DBS) is the most successful and less invasive approach. DBS involves the implantation of an electrical node typically in either the subthalamic nucleus (STN) or the globus pallidus (GP) region, associated with brain motor circuitry. The electrical stimulation blocks aberrant oscillatory activity from the GP and thalamus, thus reducing tremor, rigidity, and balance defects. Even though DBS significantly improves specific motor symptoms, cognitive deterioration and non-motor symptoms remain unaffected or could even worsen in some cases [35].

Cell replacement therapy (CRT) is another surgical approach based on the rationale that transplantation of midbrain DAergic neurons into the DA-depleted striatum could restore the lost neurotransmission in the nigrostriatal pathway [36]. CRT started nearly three decades ago with the transplantation of allografts of fetal ventral mesencephalic tissue, rich in DAergic neuroblasts, as a treatment to replace DAergic neurons in PD patients [37, 38]. Several post-grafting follow-up reports have recounted that fetal DAergic neuron grafts can reinnervate the striatum, release dopamine, and become integrated into the patient's brain, facilitating long-term survival [39–41]. Furthermore, the histopathological success of fetal DAergic neuron grafts is accompanied by sustained motor function improvement and, in the most successful cases, discontinuation of all pharmacological DA replacement agents [42, 43]. However, the occurrence of postoperative graft-induced dyskinesias, presumably caused by the excessive, uncontrolled production and release of DA in a significant number of patients (i.e., 13 of 23 patients (57%)) is one of the major obstacles to use this approach as the gold standard for treating PD [44].

Other potential DA cell-replacement approaches have emerged as we move into an era when DAergic neurons are derived from stem cell sources such as human-induced pluripotent stem cells (iPSCs) generated from patients' own fibroblasts. Recent studies on autologous iPSC-derived DAergic neuron transplantation in primate models demonstrate improved long-term survival as well as improved motor and behavioral symptoms, without requiring immunosuppression [41, 45]. These important proof-of-concept discoveries advocate great potential for stem cell therapy as a viable alternative treatment strategy for PD. However, it is important to mention that this approach may not be applied for familial PD patients.

Gene therapy (GT) has recently been considered to modulate DA deficiency in the nigrostriatal pathway. GT is based on altering the expression of genes by using a viral vector to deliver genetic material to affected cells, thus compensating for abnormal protein production. To date, GT approaches adopted to treat motor symptoms in

PD involve the delivery of neurotransmitter-producing enzymes and neurotrophic factors into the basal ganglia (SNpc, putamen, GP, and STN). The results of completed GT trials for PD have confirmed the successfully targeted expression of bioactive molecules such as gamma-aminobutyric acid (GABA), dopamine, and glial cell-derived neurotrophic factor (GDNF) [46–48]. Yet, the lack of appropriate controls or the small number of treated subjects in these studies limits the generation of robust data required for the regulatory approval of GT for PD [49].

5.2 Oxidative Stress and Aging Brain

As the brain ages, structural changes and progressively declining cellular functions limit its tolerance to internal and external damages such as OS. While many biological changes may contribute to the age-associated deterioration of brain functions, OS has been considered as one of the critical risk factors associated with neurodegeneration and cognitive decline in older individuals. OS is a result of an imbalance between prooxidant species production and their enzymatic or nonenzymatic removal [50]. Cellular pro-/antioxidant imbalance is attributed to the overproduction of ROS/reactive nitrogen species (RNS) and the breakdown of cell buffering mechanisms [51]. The major sites of ROS/RNS production in aerobic cells are the mitochondria and peroxisome. Both organelles contain several enzymes that generate ROS, such as the superoxide anion radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which serve as precursors for hydroxyl radicals ($^{\bullet}OH$) (Eq. (5.1)). In the brain, constitutively expressed enzymes such as the neuronal isoform of nitric oxide synthase (nNOS) contribute to the production of nitric oxide ($NO^{\bullet}$), an important signaling molecule that also reacts with $O_2^{\bullet -}$ to produce peroxynitrite $\left(ONOO^-\right)$ (Eq. (5.2)) [52]. Trace levels of ROS/RNS are required for the normal functions of the central nervous system (CNS), serving as signaling molecules involved in the regulation of synaptic plasticity, adult neurogenesis, and apoptosis [53–55]. However, the abnormal production/accumulation of ROS/RNS, as well as any defects in the antioxidant/salvage machinery, could severely impact specific brain regions that are inherently more prone to OS damage.

$$O_2 \xrightarrow{e-} O_2^{\bullet -} \xrightarrow{H^+} HO_2 \xrightarrow{e^-,\ H^+} H_2O_2 \xrightarrow{e^+} HO^{\bullet} \tag{5.1}$$

$$NOS \xrightarrow{L-\text{arginine} + O_2} NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \rightarrow HO^{\bullet} \tag{5.2}$$

The OS burden in the brain is associated with increased levels of oxidized lipids, proteins, and DNA [56]. Thus, OS-induced cellular damage in specific brain regions indicates a deficient neuronal antioxidant defense mechanism that progressively declines with aging. The low activity of important endogenous antioxidant systems in neurons such as superoxide dismutase (SOD), glutathione peroxidase (*GPx*), and reduced glutathione (GSH) levels has been connected to OS occurring during aging [49]. Further support for this view is provided by the observation that

the increased OS in neurodegenerative conditions is accompanied by reduced levels of GSH, which among its many functions acts as a substrate for antioxidant enzymes such as *GPx* [57]. GSH depletion in the midbrain region of PD patients suggests that breakdown of the antioxidant defense system contributes to PD pathogenesis [58, 59]. In contrast to this observation, transcriptomic studies reveal that antioxidant genes like *Gpx4* involved in H_2O_2 reduction are highly active in A9 DAergic neurons [60]. In PD, DAergic neurons of the midbrain regions and A9 neurons in the SNpc are highly vulnerable to oxidative damage in comparison to the A10 neurons in the nearby ventral tegmental area [53, 61]. One of the possible reasons is that nigral neurons involved in DA metabolism require high-energy input and a robust antioxidant defense mechanism, as they are predominantly exposed to the oxidative by-products of DA metabolism. While *Gpx4* gene upregulation in A9 neurons demonstrates that they possess an active antioxidant enzymatic pathway, reduced GSH levels in the SNpc can limit the capacity of *GPx* to ameliorate OS in DAergic neurons.

5.3 Sources of ROS in Dopaminergic Neurons

5.3.1 Neuronal Dopamine Metabolism

The DA biosynthesis pathway is a two-step process that initiates with the hydroxylation of L-tyrosine by tyrosine hydroxylase (TH) to L-DOPA. This is regulated by tetrahydrobiopterin (BH4), a cofactor synthesized by GTP cyclohydrolase (GTPCH) and required for the activation of TH. L-DOPA is then decarboxylated by L-aromatic amino acid decarboxylase (L-AADC) to yield DA [62]. Though DA itself is not harmful, its metabolism releases a series of derivatives/by-products that are highly reactive in the presence of ROS/RNS, leading to the generation of harmful neurotoxins.

Since neurons maintain only a small DA pool, continual synthesis/regulation of DA occurs in DAergic neurons. However, excess DA synthesis can lead to severe cellular oxidative damage. DA and its metabolite L-DOPA act as a source of OS by oxidizing into cytotoxic quinones (DA-Q and L-DOPA-Q) (Fig. 5.1) [62]. This is promoted by prooxidant metals–DA complex formation, DA auto-oxidation, or two-electron DA oxidation by tyrosinase [63, 64]. Quinones like DA-Q are precursors to endogenous neurotoxins like 6-hydroxy dopamine (6-OHDA) and *salsolinol*, which cause OS, mitochondrial damage, and TH inhibition [65]. The oxidation of DA via MAO also generates H_2O_2 and the reactive aldehyde DOPAL (3,4-dihyd roxyphenylacetaldehyde) that is 100–1000-fold more toxic than DA both in vivo and in vitro [66]. Deregulated DA oxidative metabolism could thus result in excess ROS production and thereby affect redox cycling reactions. Therefore, efficient antioxidant machinery to counter this and maintain redox homeostasis is essential to prevent OS damage to DA neurons.

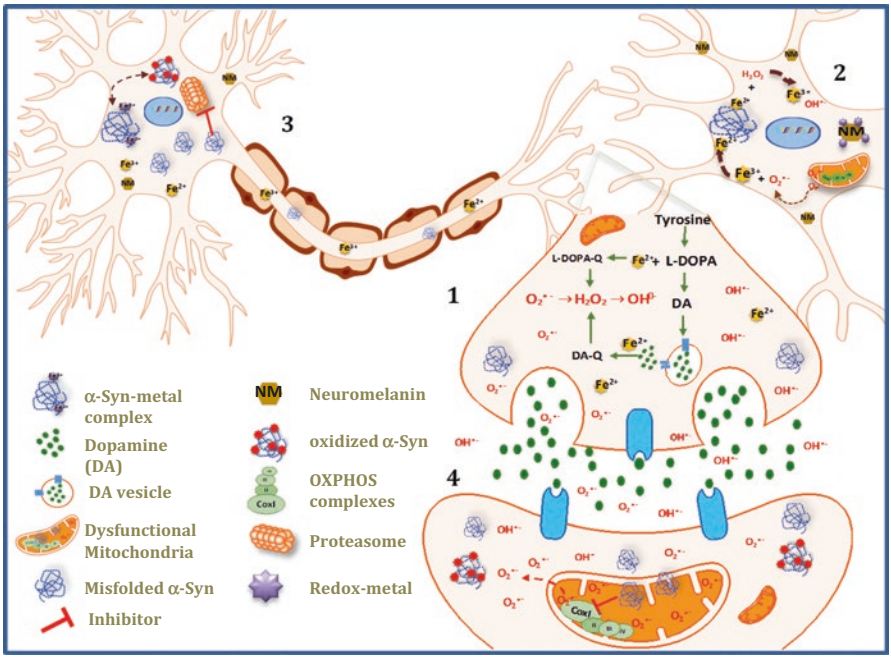

Fig. 5.1 Sources of ROS in DAergic neurons: (*1*) Dopamine and its metabolite L-DOPA are autoxidized spontaneously or by iron (Fe^{2+}/Fe^{3+}) resulting in the formation of cytotoxic quinones (L-DOPA-quinone [L-DOPA-Q] and dopamine-quinone [DA-Q]) that further generate ROS. (*2*) High levels of prooxidant metals in SNpc DAergic neurons promote Fenton-like reactions with $O_2^{\bullet-}$ and H_2O_2, generating highly toxic $^{\bullet}OH$ radicals. Recurrent cycles of these reactions increase the labile Fe pool, leading to the saturation of iron-chelating sites on NM. (*3*) Mitochondrial dysfunction is one of the primary sites of $O_2^{\bullet-}$ and H_2O_2 generation, which further increases following the inhibition of COXI by neurotoxin chemicals (MPTP) or proteins (α-Syn). Excess of extramitochondrial $O_2^{\bullet-}$ leads to the oxidation of proteins and other biomolecules. (*4*) α-Syn toxicity may directly generate ROS via the formation of a redox metal–α-Syn complex that can further catalyze Fenton-like reactions or by inhibiting ubiquitin–proteasome system (UPS) activity leading to the accumulation of oxidized proteins

5.3.2 Prooxidant Metal Ions

The accumulation of prooxidant transition metals [Fe, Cu, Mn, calcium (Ca), mercury (Hg), etc.] is considered as one of the key mechanisms underlying progressive DAergic neuronal death in PD. These metals cause intricate oxidative damage by promoting DA oxidation and lipid peroxidation in PD [67–69]. In addition, redox metal-induced oxidation of cysteine (Cys) can inhibit the biogenesis of reduced GSH at the intracellular level, thereby attenuating the neuronal antioxidant machinery and perturbing mitochondrial function via inhibition of mitochondrial complex I activity [70–73].

In DAergic neurons, Fenton-like reactions (Eqs. (5.3)–(5.5)) between redox-active H_2O_2 molecules generated by both enzymatic and nonenzymatic metabolism of DA and metals aggravate the ROS load (Fig. 5.1) [74, 75].

$$\mathrm{Fe(III)} + \mathrm{O_2^{\bullet -}} \leftrightarrow \mathrm{Fe(II)} + \mathrm{O_2} \tag{5.3}$$

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(III)} + {}^{\bullet}\mathrm{OH} + {}^{-}\mathrm{OH} \tag{5.4}$$

$$\mathrm{Cu(I)} + \mathrm{H_2O_2} \rightarrow \mathrm{Cu(II)} + {}^{\bullet}\mathrm{OH} + {}^{-}\mathrm{OH} \tag{5.5}$$

In addition, hydroxyl radicals generated by Fenton reaction further react with H_2O_2 to produce more superoxide radicals via the Haber–Weiss reaction, and the reaction continues in a cyclic fashion (Eqs. (5.6) and (5.7)) [76].

$$\mathrm{O_2^{\bullet -}} + \mathrm{H_2O_2} \leftrightarrow \mathrm{O_2} + {}^{\bullet}\mathrm{OH} + {}^{-}\mathrm{OH} \tag{5.6}$$

$${}^{\bullet}\mathrm{OH} + \mathrm{H_2O_2} \rightarrow \mathrm{H_2O} + \mathrm{H^+} + \mathrm{O_2^{\bullet -}} \tag{5.7}$$

DA quinones produced by transition metal-induced oxidation of free DA have also been suggested to carry out multimodal toxic reactions in the DAergic neurons in PD [77–79]. They covalently bind to Cys residues of the DA transporter, consequently inhibiting the uptake of DA [80].

The highly redox-active neuromelanin (NM) is another source of ROS/RNS in DAergic neurons. NM normally sequesters paramagnetic transition metal ions [81]. NM could be considered as double edged in SNpc neurons. Its metal chelating free radical scavenging property includes neutralization of $^{\bullet}OH$ radicals, protecting SNpc neurons against OS. Conversely, NM can be cytotoxic when overloaded with free metal ions. NM released by degenerating neurons activates the inflammatory response in the surrounding healthy neurons, triggering more OS [82, 83].

Studies have shown that Fe is the predominant metal ion bound to NM granules, consistent with the spectroscopic analysis of human SNpc, which revealed a marked increase in Fe^{3+} levels in NM granules of PD patients compared to control specimens [84–86]. Interestingly, NM granules in SNpc contain L-ferritin, which is known to be common in glial cells rather than neurons, indicating an alternative Fe storage mechanism other than the Fe–NM complex in human [87]. The role of ferritin in inhibiting Fe^{3+} ions from participating in the Fenton reaction is contentious. Connor et al. observed no compensatory increase of SNpc ferritin in response to iron overload in a quantitative analysis using a monoclonal antibody against the neuronal isoform of brain ferritin [88]. However, NM released from affected DAergic neurons activates microglia, generating superoxide radicals that may in turn induce the release of Fe from ferritin [89, 90].

Like Fe, Cu is an essential transition metal for normal cellular functioning. Cu is a cofactor for antioxidant copper/zinc SOD (Cu/Zn SOD) and a number of CNS-specific enzymes such as tyrosinase, dopamine-β-hydroxylase, ceruloplasmin, monooxygenase and cytochrome *c* (Cyt *c*) oxidase, etc. [91, 92]. Cu also contributes to the electron transport chain between complex III (COXIII) and complex IV (COXIV) at the inner mitochondrial membrane. Analyses of sporadic PD-affected brains have revealed an accumulation of Fe and decreased Cu levels in SNpc com-

Table 5.1 Dyshomeostasis of metal ions in the brain and peripheral fluids (blood) in PD patients

Metal ion	[a]Percent change of trace metals in total *SN tissue*	[b]Percent change of trace metal in human *serum sample*	References
Fe	↑ (~35–45)	↓ (~30)	[a]Dexter et al. [68]
			[a]Howitt et al. [94]
			[b]Hegde et al. [95]
Cu	↓ (~35–45)	↑ (~62)	[a]Dexter et al. [68]
			[a]Davies et al. [96]
			[b]Hegde et al. [95]
Mn	↑ (~127)	↑ (~1.5)	[a]Uitti et al. [97]
			[a]Dexter et al. [68]
			[b]Forte et al. [98]
Zn	↑ (~50)	↓ (~19)	[a]Dexter et al. [68]
			[b]Hegde et al. [95]
Hg	–	↑ (~43)	[b]Forte et al. [98]
Pb	↓ (~10)	↑ (~19)	[a]Dexter et al. [68]
			[b]Forte et al. [98]

A distinct inverse correlation was observed in most metal levels in the brain vs. blood/serum. Related references are indicated

pared to healthy individuals [68, 93]. Interestingly, opposite pattern of metal imbalance is observed in PD patient's blood plasma, who present reduced Fe levels and increased Cu levels (Table 5.1). Furthermore, several epidemiological studies suggest an association between increased PD incidences and chronic occupational exposure to Cu [67, 99, 100]. While the pathogenesis underlying Cu toxicity-induced PD remains elusive, it is known that Cu as a redox-active transition metal participates in the Fenton reaction when it is in free ionic state. Metal ions, particularly Cu^{2+}, can induce α-Syn misfolding in vitro, suggesting a linkage between Cu accumulation and accelerated α-Syn aggregation as an initial event in LB formation [101]. This is in agreement with evidence demonstrating early accumulation of α-Syn in SN A9 neurons' NM, which is rich in heavy metals like Cu. Thus, it is proposed that A9 neurons may have a high propensity to form α-Syn precipitate around NM lipid under OS conditions [96, 102–104].

α-Syn presents a high binding affinity to Cu at His50 and D121, allowing the formation of α-Syn–Cu complexes. Thus, it is hypothesized that readily oxidizable cellular species could react with α-Syn–Cu^{2+} complexes (Eq. (5.8)). In this model, the oxidation of α-Syn–Cu^{+} into α-Syn–Cu^{2+} can facilitate the generation of H_2O_2, which can react with a variety of cellular and chemical species [78]. Indeed, α-Syn–Cu^{2+} complex of has been implicated in oxidation of DA to *O*-quinone via a free radical chain reaction [105]. In this study, authors have shown that the incubation of this complex with the metallothionein III enzyme, an enzyme abundantly expressed in neurons, transfers the Cu^{2+} ion from the α-Syn complex to the enzyme, indicating a possible therapeutic intervention in PD.

$$2\alpha\ \text{Syn}-\text{Cu}^{+}+\text{O}_2+2\text{H}^{+}\ \rightarrow\ 2\alpha\ \text{Syn}-\text{Cu}^{2+}+\text{H}_2\text{O}_2 \tag{5.8}$$

Manganese (Mn) is also required for the normal body functions in trace levels as a cofactor for many essential enzymes involved in antioxidant machinery function. In the brain, it plays a regulatory role in association (~80 % of total Mn) with the astrocyte-specific enzyme glutamine synthetase [106]. The ability of Mn to cross the blood–brain barrier (BBB) and its limited excretion, particularly in infants, makes it highly neurotoxic [107, 108]. Mn neurotoxicity is common among miners and welders. Welding fumes contain complex metal aerosols with various Mn compounds that have been suspected to predispose welders to PD [109]. High Mn levels are correlated with loss of striatal DA neurons in Mn-exposed animal and cell PD models [110]. According to this model, Mn alters DA transport by promoting the internalization of the cell-surface DA transporter (DAT) [111]. Inhibition of DA influx/efflux can increase the intracellular DA pool, increasing the chances of two-electron DA oxidation to DA-Q by redox-active metals already present in neurons. Indeed, it has been demonstrated that Fe^{2+}/Fe^{3+} and Mn^{2+} can also catalyze the autoxidation of DA. Furthermore, Mn accumulation in the brain causes the depletion of TH and mitochondrial dysfunction, resulting in DAergic neuron injury [112]. Although it is known that Mn overloading causes neurotoxicity via OS, the precise mechanism is not clearly understood [113]. Stephenson et al. showed that Mn-induced free radical toxicity causes the accumulation of single-strand breaks in DNA and oxidatively damaged thymine bases in a neuroblastoma cell model [114]. These authors also rescued Mn-induced oxidative damage by treating cells with reduced GSH and *N*-acetylcysteine as antioxidant agents, reaffirming the involvement of free radicals in Mn toxicity.

Mercury (Hg) poisoning, although rare, has been considered a potential risk factor for PD and neurodegenerative diseases. Nylander et al. reported that chronic Hg toxicity could be caused by exposure to dental amalgamation [115]. Hg vapor also causes damage to the CNS among miners [116, 117]. Highly diffusible Hg compounds cross the BBB in the pre- and postnatal developing human brain, causing neurological abnormalities, cognitive impairment, and behavioral disturbances [118, 119]. Furthermore, methyl–mercury (Me–Hg) is one of the widespread toxic forms of Hg and has been shown to induce significant chromosomal aberration in affected individuals [120]. Unlike other heavy metals, which normally cause acute toxicity, Hg does not affect cell viability; instead, it alters gene expression by epigenetic modifications and crucial signaling pathways, causing long-term adverse effects. Animal and stem cell models exposed to low doses of Me–Hg to mimic chronic toxicity conditions revealed perturbed cell proliferation rate of progenitor cells, inhibition of differentiation of DAergic neurons, and adolescent memory deficiency [121–124]. Hg attenuates the antioxidant defense machinery due to its strong affinity to the thiol group (–SH) of GSH leading to its inactivation [125, 126].

5.3.2.1 Homeostatic Imbalance: Primary Molecular Basis of Metal Toxicity

The intracellular versus extracellular levels of essential metals are highly regulated and actively maintained in healthy cells via a complex network of metal storage proteins (e.g., ferritin, transferrin, and ceruloplasmin) and cellular signaling.

The metals sequestered with storage proteins are released only in response to metabolic needs. Remarkably, studies have revealed unique charge-/valence-dependent changes in brain metal homeostasis with the progression of disease severity in PD and AD [127]. For example, the level of divalent Fe(II) or Cu(II) increases in the brain during the early disease phase, whereas trivalent metals increase at late disease stages [128]. Furthermore, in AD and PD cases with no evident dietary/occupational metal exposure, the overall brain metal burden was found unaltered; instead, there is a charge-dependent redistribution of specific metals in the affected brain regions. For example, Fe increase in PD patients is simultaneously associated with decrease in Zn [95]. Thus regardless of whether metals are primary risk factors or their imbalance is a consequence of pathological mechanisms, a moderate change in a single metal ion concentration will result in significant metal level imbalances in the whole system (serum, CSF, and brain). This may imply that the impact of an increase or decrease of an individual metal is not restricted to that metal alone, but causes a more dramatic overall homeostatic imbalance of metals, presumably due to loss of regulation of metals across cell membranes. This may be critical for formulating metal chelation therapy, which should include supplementation of the depleted metal, in addition to chelating the increased metal ions.

Furthermore, studies suggest an inverse correlation of metal levels in serum vs. brain or CFS in PD patients. For example, increased Cu levels in the CSF and SNpc of PD patients are normally associated with its decrease in serum [95]. A similar trend was observed in many cases for Zn, Fe, and other metals (Table 5.1). Thus, the levels of trace metals in serum may be related to the levels in brain with reference to essential elements. Moreover, the source for increased brain metals in PD may come from serum in many sporadic PD cases. The molecular events leading to such a migration pattern of metals between body fluid/organs in PD and other neurological disorders need further investigation.

5.3.3 Weak Antioxidant Machinery

Enhanced OS in the aging or neurodegenerative brain is also alleged to be due to deficient antioxidant defense, resulting in a homeostatic imbalance between the generation and detoxification of excessive ROS/RNS. Multiple antioxidant mechanisms exist in cells to maintain free radical levels below the toxic threshold; these include the enzymes GSH reductase, *GPx*, Cu/Zn and Mn SOD, and catalase, as well as the employment of low molecular weight antioxidants (GSH, ascorbic acid, vitamin E, etc.) [54]. Brain OS vulnerability can be related to reduced levels of SOD and catalase, especially during aging and neurodegeneration. Alternatively, the brain presents high levels of other enzymes like *GPx* to compensate for reduced catalase activity [129]. However, as the brain ages, this backup pathway is limited by the depletion of GSH, used as a substrate by GP*x* to convert O_2^- into H_2O_2. Therefore, GSH is considered the major brain antioxidant defense mechanism preventing OS-induced neuronal injury.

Postmortem brain analysis reveals depletion of SNpc's GSH, while in the frontal cortex (FC), GSH levels and related activities are elevated [130]. This suggests that GSH loss could be an early indicator of SNpc degeneration, whereas elevated GSH in non-SNpc regions might serve as a protective mechanism to prevent OS damage in these regions [131]. The causes of GSH depletion in PD remain unknown, but its absence can lead to severe alterations such as inhibition of proteasome activity, OS exacerbation, and inflammatory response via activation of the c-Jun N-terminal kinase (JNK) pathway. OS-induced DNA and protein damage in neurons can further affect the activation/regulation of important antioxidant genes in senescent cells, debilitating the antioxidant defense machinery of the aging brain.

5.3.4 Mitochondrial Dysfunction: A Primary Source of ROS

Mitochondrial dysfunction is intrinsically coupled to excessive ROS formation in DAergic neurons. The high-energy demand of neurons requires more active OXPHOS machinery, which would also generate H_2O_2 and $O_2^{\bullet-}$ radicals under physiological conditions.

Compelling evidence demonstrates decreased levels of specific OXPHOS activity associated with the increased generation of ROS in brain tissue from PD patients and animal models [132]. Among the four complexes of the mitochondrial respiratory chain, complex I (COXI) and complex III (COXIII) are the most affected. Under normal physiological conditions, COXI and COXIII maintain trace levels of $O_2^{\bullet-}$ and H_2O_2 [132]. In situ studies in mitochondria isolated from synaptic nerve terminals revealed that inhibition of OXPHOS complexes favor the formation of H_2O_2 in a dose-dependent fashion. Moderate inhibition (16–20 %) of COXI resulted in a significant increase in ROS formation, in contrast to COXIII and COXIV, which required ~70–80 % inhibition to cause similar ROS generation [133]. These findings, along with reports of a 25–30 % decrease in COXI activity in the SNpc of PD patients, suggest that partial COXI inhibition is enough to exacerbate ROS formation in DAergic neurons [134]. However, evidence from Ndufs4 conditional knockout mice and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD cell/animal models reveals that COXI failure is not enough to account for the ROS levels required to trigger the extent of mitochondrial dysfunction and DA neuronal toxicity observed in PD [135–137].

A PD-like syndrome presented in MPTP drug abusers linked mitochondrial dysfunction for the first time to PD. MPTP is metabolized in astrocytes by MAO-B into its ionic form MPP^{+-}, which is selectively acquired by DAergic neurons where it inhibits COXI and α-ketoglutarate dehydrogenase (α-KGDH) [132, 138]. α-KGDH is a rate-regulating enzyme of the TCA cycle as it catalyzes the conversion of α-ketoglutarate mediators (coenzyme A and NAD^+) into succinyl-COA, NADH, and CO_2 [139]. α-KGDH deficiency is associated with the impaired brain energy metabolism observed in several neurodegenerative conditions, including PD and AD [139, 140]. Evidence of decreased α-KGDH activity in brain SNpc has been

observed in both sporadic and familial PD [140, 141]. Studies involving in situ mitochondria within isolated nerve terminals demonstrate that inhibition of α-KGDH catalytic activity by a high NADH/NAD^+ ratio promotes ROS generation [142]. Furthermore, inhibition of α-KGDH may severely affect the electron pathway by limiting the mandatory succinate supply for COXII activity. Even though there is no report of COXII deficiency in PD, electron transfer via COXII may not compensate for the COXI deficiency observed in parkinsonism [139].

5.3.5 Alpha-Synuclein Toxicity and ROS Generation

α-Syn is a 140 amino acid brain-enriched protein whose intracellular aggregation has been etiologically implicated in three main types of α-synucleinopathies: PD, dementia with LBs (DLBs), and multiple system atrophy (MSA) [143, 144]. Immune-gold electron microscopy in a PD rat model showed that α-Syn is unevenly distributed in the axons, presynaptic terminals, cytoplasm, and nucleus of neurons. The same analysis also detected high levels of mitochondrial α-Syn in hippocampus, striatum, and SNpc neurons [143]. However, the physiological function(s) of α-Syn and its role in the pathophysiological process of neurodegenerative disease remain unclear.

The intrinsically disordered property of α-Syn renders it prone to become misfolded and form antiparallel β-sheet oligomers, resulting in the eventual formation of protofibrils and insoluble aggregate inclusions. Self-aggregation of α-Syn into amyloidogenic fibrils via toxic intermediate oligomer forms is considered one of the toxic mechanisms that might impair basic neuronal processes. However, the multiple factors influencing α-Syn aggregation make it difficult to establish a definite mechanism that explains α-Syn species-induced toxicity in the brain. Extensive research has demonstrated that metal toxicity, as well as neurotoxins such MPTP and 6-OHDA, can promote α-Syn aggregation accompanied by ROS generation, providing an insight into the possible mode by which α-Syn can promote neurotoxicity.

As previously mentioned, the presence of redox-active metals (Fe^{2+}, Cu^{2+}) induces α-Syn aggregation and formation of α-Syn–metal complexes (Fig. 5.1). Increased OS burden due to metal–α-Syn complexes can induce the oxidation of endogenous antioxidants (GSH) and mediate Fenton-like reactions, resulting in the accumulation of toxic ROS that can further oxidize important macromolecules. This is in agreement with the evidence of nuclear and mtDNA damage in α-Syn-overexpressing PD-affected brain samples. Mitochondrial DNA damage, in particular, can intensify ROS/RNS load by altering the expression of the OXPHOS complexes leading to cellular respiration deficiency [145]. Likewise, MPTP PD models suggest that COXI inhibition is an important step underlying mitochondrial dysfunction, a phenomenon also observed in mitochondrial accumulation of α-Syn. A study on the effects of aggregated α-Syn in control and COX1-deficient neurons revealed that H_2O_2 production is the same in both types of cells [145].

Another possible mode by which α-Syn aggregation can promote ROS generation is through impairment of the ubiquitin–proteasome system (UPS) (Fig. 5.1). The UPS is one of the defense mechanisms that abrogate the deleterious effects of OS in cells [146]. Under normal conditions, the UPS is involved in the degradation of oxidized and misfolded proteins. However, interference in this pathway results in the accumulation of oxidized/misfolded proteins and subsequent aggregate formation. The binding of α-Syn protofibrils to the 26S UPS subunit and UPS substrates blocks the translocation of substrates required for proper UPS functionality, providing an insight into how UPS dysfunction is mediated in PD [25, 147]. Dysfunctional UPS in an OS-rich environment can increase the pool of oxidized proteins targeted for degradation. Such unrestricted accumulation of oxidized species can further initiate the dissociation of the 20S core particle from the 19S regulatory particle of the 26S UPS, inhibiting 26S proteasome activity [148].

5.4 Consequences of Ion-Catalyzed Oxidative Insults in DAergic Neurons

5.4.1 Genome Damage

The fidelity of the genome in an aerobic cell is at constant assault from endogenous (i.e., ROS/RNS) as well as exogenous (i.e., UV, ionizing radiation, drugs, etc.) sources. Oxidative DNA damage represents the major class of DNA lesions induced by ROS/RNS, generated as by-products of respiration, inflammatory response, or aberrant antioxidant reactions [149]. These lesions include a wide range of oxidized DNA bases, oxidized abasic (AP) sites, oxidized sugar fragments, and DNA single-strand breaks (SSBs) that are repaired via the base excision/SSB repair (BER/SSBR) pathway in both the nuclear and the mitochondrial genomes [149]. DNA base oxidation occurs most readily at guanine bases forming 8-oxo-7,8-dihydroguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G) lesions. These and other common base lesions, such as 5-hydroxyuracil (5-OHU), thymine glycol, and 4,6-diamino-5-formamidopyrimidine (Fapy-A), are both cytotoxic and mutagenic. SSBs are not only induced by ROS directly but also as intermediates during the repair of oxidized base lesions or AP sites. ROS-induced SSBs invariably contain blocked termini such as 3′-phosphoglycolate, 3′-phosphate, and 5′-deoxyribose phosphate [150, 151]. The persistent generation of ROS-induced SSBs and their defective repair could generate secondary double-strand breaks (DSBs), which are the most toxic lesions in cells [152].

In addition to ROS, redox cycling transition metals that accumulate in the PD brain contribute to genome damage not only by their direct binding to nicked DNA but also via increasing the generation of ROS and oxidized proteins/lipids [149, 153]. Furthermore, these metals bind to proteins that have a propensity for misfolding, including α-Syn, and could promote their abnormal nuclear as well as mitochondrial localization. Cumulative evidence suggests that presence of misfolded α-Syn in

the neuronal nuclei may be genotoxic by altering the chromatin organization or directly binding/damaging DNA [30, 154, 155]. Studies indicate that α-Syn forms a tight complex with histone proteins, which are involved in the maintenance of chromatin conformation integrity and whose coupling/uncoupling dynamics regulates various DNA transactions including gene expression [156]. The α-Syn–histone interaction was shown to regulate histone acetylation patterns, possibly leading to the dechromatinization of genome sequences, making them vulnerable to oxidative insults [154]. Our lab reported that α-Syn binds GC-rich sequences in DNA, altering the B-form conformation as well as superhelicity of the DNA [155, 157, 158].

CNS cells' genomes are generally more susceptible to oxidative damage due to their high O_2 consumption, high metabolic rate coupled with relatively low antioxidant activity, and the presence of high levels of polyunsaturated fatty acids that are labile to oxidative modifications [159]. Consistently, nuclear (nuDNA) isolated from the midbrain region of PD patients showed a high level of strand breaks compared to age-matched controls [29]. However, there have been conflicting reports on the presence of DNA lesions in the SNpc of PD patients. Alam and colleagues reported that guanine damage in nuDNA was not higher in PD brains in comparison to age-matched controls [160]. In contrast, Zhang and colleagues utilized an antibody that recognized the DNA and RNA damaged products of 8-oxo-dG and 8-oxoG and found increased reactivity in PD midbrain region samples when compared to age-matched controls [161]. This reactivity was particularly higher in the cytoplasm than in the nucleus, suggesting more oxidative damage in mtDNA and/or RNA. Although little is known about oxidative RNA damage, RNA is likely to be equally subjected to oxidative damage; further investigation into oxidative RNA damage in PD brains is required [162].

Similar to nuDNA, extensive damage was observed in mtDNA in PD, including oxidative lesions as well as sequence deletions [163]. Moreover, a recent study showed the presence of DSBs in mtDNA in mice with DAergic neurodegeneration [164]. There is evidence of impaired energy metabolism and 50 % reduction of mtDNA/RNA content in PD [165]. Mitochondrial DNA damage may also be caused by external insults such as paraquat, cadmium chloride, and aflatoxin, which are common etiological factors implicated in PD and cause DAergic neurodegeneration in *Caenorhabditis elegans* and other animal models [166]. As expected, mtDNA is reported to be more susceptible to oxidative damage than nuDNA, due to its proximity to electron chain-mediated ROS production and the absence of chromatin [167]. Although both nuclear and mtDNA are extensively damaged in the PD brain, their relative contribution to PD and the sequence of their occurrence remains unclear.

5.4.2 DNA Repair Inhibition

Maintaining genomic fidelity via highly evolved DNA repair systems is essential for the normal functioning and survival of a cell. Defects in repair leading to the accumulation of unrepaired genomic damage have been associated with a range of

human disorders, including cancer susceptibility, aging, and various neurodegenerative diseases. There is a growing interest in understanding the possible role of DNA repair defects in PD, other age-associated neurodegenerative diseases, and accelerated aging. The major limitation of neuronal DNA repair is that neurons are terminally differentiated, and no replication-associated DNA repair is possible [168]. Damage to both nuDNA and mtDNA normally elicits a DNA damage response (DDR) signaling cascade, which is crucial for maintaining genomic stability by activating/recruiting efficient repair machinery. Because the brain is generally protected from external genotoxins by the BBB, intrinsic ROS-induced damage is the most common threat to neuronal genomes, and consistently robust BER/SSBR activities have been characterized in brain cells [149, 169–171]. Among the oxidized DNA base repair-initiating DNA glycosylases, oxoguanine glycosylase (OGG1) expression was shown to decrease during development in rat brain, while Nei endonuclease-like (NEIL)1 and NEIL2 levels increased [172]. This is consistent with high transcriptional activity in the brain [173] and the likely involvement of the NEILs in transcription-associated repair [174–178]. Among other BER proteins, the strand-break end-processing enzymes polynucleotide kinase 3′phosphatase (PNKP) and apurinic/apyrimidinic endonuclease 1 (APE1) are generally highly expressed in the brain. DNA polymerase β (Polβ) appears to be the major DNA polymerase in neurons and is ubiquitously expressed in different brain regions [179]; however, its activity decreases with age [180]. Further, the DNA replication/repair proteins like flap structure-specific endonuclease 1 (FEN-1), Polδ/ε, proliferating cell nuclear antigen (PCNA), and DNA Ligase I levels are low in adult neurons, while X-ray repair cross-complementing protein 1 (XRCC1) and DNA Ligase IIIα are highly expressed in the brain compared to other tissue types [172]. Even though these studies confirm the presence of BER/SSBR proteins/activity in the neurons, their expression levels by themselves may not provide insight into their repair capacity. Furthermore, the lack of a detailed picture of genome repair in the brain could be attributed to the fact that human brain genome damage/repair can only be studied in postmortem samples, and to the difficulty in examining these processes in individual cell type-specific manner, which would be critical because of potential differences in damage tolerance in these cell types.

The nervous system is often profoundly affected by DNA repair deficiency, contributing to neurodegeneration, microcephaly, or brain tumors. Defective DNA repair in adult neural tissues has been linked to aging and, more recently, to common neurodegenerative syndromes including PD, AD, and ALS. Several reports have suggested a role of defective nuDNA and mtDNA repair pathways, particularly of the BER in the etiology of PD [181]. Inactive genetic variants of APE1 and XRCC1 have been implicated as risk factors for PD [182]. Although the inhibition or defective DNA repair activity has been consistently associated with PD and other neurodegenerative diseases, the molecular events leading to such deficiencies in DNA repair have not been characterized. Furthermore, lack of a direct correlation between repair deficiencies with the expression, modification, or activation of repair enzymes in most cases suggests the involvement of additional mechanisms.

We and others have recently demonstrated the oxidative inactivation of certain DNA repair pathways by ROS and prooxidant metals in neuronal cells [183, 184]. Heavy metals implicated in neuronal dysfunction not only induce DNA strand breaks but also interfere with damage scanning and the recruitment of repair machinery. Nickel (Ni) and cadmium (Cd) have been shown to inhibit SSB end joining in the presence of ROS, presumably by interfering with ligation activity [185]. We have demonstrated that the prooxidant transition metals Cu and Fe specifically inhibit the activities of key BER-initiating enzymes NEIL1 and NEIL2 by forming stable complexes with these proteins and oxidizing Cys residues at nanomolar (nM) to low micromolar (μM) levels. The inhibition was found to be very specific, as demonstrated by the lack of similar inhibition of OGG1 with 8-oxoG substrates [183, 184].

Further, ROS and metal ions could also disrupt protein–protein interactions among the BER proteins. DNA damage repair is a highly coordinated dynamic process that largely depends on myriad of protein–protein interactions to multiple repair subcomplexes. We have shown that Fe^{2+} affects the interaction of NEIL1 with Polβ and FEN-1, which is critical for overall BER [183, 184].

Other reports show that Cd^{2+}, Ni^{2+}, and Zn^{2+} inhibit the activity of the recombinant human *N*-methylpurine-DNA glycosylase (MPG), a monofunctional DNA glycosylase that removes a variety of alkylated bases [186, 187]. Whiteside et al. showed that Cd and Cu inhibit both the phosphatase and kinase activities of PNKP, a key end-processing enzyme for both BER and SSBR [188]. Furthermore, elevated Fe levels caused a reduction in FEN-1 and LigIII activities due to interference with the binding of these repair proteins to their DNA substrates [183, 184]. The vulnerability of DAergic neurons in PD to ROS generation and metal toxicity, together with this reduced DNA repair capacity, strongly suggests a role for these factors; however, a direct link between ion-mediated DNA repair inhibition and DAergic neuronal death in PD remains to be established.

Many DNA repair enzymes are either Zn-finger domain-containing proteins or proteins that normally utilize metal ions as cofactors. However, excess exposure to metal ions could replace Zn and cause structural and functional alterations in repair proteins. For example, human APE1 involved in SSB end processing has two Mg-binding sites and requires one Mg ion to shuttle between the sites for its AP site cleavage activity. APE1 is inhibited if both the sites are saturated with Mg [189]. APE1 and NEIL1 also have been found to be inactive upon oxidation by H_2O_2/ROS [183, 190].

As mentioned already, significant aberrations in mtDNA, including deletions and mutations, have been observed in the PD-affected brain, and thus similar metal-mediated repair inhibition is expected to occur in mitochondria. However, the role of defective mtDNA repair machinery in PD remains to be investigated. Compelling evidence linking DNA repair defects in the manifestation of the neurological phenotype comes from studies in inherited neurological diseases, many of which have been associated with defects in various DNA repair pathways [149]. Mutations or altered expression of proteins involved in the BER (e.g., OGG1, XRCC1), SSBR (e.g., TDP1, aprataxin, PNKP), and DSBR (e.g., ATM, NBS1) pathways have been

observed in humans predisposed to various hereditary neurodegenerative diseases [191–197].

These studies thus suggest that excessive levels of ROS and/or prooxidant metals in neurons act as a "double whammy" by inducing genome damage and also inhibiting damage repair at the same time [152, 184]. Understanding ion-mediated repair inhibition at the molecular level is critical for developing effective therapeutic interventions.

5.4.3 Protein Oxidation

Protein oxidation is particularly linked to OS and could be induced directly by ROS/RNS or indirectly by reactions with secondary by-products of OS (i.e., lipid peroxidation); these are generally characterized by the oxidation/nitration of amino acid residue side chains and protein backbone [198]. For instance, the presence of nitrated α-Syn (n-α-Syn) in LBs from postmortem brain tissue suggests that tyrosine residues Y_{39}, Y_{125}, Y_{133}, and Y_{136} in α-Syn are susceptible to nitration in the presence of oxidizing agents such as peroxynitrite ($ONOO^-$) [6]. OS-induced peroxidation of membrane-associated lipids and lipoproteins generates secondary products such as 4-hydroxy-2-nonenal (HNE) that can form cytotoxic protein adducts [199]. High levels of HNE-modified proteins in the brainstem and cortical-type LBs in PD suggest that abundant proteins like α-Syn are likely to be modified by reactive aldehydes [200–202]. This is supported by recent evidence suggesting that α-Syn is capable of binding to modified/defective lipid membranes resulting from lipid peroxidation by-products and is oligomerized in the presence of HNE via adduct formation at the histidine and lysine residues [202–204].

Oxidative modifications may affect proteins' key functions via inhibition of their binding activity. Reports show that α-Syn aggregation reduces upon binding to negatively charged phospholipids, suggesting a possible preventive mechanism against the misfolding/aggregation of α-Syn [205]. In addition, interaction of α-Syn with negatively charged lipid vesicles revealed that α-Syn adopts α-helical structures that could provide a lipid ordering effect and vesicles' thermal stability [206]. However, this interaction is affected when α-Syn undergoes oxidative modifications such as tyrosine nitration or methionine oxidations. For example, α-Syn-induced nitration with tetranitromethane (TMN) reduces its binding affinity to negatively charged lipid vesicles [199]. Hence, defective binding of oxidatively modified α-Syn with membranes could lead to the destabilization of synaptic vesicles. In addition, HNE-α-Syn and n-α-Syn promote the formation of soluble oligomers that are significantly toxic to differentiated DAergic cells [207]. As a consequence, free oxidative modified α-Syn can increase the formation of toxic oligomeric forms that can contribute to the mechanisms leading to neuronal cell death in PD.

The intracellular accumulation of oxidized proteins reflects the balance between prooxidant species and the activities of the proteases that degrade oxidatively dam-

aged proteins [208]. Although the mechanism underlying the imbalance in oxidized protein degradation in PD is not clear, it is possible that UPS complexes may be altered by interaction with toxic oligomeric/protofibrillar α-Syn species generated from oxidatively modified α-Syn [207, 209, 210]. This is in agreement with evidence demonstrating that binding of α-Syn protofibrils to the 26S proteasome inhibits the proteasomal degradation of polyubiquitinated proteins [209]. Furthermore, the interaction of oxidized α-Syn with UPS complexes could promote direct oxidative modifications of the proteasome subunits, inhibiting the proteasome catalytic peptidase activity [211]. Thus, the disruption of UPS pathway can lead to the accumulation of nondegraded oxidized proteins that may further enhance the formation of ROS/RNS. In addition, S-nitrosylation of *Parkin* Cys residues decreases its E3-ubiquitin-ligase activity as well as its protective function in α-Syn-overexpressing cells that were exposed to proteasome inhibitors [212]. Loss of function of E3-ubiquitin-ligase activity is considered as one of the causes of UPS impairment in familial PD cases with mutations in the *PARK2* gene. Thus, this evidence suggests that protein oxidation could be a possible mechanism by which PD-related proteins, α-Syn and *Parkin*, function may be affected in sporadic PD.

5.4.4 ROS/Metals Promote α-Synuclein Misfolding/Aggregation

The binding of redox-active transition metals to amyloidogenic proteins is an important event underlying the pathogenesis associated with several neurodegenerative conditions. Multiple lines of evidence demonstrate that the formation of metal–amyloid complexes could be more toxic, because amyloidogenic proteins are prone to form toxic oligomers and aggregates in the presence of redox-active metals [30, 213]. For instance, the interaction of Cu at α-Syn N-terminus or of Fe at α-Syn C-terminus is accompanied by large conformational changes that accelerate fibril assembly [214, 215]. Cu in particular has been identified to significantly exacerbate the toxicity of α-Syn oligomeric species and aggregates. Soluble α-Syn oligomers tend to adopt a unique stellate form in the presence of Cu^{2+}. The exogenous application of these stellate oligomers into a DAergic cell culture model significantly decreased cell viability by >50 % [215]. While the implications of metal-induced oligomeric α-Syn toxicity in PD is not clear, experimental evidence suggests that the formation of amyloid metal complexes alters the redox properties of transition metals, which could lead to the further generation of toxic ROS. Electrospray mass spectrometry (ES–MS) studies have revealed that α-Syn–Fe^{2+} can be readily oxidized to the unstable α-Syn–Fe^{3+} complex, which in turn rapidly dissociates because of the low binding affinity of the Fe^{3+} with α-Syn, yielding H_2O_2 and $Fe(OH)_3$ as coproducts (Eq. (5.9)) [216]. These studies suggest a possible mechanism in which metal–amyloid oligomer/aggregate complex-induced generation of ROS can further cause detrimental cellular damage (Fig. 5.1).

$$2\alpha - \text{Syn} - \text{Fe}(\text{II}) + \text{O}_2 + 6\text{H}_2\text{O} \rightarrow 2\alpha - \text{Syn} + \text{H}_2\text{O}_2 + 4\text{H}^+ + 2\text{Fe}(\text{OH})_3 \quad (5.9)$$

Furthermore, redox-active transition metals can contribute to amyloid protein aggregation by inhibiting transcription factors such as nuclear factor E2-related factor 2 (Nrf2), which is critical for the expression of cytoprotective, antixenobiotic, and antioxidant enzymes [217], including heme oxygenase 1 (HO-1), a cellular stress response enzyme involved in the degradation of heme to carbon monoxide (CO), Fe^{2+}, and biliverdin/bilirubin. Studies in SK-N-SH neuroblastoma cells reveal that Fe^{2+} promotes α-Syn aggregation by inhibiting the Nrf2/HO-1 pathway [7]. Although it is not yet clear how Fe^{2+} inactivates the Nrf2/HO-1 pathway in DAergic neurons, reports indicate that HO-1 inhibition affects the intracellular proteasomal degradation of α-Syn in human neuroblastoma M17 cells, suggesting that α-Syn catabolism in these cells is dependent on HO-1 enzymatic activity [218]. This correlates to the fact that HO-1 or HSP32, a member of the heat shock proteins (HSP), is important for preventing protein misfolding by accelerating the degradation of misfolded protein in neurons [7, 219]. Additionally, the presence of a significant fraction of GFAP-positive astroglia expressing HO-1 in the SNpc of PD patients suggests that HO-1 regulation is essential to overcome chronic OS in the affected tissue [220]. However, high levels of HO-1 activity in glial cells can exacerbate intracellular levels of free Fe^{2+}, promoting further amyloid protein aggregation and the generation of neurotoxic ROS that could damage nearby neurons [221, 222].

Collectively, these lines of evidence suggest that increased levels of metals/ROS compromise neuronal cell proteostasis. Moreover, the physiological functions of many proteins implicated in neurodegeneration are affected upon acquiring an unstable structural conformation. In vitro studies demonstrate that α-Syn is able to function as a cellular ferri-reductase by regulating the conversion of Fe^{3+} to the biologically active Fe^{2+} in the presence of Cu and NADH [223]. This finding suggests that α-Syn uses Cu as its catalytic center to transfer electrons between a donor such as NADH and an acceptor such as Fe^{3+} [223, 224]. However, aggregated α-Syn loses its iron-reducing ability. Though the physiological relevance of the ferri-reductase activity of α-Syn in normal and PD conditions remains obscure, it is possible that a decrease in Fe^{2+} could compromise normal cell reactions. In DAergic cells, Fe^{2+} plays a central role in regulating the TH activity necessary for the biosynthesis of the DA precursor L-DOPA [223, 225]. The synthesis/regulation of DA and its metabolite L-DOPA is essential for pro-/antioxidant homeostasis in DAergic neurons. Since these molecules are easily oxidized, DAergic cells maintain a small DA pool to prevent further OS. Consequently, the inhibition of TH activity by low levels of Fe^{2+} may alter DA biosynthesis. However, the in vivo relevance of α-Syn ferri-reductase activity needs to be investigated to determine its impact on DAergic neuron survival. It is important to mention here that α-Syn knockout mice present reduced DA levels in striatal DAergic neurons, demonstrating the role of α-Syn as a regulator of DA biosynthesis [226]. Furthermore, soluble α-Syn binding to TH also regulates DA levels in striatum DAergic cells by reducing TH phosphorylation, resulting in reduced TH activity [227, 228]. Thus, the loss of soluble α-Syn, due to

its conversion to an aggregate form, can result in a positive feedback loop leading to an imbalance in DA synthesis.

5.4.5 *Mitochondrial Pathology in PD: Cross Talk Between ROS, Metals, and α-Syn Toxicity*

Defective scavenging of mitochondrion-generated ROS can affect the maintenance of mitochondrial homeostasis required for normal cellular functions. Under basal conditions, mitochondrial ROS can function as signaling mediators of the stress response linked to the activation of mitochondrial maintenance pathways such as mitophagy [229]. Mitophagy involves the selective degradation of dysfunctional mitochondria, which is considered to be a cytoprotective response that prevents excessive ROS generation, and the early release of pro-apoptotic proteins such as Cyt *c*. In neurons, PINK1–Parkin-mediated mitophagy of damaged mitochondria has been shown to take place in distal axons to prevent the further spread of oxidative damage [230]. Thus, this local mitophagy response provides rapid neuroprotection against axonal degeneration, which precedes the loss of the cell body in neurodegenerative diseases. Further studies reveal that increased levels of ROS in the mitochondrial matrix resulted in loss of mitochondria, mitochondrial depolarization, and subsequent activation of the Parkin-dependent mitophagy pathway [231]. Hence, failure of dysfunctional mitochondria clearance by the Parkin-dependent mitophagy pathway can have detrimental cytotoxic effects. In addition, SOD overexpression inhibits mitochondrial fragmentation and Parkin-dependent mitophagy activation induced by the acute elevation of mitochondrial ROS. Hence, mitochondrial inner membrane depolarization and further mitophagy activation could be attributed to accumulation of specific ROS, like $O_2^{\bullet -}$, in neurons [232]. Furthermore, if neurons spend most of their energy undergoing continuous mitophagy cycles without proper mitochondrial biogenesis, the bioenergetic reserve needed for other essential cellular functions can be altered. Mitochondrial biogenesis in PD could be reduced, as the expression of the mitochondrial gene expression activator PGC-1α has been reported to be downregulated in the human PD brain, in A30P α-Syn transgenic mice, and in a cell culture model for α-Syn oligomerization [233]. In addition, reduced PGC-1α activity is associated with α-Syn oligomerization. Interestingly, nuclear α-Syn has been found to bind to the PGC-1α DNA promoter, downregulating its expression in response to OS [234].

Mitochondrial dysfunction induced by excessive ROS production may initiate/contribute to Fe dyshomeostasis, leading to mitochondrial Fe overload. Proper mitochondrial function is also necessary for sustaining the generation of iron–sulfur clusters (ISC) and heme, which are prosthetic groups involved in the intracellular Fe homeostasis. Biosynthesis of ISC and heme requires a constant influx of Fe^{2+} into the mitochondria, leading to the possibility of further ROS generation through Fenton chemistry [235]. However, how Fe gets transported into the mitochondria,

especially in neuronal cells, remains unclear. A novel study in PD rotenone mouse and cell models suggests that one possible mechanism for neuronal mitochondrial Fe uptake involves the transferrin (Tf) and transferrin receptor 2 (TfR2) pathway [236]. In this model, Tf accumulates in DA neuronal mitochondria and is partly oxidized at Cys26, possibly leading to the formation of an intermolecular disulfide bond. The oxidation of thiols to disulfide bonds could promote Tf conformational changes, which might facilitate the release of labile Fe. Furthermore, mitochondrial Tf import is increased in cells overexpressing exogenous TfR2 and exposed to rotenone. Based on these data, the authors of this study proposed that oxidation of the mitochondrial ISC protein complex inhibits proper Fe binding, which could lead to the accumulation of labile Fe in the mitochondria [236].

5.4.5.1 Mitochondrial Accumulation of α-Syn and Protein Import Defects: Emerging Evidence

As mentioned previously, increased accumulation of α-Syn has been observed in affected neurons in PD patients as well as in model organisms/cell lines. Moreover, the mitochondrial accumulation of α-Syn coincides with decreased membrane potential, COXI inhibition, altered composition of outer mitochondrial membrane proteins (OMM), and ATP reduction. Thus, mitochondrial dysfunction in sporadic and *SNCA* gene-mutated PD human samples could be linked to the upregulation of mitochondrial α-Syn along with increased OS in these cases. Furthermore, the accumulation of defective mitochondria in the PD brain indicates a possible impairment in the mitophagy pathway. Interestingly, similar mitochondrial impairment is also observed in iPSC lines derived from early-onset PD patients carrying mutations in the *Parkin* or *PINK1* genes [237, 238]. These data support the notion that the PINK1–Parkin-mediated mitochondrial clearance mechanism is compromised in autosomal recessive PD patients with *PARK6* (*PINK1*) or *PARK2* (*parkin*) gene mutations or deletions.

Recent studies suggest that the PINK1–Parkin pathway governs the turnover/removal of dysfunctional mitochondria in response to impaired translocases of the OMM (TOMM) machinery [239–241]. PINK1 accumulates in the OMM in response to collapsed mitochondrial membrane potential or mitochondrial protein import capacity, mediating Parkin recruitment into the OMM. Subsequent activation of Parkin in the OMM stimulates the removal of dysfunctional mitochondria by ubiquitinating OMM proteins, such as the TOMM-complex proteins [237, 238]. While the relevance of TOMM-complex degradation remains unclear, it has been observed that a reduction in the levels of TOMM proteins like TOM40 is accompanied by increased OS, mtDNA deletions, oxidative DNA damage, and altered levels of COXI in PD brain samples and α-Syn transgenic mice [26, 28]. Amelioration of the defective molecular phenotype observed in these mice after TOM40 lentiviral delivery confirmed the role of TOM40 in α-Syn-mediated mitochondrial toxicity [28]. Thus, TOM40 decay could be a possible pathomechanism by which α-Syn can interfere with mitochondrial function. However, it remains unclear what triggers the specific degradation of TOM40, as no other TOM proteins, such as TOM20, are degraded

[28]. Further research is required to understand the precise role of this phenomenon in early or late PD pathology and its potential as a target for therapeutic intervention.

5.4.6 Neuroinflammation

The accumulation of toxic products of oxidative insult induces the activation of astrocytes and microglial cells and subsequent inflammatory reactions. Under basal conditions, innate immune responses (i.e., microglia activation) are considered beneficial for neural tissue, since it promotes the clearance of cell debris and the secretion of neurotrophic factors that are required for neural survival and maintenance [53]. However, the persistent activation of microglial cells and astrocytes leads to ROS/RNS buildup, promoting further neuronal damage. Activation of microglia relies on the regulated induction of enzymes such as NADPH oxidase (NOX) or nNOS, which are involved in the production and release of $NO^{\bullet}$ and $O_2^{\bullet -}$, further increasing the pool of toxic ROS/RNS products. In addition, upregulated myeloperoxidase (MPO) in active astrocytes catalyzes the conversion of NO_2 to $NO_2^{\bullet -}$ radical, which contributes to protein nitrosylation [242].

Interestingly, several factors known to induce the degeneration of DAergic neurons also trigger the activation of microglia, leading to the premise that neuroinflammatory responses are triggered secondarily to cellular damage and/or nervous system degeneration. Among these factors, α-Syn, neuromelanin, and redox-active metals are most associated with microglia activation. Cumulative evidence points toward a connection between oxidized α-Syn species and microglial activation in PD [207, 243, 244]. Administration of n-α-Syn and HNE-α-Syn to a DAergic cell culture model revealed that extracellular oxidized α-Syn induces toxicity, triggering the activation of microglia and neuronal cell death [207]. Therefore, it is hypothesized that microglial activation could occur via the release of protein aggregates from apoptotic neurons or LBs.

Alternatively, neuronal death fosters the activation of inflammatory processes, resulting in a feed-forward cycle. Primary neuronal culture from the rodent midbrain suggests that released Fe from degenerated/dead DAergic neurons triggers the activation of microglial cells in particular. Environmental substances such as rotenone and MPTP also activate microglia, supporting the role of inflammation in a PD-like phenotype.

5.5 Overview of Mechanisms of Nigral Cell Death in PD

5.5.1 Role of Mitochondrial vs. Nuclear Pathology

Programmed cell death associated with neurodegeneration typically involves two major pathways, namely, the extrinsic and intrinsic pathways. The intrinsic or mitochondrial apoptotic pathway is triggered by intracellular stimuli such as increased

Ca^{2+} or ROS levels, whereas the extrinsic or death receptor pathway initiates with the activation of cell-surface receptors, such as Fas (CD95) and tumor necrosis factor receptor (TNF-R) 1. Both pathways mediate cell death through caspase-8 and caspase-9, activated first by upstream signals, which later activate the death-effector caspase-3 and caspase-7 [245]. Several components of mitochondrial apoptosis have been implicated in the pathogenesis of PD, since ROS/RNS accumulation is an important pathological feature underlying nigral cell death. This is supported by many cell culture and animal models demonstrating that ROS trigger mitochondrial dysfunction by inducing mtDNA damage that could result in the defective assembly of mitochondrial electron transport complexes. In addition, other PD-related etiological factors like neurotoxins MPTP, rotenone, and α-Syn accumulation are known to inhibit/disrupt COXI activity, increasing internal mitochondrial ROS levels. Thus high mitochondrial ROS levels can result in the oxidation of mitochondrial internal elements such as the lipid cardiolipin, which disrupts the binding of Cyt *c* from the inner mitochondrial membrane (IMM) [245]. Cyt *c* release enhances downstream mitochondrial ROS by activating caspase-3/7, which mediates the cleavage of the p75 NDUFS1 subunit of COXI [245, 246]. Further, COXI activity blockade results in ROS production, amplifying the apoptotic-signaling pathway. Thus, COXI deficiency is proposed to sensitize neurons to mitochondrion-dependent apoptosis through oxidative damage and activation of members of the pro-apoptotic Bcl2 family (BAX/BAK) proteins [247]. In DAergic neurons, BAX upregulation/posttranslational activation is mediated by the activation of the transcriptional factor p53, which is activated upon DNA damage or other stresses [248]. Pharmacological inhibition of p53 translocation into the nucleus or BAX downregulation significantly reduces rotenone-induced BAX upregulation and apoptotic cell death in rotenone-induced PD animal and cell model [249]. However, BAX translocation into the mitochondria does not rely on p53, but rather on JNK activation of the BH3-only protein (Bim) [248]. The activation of BAX initiates mitochondrial outer membrane permeabilization, resulting in the release of soluble Cyt *c* [245]. Once released in the cytosol, Cyt *c* forms the apoptosome complex with protease-activating factor 1 (Apaf1) and caspase-9 [245]. The formation of this complex triggers caspase-9 and caspase-3, eventually leading to cell death [250].

Although the participation of the extrinsic extracellular pathway in PD is not well characterized, its activation is associated with neuroinflammatory responses. Apoptotic DAergic neurons release several cytotoxic pro-inflammatory factors, such as protein aggregates, neuromelanin, and ROS/RNS. The interaction/binding of these factors with different pattern-recognition receptors (TLRs 2 and 4 and Fas) activates microglia to promote the release of pro-inflammatory cytokines TNFα, interleukin β, and interferon γ. These cytokines, in turn, could induce the activation of cell-surface receptors expressed on DAergic neurons that are coupled with a pro-apoptotic cell death pathway [242]. This model is supported by evidence demonstrating that neuron-released oligomeric α-Syn, in particular, acts as an endogenous agonist for Toll-like receptor 2 (TLR2), which is involved in the activation of inflammatory responses for microglia [251]. Once active, microglia cells release cytokines such as TNFα associated with the activation of members of the TNF-R family, such as TNF-R1,

within DAergic neurons [252]. Upon TNF-R activation, the adaptor proteins Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) and Fas-associated with death domain (FADD) are recruited into the TNF-R cytoplasmic death domain, leading to the activation of the initiator caspase-8. Caspase-8 mediates the activation of the intrinsic apoptotic pathway via either cleavage of effector caspase-3 or cleavage of cytosolic BH3 domain-only death agonist (BID) into truncated BID (tBID) [253]. Activated tBID results in the allosteric activation of BAX, triggering the intrinsic/mitochondrial apoptotic pathway. Thus, caspase-8-mediated activation of caspase-3 and BID results in Cyt *c* release [250]. In addition, the activation of TNF-R might have a deleterious effect on neurons by directly activating other intrinsic death pathways mediated by NF-kB, JNK, and p38 [242].

5.5.2 Lessons Learned from Parkinsonism Animal Models

The current experimental PD animal models are limited to the study of specific pathological aspects of the disease, failing to recapitulate the cross talk between neurodegeneration and clinical symptoms. At present, there are two categories of PD animal models: genetic and neurotoxic. Genetic PD animal models are mainly used to understand the function of genes that are associated with familial PD forms, but do not significantly manifest selective SNpc cell death. Conversely, the use of neurotoxins such as MPTP, rotenone, 6-OHDA, and paraquat (PQ) has demonstrated a significant cell death phenotype that is absent in transgenic models. In addition, some of these models, such as MPTP and rotenone, develop cell-alteration patterns similar to those observed in sporadic PD cases, such as reduced COXI activity, increased ROS, accumulation of oxidized biomolecules, and accumulation of α-Syn. The use of transgenic animal models in combination with neurotoxin models is an approach to study the apoptotic pathways involved in DAergic neuron degeneration/death observed in both sporadic and familial PD.

MPTP- and 6-OHDA-based PD cell/animal models have provided important insights with respect to the ROS-mediated activation of intrinsic apoptotic pathways. Both models present consistent activation of mitogen-activated protein kinases (MAPKs), which can mediate intracellular signaling pathways associated with cell proliferation, differentiation, survival, and death. The MAPK family consists of extracellular-signal kinase (ERK1), JNK, and p38 kinase. MPTP-treated mice consistently show selective activation of the p38-MAPK death-signaling pathway in SNpc DAergic neurons, and of JNK in microglia cells, demonstrating that the mode of MAPK pathway activation is neuron type dependent [254]. Active p38-MAPK can phosphorylate p53, facilitating its nuclear translocation and inducing the transcription of *BAX* and *PUMA*. Alternatively, MPTP-induced p38-MAPK activation can initiate p53 transcription by mediating NF-kB nuclear translocation [255]. In both cases, p38 inhibition prevented p53 and NF-kB nuclear activation, indicating the role of p38 in pro-apoptotic signal activation in damaged midbrain DAergic neurons.

The activation of p53-dependent pathways is a persistent phenomenon in genetic/neurotoxin PD animal models, as well as in PD human brain samples. A53T mutant α-Syn transgenic mice present p53 accumulation in the OMM of mitochondria from spinal motor neurons, suggesting that the neuronal cell death observed in this model involves p53 activation [256]. Furthermore, the application of 6-OHDA to p53 upregulated modulator of apoptosis (PUMA) and p53 knockout mice reveals DNA damage accumulation accompanied by p53 activation, triggering PUMA-mediated cell death, thus suggesting that 6-OHDA-generated ROS upregulates p53 expression, which is necessary for activating DNA damage/response pathways [257]. Interestingly, the PQ/fungicide maneb model proposes that S-nitrosylation of Parkin regulates p53-mediated neuronal death. In this model, Parkin plays a neuroprotective role by repressing p53 gene transcription under physiological conditions [258]. Upon nitrosative stress, Parkin becomes nitrosylated and loses its capacity to regulate p53 activity, resulting in constitutive p53 gene activation and subsequent p53-mediated cell death. All these models correlate with PD human brain sample evidence, demonstrating increased p53 protein levels accompanied by neuronal loss [258]. Therefore, a combination of neurotoxin and genetic PD models is a fundamental approach to elucidate how the mechanism of neuronal cell death relates to the clinical symptoms as the disease progresses.

5.6 Conclusions: Challenges and Future Perspectives

Cumulative evidence suggests that OS is one of the major events involved in PD pathogenesis. Dyshomeostasis of both production and salvage of ROS, naturally generated endogenously as a by-product of mitochondrial respiration/other cellular processes as well as multiple exogenous insults including prooxidant metal toxicity, has been consistently implicated in both sporadic and familial parkinsonism phenotypes. The key pathological markers in PD such as α-Syn/LBs, mitochondrial dysfunction, disruption in DA metabolism, and its salvage pathway all lead to disruption in maintenance of ROS/metal ion homeostasis to variable extents. The abnormally processed non-sequestered ions (ROS/metal salts) in turn make the cross talk with α-Syn/LBs, DA metabolism, and mitochondria in the affected neurons, further exasperating the pathogenesis. Thus, sporadic PD appears to be primarily associated with ROS/metal ion-mediated mitochondrial toxicity, regardless of the initial cause or trigger, under meaning of which is still obscure. This raises the question as to why ROS-/ion-targeted interventions have not been adequately successful although effective transiently in animal models. As discussed comprehensively in this article, further understanding of ROS/metal toxicity at molecular and cellular sub-organelle level is required to revisit/explore ion-targeted mechanism-based therapeutic approaches. The critical gaps that need to be addressed include (a) characterization of the impact of ROS/specific metal overload of overall ion homeostasis in various cellular compartments, (b) concerted/global impact of ion-mediated toxicity in distinct cellular organelles—nucleus vs. mitochondria, (c) ion-mediated oxidative modifications in enzymes and their reversibility, and (d) mechanism of activation of

specific antioxidant machinery/pathway in response to ROS- or ion-mediated toxicity in order to develop antioxidant-targeted therapy. Addressing these pertinent challenges concerning ROS/metal ion toxicity in neurons would not only provide avenues to improve our ability to comprehensively target ion-mediated pathology in PD but also in other neurodegenerative diseases.

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Chapter 6
Toxin-Mediated Complex I Inhibition and Parkinson's Disease

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6.1 Introduction

Understanding the relationship between the molecular pathology of Parkinson's disease (PD) and mitochondrial dysfunction has driven significant momenta in understanding the etiology of the disease. The consequences of disrupted mitochondria function are manifold; however, the ability of chemical toxins to produce parkinsonian symptoms indicates a central role for complex I inhibition in PD. As the largest and most complicated of the electron transport chain protein complexes, complex I is vulnerable to endogenous stress as well as a number of exogenous toxins, including the prototypical parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone. Loss of complex I activity via direct inhibition by toxins mimics several pathological features that are present in the brain and peripheral tissue of PD patients, providing evidence that reduced complex I function may be a global phenomenon in the pathophysiology of PD etiopathogenesis. In addition, dopamine neurons appear selectively vulnerable to complex I inhibition and the sequelae of oxidative damage that occurs therein. Here, we discuss the intersection between complex I inhibition, pathologies induced by the resulting oxidative stress and energy dysfunction, and toxin-induced parkinsonism as an environmental manifestation as well as a model for PD research.

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6.2 Complex I

Complex I (also referred to as NADH: ubiquinone/CoQ10 oxidoreductase) is a multimeric protein group responsible for catalyzing electron transfer from NADH to coenzyme Q10 (CoQ10), a redox reaction involving the translocation of four protons across the inner mitochondrial membrane [1–4]. As NADH binds complex I, two electrons are transferred via iron-sulfur (Fe-S) clusters to the accepting CoQ10 molecule, assisting in the formation of an electrochemical gradient, ultimately used to produce ATP. In mammals complex I is the largest of the electron transport chain (ETC) complexes and is comprised of 45 subunits with a combined mass of 1 MDa that span membrane and hydrophilic domains; seven of the subunits are encoded by mitochondrial DNA (mtDNA) and the remaining encoded by nuclear DNA [5–10]. This unique arrangement requires an elegant interplay between mtDNA and the molecular machinery of the cellular nucleus, where mtDNA relies on nuclear-encoded proteins for DNA replication, DNA repair, transcription, and translation of 13 polypeptides. Furthermore, mammalian mitochondria lack several key DNA repair pathways that work in the nucleus, including nucleotide excision repair and double-strand break repair. While mitochondria have active base excision repair that acts on a wide range of oxidative lesions, increased mitochondrial iron and the intrinsic production of reaction oxygen species [11] render mitochondrial DNA particularly sensitive to oxidative damage [12, 13].

6.2.1 Production of Reactive Oxygen Species Within Complex I

Even under healthy basal conditions, the mitochondria is widely accepted as the predominant site for ROS within a mammalian cell, with leakage of a very small proportion of electrons from the ETC directly to O_2 to produce the superoxide radical ($O_2^{\bullet-}$). The production of superoxide within complex I has been described at two sites: the NADH-oxidizing site (flavin site; I_F) and the ubiquinone-reducing site (I_Q), both of which have been linked with the etiology of Parkinson's disease [1, 3, 14]. The I_F site of superoxide production has been the most clearly detailed source of reactive oxygen species (ROS) within neuronal systems (Fig. 6.1) [5, 7, 15]. Within rat brain mitochondria, it was shown that ROS production at complex I under physiological conditions is dependent upon mitochondrial membrane potential ($\Delta\psi$) and the NAD(P)H redox state, a condition important for neurons due to wide fluctuations in cellular energy demand [14, 16]. This reaction based at the I_F site with NADH is dependent on the ratio of reduced NAD^+ to NADH, with increased levels of NADH over NAD^+ favoring superoxide production [15, 17, 18]. Production of superoxide and hydrogen peroxide at complex I and at different sites is also affected by rest or exercise conditions [19].

The second putative site of ROS production within complex I (I_Q) has been recently discussed based on the observation that maximal ROS production at the I_F

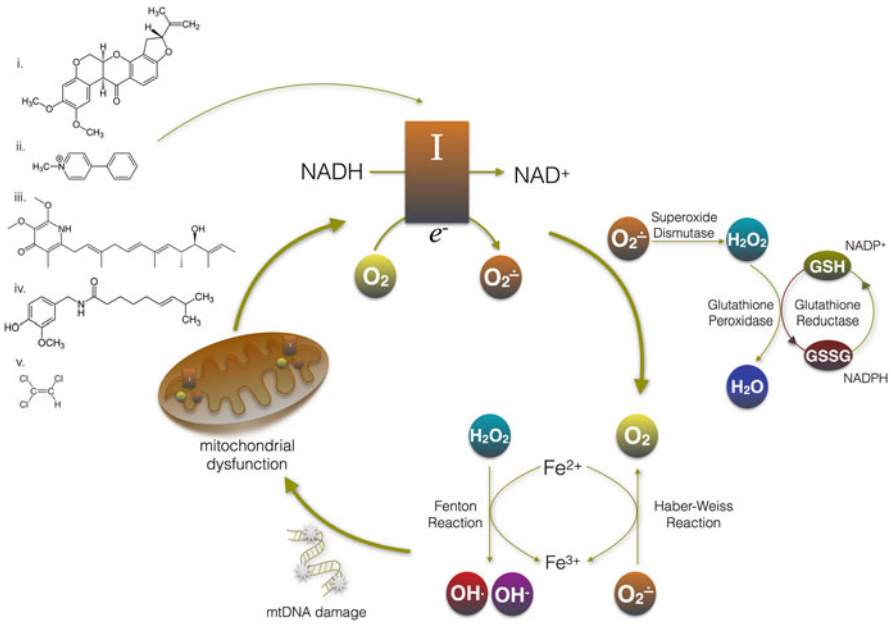

Fig. 6.1 Toxin-mediated production of superoxide causes self-propagating cycle of oxidative stress. Complex I inhibitors such as rotenone (*i*), 1-methyl-4-phenylpyridinium cation (MPP^+; *ii*), piericidins (*iii*), capsaicin (*iv*), or trichloroethylene (TCE; *v*) bind and inhibit electron flow resulting in the increased production of superoxide ($O_2^{\bullet-}$). Superoxide is detoxified via superoxide dismutase (MnSOD in the matrix and CuZnSOD in the intermembrane space and cytoplasm) to hydrogen peroxide (H_2O_2). Glutathione peroxidase breaks down H_2O_2 while oxidizing two molecules of glutathione to oxidized glutathione (GSSG). Glutathione reductase then acts to reduce GSSG to glutathione (GSH) using NADPH. High levels of superoxide may overwhelm detoxifying enzymes and in the presence of iron undergo the Fenton reaction to produce the hydroxyl radical ($OH^{\cdot}$), responsible for damaging cellular macromolecules (e.g., mtDNA) and the mitochondria. Damaged mitochondrial DNA cause loss of key mitochondrial proteins resulting in elevated levels of electron leakage as well as enhanced superoxide production

site does not fully explain the rate of superoxide generation from the available NAD^+ pool [15, 17]. In fact, superoxide is rapidly produced during reverse electron transport by the reduction of NAD^+ at site I_Q, suggesting that NAD redox state is important in the production of superoxide in the mitochondria [14, 17, 18]. Therefore, it is likely that both I_F and I_Q sites contribute to ROS generation following complex I inhibition, depending on both the amount of NAD as well as its redox state [17, 20]. The discovery of new compounds that are site-selective inhibitors of mitochondrial superoxide/H_2O_2 will now allow for the investigation of the role of mitochondrial ROS production in physiological and disease states [1, 21, 22].

Superoxide detoxification is achieved either within the mitochondrial matrix by manganese superoxide dismutase (MnSOD) or within the cytosol by copper/zinc superoxide dismutase (Cu/Zn SOD), which converts superoxide to hydrogen peroxide. Within the mitochondria hydrogen peroxide is rapidly converted to glutathione peroxidase or peroxiredoxins [14, 23]. Superoxide that is not detoxified either due

to overwhelming demand of antioxidant proteins or uncoupling of the electron transport chain may attack Fe-S centers releasing Fe that can further undergo Fenton reaction cycling to produce the highly reactive hydroxyl radical ($OH^{\cdot}$), a highly reactive species that can damage all macromolecules (Fig. 6.1).

6.3 Managing Energy Demands in Neurons

The brain is responsible for approximately 20 % of the body's oxygen consumption, however contributing to only a small percentage of mass [24, 25]. It is widely accepted that this energy demand is founded in the ATP consumption of neurons required to maintain membrane potential via Na/K ATPases, tantamount to the action potential used in neuron synapses [20, 26]. These energy requirements also determine the location of neuronal mitochondria, which are found to be favorably located within the pre- and postsynaptic terminals near sites of large ATP consumption [27–30]. Neurons derive their ATP almost entirely by mitochondrial respiration, leaving the cells highly dependent upon proper mitochondrial function. Highly specialized neurons, such as the dopamine neurons of the substantia nigra, are considered especially vulnerable to disruptions in metabolic demand, given their requirement to produce the dopamine neurotransmitter as well as maintain a resting membrane potential along the long length of their projection axons to the striatum [31]. Likewise, dopamine neurons of the substantia nigra appear to be particularly susceptible to inhibition of complex I, exhibiting a low threshold for dysfunction and cell death to the molecular blockade of complex I by exogenous and endogenous compounds [32–35]. Several toxins (discussed in detail below) with inhibitory action at complex I, readily produce dopaminergic neuron damage and elicit a parkinsonian phenotype in animal models of the disease.

6.4 Molecular Pathology of Parkinson's Disease

PD is well characterized by the chronic, progressive loss of dopamine-producing neurons of the substantia nigra and their terminal projections to the striatum. Decades of research have led to an immense wealth of information regarding the pathological mechanisms implicated in the disease; however, the etiology of idiopathic PD remains elusive, and the demand for a disease-modifying therapy grows more critical as the global aging population increases. Still, the reasons behind the selective loss of dopaminergic neurons and brain regions affected in PD remain unclear [36].

With a small minority, approximately 5–10 % of cases originating from familial inheritance of autosomal dominant or recessive mutations in known PD-related genes, a disproportionate majority of the disease is considered idiopathic. Given the wide distribution of nonfamilial or sporadic PD, gene–environment interactions that

include susceptibility genes with low penetrance in populations are thought to interact with environmental factors leading to PD [23, 37, 38]. Common pathogenic mechanisms may underlie familial and idiopathic forms of PD. For example, evidence of α-synuclein pathology within the SN, and the propagation of α-synuclein as proposed in the Braak hypothesis of Lewy pathology, is a feature common to many forms of PD at postmortem analysis [26, 39]. While it is yet unclear whether α-synuclein pathology is a cause or effect from PD pathogenesis, abundant evidence suggests that oligomerization of the protein plays at least in part a major role in the intracellular dysfunction of mitochondria and lysosomes [37, 38, 40]. Duplication or triplication of the *α-synuclein* (SNCA) gene in humans results in sufficient pathology to cause an early-onset form of PD [41] and overexpression of α-synuclein in transgenic mice, and vector-mediated expression in rats causes neuronal loss in animal models [39, 42, 43]. Conversely, the blockade of α-synuclein is protective in animal models of PD [44, 45]. Hypotheses for the role α-synuclein plays in idiopathic PD are varied (for review see [40]), and targeting α-synuclein to combat the progression of the disease is among the forefront of therapeutic strategies currently being explored [46].

Neuroinflammation, via activated microglia and reactive astrocytes in a probable response to the death of dopamine neurons, is thought to be important in the progression of PD [47, 48]. As the resident immune cells of the brain, microglia remain in an inactivated phenotype and promote neurotrophic factor production under homeostatic conditions [49, 50]. Upon stimulation by pathogen invasion or tissue damage, microglia become activated and initiate an inflammatory response to aid in repair, a process that is normally self-limiting, but under pathological conditions may become sustained if feedback inhibition fails or the inflammatory stimulus persists [48, 51, 52]. This dynamic control is a source for neurotoxic processes to occur, where uncontrolled inflammation leads to the production of cytokines (e.g., IL-1β, TNFα, IFNγ) that stimulate proinflammatory cascades in surrounding cells [50, 51, 53, 54]. As the most abundant cell type in the brain, astrocyte activation readily occurs in response to microglia reactivity, resulting in the production of, among other factors, inducible nitric oxide synthase (iNOS) producing nitric oxide (NO) that directly damages neuronal proteins and other macromolecules [52, 53, 55]. Evidence of protein nitration within dopamine neurons has been observed in both human cases of PD as well as animal models, including the complex I protein [56] and tyrosine residues of the enzyme responsible for the rate-limiting step in producing dopamine, tyrosine hydroxylase (TH) [55, 57, 58]. Both reactive types of glial cells are capable of inducing damage to neurons in the SN, as seen in cases where bacterial lipopolysaccharide (LPS) activation of gliosis causes significant loss of dopamine neurons [59, 60]. Microglia are a prominent source of ROS within the brain, as their immune function requires NADPH oxidase, used in the oxidative burst process to eliminate pathogens through superoxide production [52, 61]. It has also been shown that α-synuclein phagocytosed by microglia can directly activate NADPH oxidase and lead to an increased production of ROS [62].

Mitochondrial dysfunction, whether as a direct cause of complex I inhibition or consequence thereof, may also play a key role in the pathogenesis of PD.

Mitochondrial impairment appears to be relevant for both familial and sporadic PD [24, 63–65], and there is strong evidence for oxidative stress in human PD and particular animal models (reviewed [11]). While it has been demonstrated that one source of ROS is the NADPH oxidase production within microglia, the major contributor to ROS production in dopamine neurons is largely considered to be the mitochondria [5, 63, 66, 67]. While defects in the individual components that comprise the ETC (complexes I, II, III, IV, and V) have each been implicated in mitochondrial diseases (for review see [68]), complex I inhibition has been intrinsically tied to PD for decades [63, 69].

6.4.1 Evidence for Complex I Inhibition in PD

Complex I activity in human PD patients was first investigated by Schapira and colleagues [70] who studied the respiratory chain activity of the mitochondria in the SN of nine PD patients. They reported a specific complex I defect in these individuals and also indicated that other complexes of the ETC appeared to be unaffected [70]. Follow-up studies confirmed this initial discovery, observing specific complex I deficiencies in the SN of PD patients relative to controls [63, 71]; however, complex I deficiency has not been observed in other neurodegenerative disorders that involve the SN [72]. Although the studies by Schapira et al. [63] did not specifically elucidate differences in complex I subunit expression, a later study using an immunohistochemical analysis revealed decreased levels of complex I components in the SN of PD brain tissue [73]. Interestingly, a decreased number of subunits in complex I in the striatum of PD patients has also been reported. This does not appear to correlate with a decrease in complex I activity, and it was not distinguished whether this decrease correlated with nuclear versus mitochondrial-encoded subunits [74]. In addition, purified mitochondria from the frontal cortex of PD patients showed lowered complex I activity than age-matched controls, a phenomenon which may be due to oxidation of complex I subunits and abnormalities in assembly of mitochondria in PD cortex [75, 76]. None of these studies, however, have differentiated between synaptic and somatic mitochondria, which is significant as the source may have differing effects on ATP depletion [77]. Furthermore, whole tissue homogenates cannot distinguish between discrete neuronal populations and may not accurately represent dopaminergic neurons.

6.4.2 Complex I in the Periphery

There are several key observations that complex I deficiency in humans is not restricted to the brain in PD patients but may be observed as a systemic defect and is measurable in peripheral tissues. Human platelets have been a natural choice for PD research due to the ease of availability and aminergic neuron similarities, and

several reports of complex I deficiency from either crude or isolated mitochondria have been observed from PD-derived platelets [78–81]. It should be noted, however, that not all groups have successfully replicated these findings, and while some reports indicate a specific complex I inhibition in human platelets [78, 80], others have found impairments of the respiratory complexes (II–V) in addition to complex I. More recently there is controversy regarding the use of appropriate community-matched controls that may mitigate any findings of complex I dysfunction [27]. In addition to platelets, studies investigating muscle tissue and lymphocytes from PD patients [82, 83] have observed a measurable decrease in mitochondrial complex I activity. While these results indicate that PD may involve a system-wide decrease in complex I activity, it should be noted that these data are still controversial.

Overall less is known about complex I abnormalities from peripheral tissues in familial PD patients. Mutations in the PARK2 gene *parkin*, which are recessively inherited, are responsible for the majority of early-onset parkinsonism [84]. Age-matched healthy control fibroblasts compared to those that harbored either a homozygous or compound heterozygous *parkin* mutation demonstrated significant lower complex I activity; complex II, III, and IV activity were similar in the *parkin*-mutant patients and controls [85, 86]. These results were confirmed in a subsequent study from a different group that found *parkin*-mutant fibroblasts had lower mitochondrial complex I activity relative to healthy controls [87]. Mutations in *PINK1* (PTEN-induced kinase 1) lead to a rare autosomal recessively inherited form of PD [88]. Fibroblasts from patients homozygous for the G309D-PINK1 mutation showed a significant decrease in complex I (but not complex III or IV) activity, but differences were not detectable with heterozygous carriers [89]. In addition, no differences in complex I activity were detected in fibroblasts from a patient carrying the homozygous W437X nonsense mutation in the *PINK1* gene compared to healthy fibroblasts [90]. It is unclear whether complex I deficiency may be limited to a subset of *PINK1* mutations. Fibroblasts from a patient with a triplication in the *SNCA* gene showed a dramatic reduction in complex I activity [91]. In contrast, a specific decrease in respiratory chain complex IV but not complex I activity was found in fibroblasts derived from PD patients that carry the LRRK2 G2019S mutation [92]. To date, fibroblasts from human patients that carry mutations in DJ-1 have not been investigated for complex I activity. Collectively these studies suggest that complex I is inhibited in genetic forms of PD, but future studies should include additional peripheral tissues to complement the data in fibroblasts.

In summary, there is solid evidence for a complex I defect in the mitochondria of the SN as well as the cortex of sporadic PD brains. While the systemic defect is highly variable, there appears to be a mild complex I defect in at least some cases in platelets, muscle, and lymphocytes, derived from PD patients that may play an important role in PD pathogenesis. The inconsistencies from study to study may be due to several reasons. First, there may be methodological differences in tissue sample selection. When measuring complex I activity, use of crude or isolated mitochondria could perhaps be a key variable. Additionally, many of the studies do not distinguish between familial or sporadic PD nor do they indicate treatment status in the reported data. Lastly, the stage of the disease—either early or late—may certainly

influence which brain regions are affected and the extent of the complex I deficiency. Overall, complex I deficiency in the brain and periphery and its relevance to PD pathogenesis and etiology need to be further investigated.

6.5 Toxin-Mediated Complex I Inhibition and Modeling PD

There are several mitochondrial toxins that are capable of reproducing at least in part the cellular and molecular pathology observed in human PD. Further still, many of these neurotoxins appear in their action to only exert complex I inhibition, a significance that has yet to be fully explained in the context of environmental exposures, genetic polymorphisms, and the susceptibility of the dopaminergic system. Here, we discuss exogenous toxins that specifically target complex I and their use in reproducing key features of PD, as well the risk they pose as etiological factors in the pathogenesis of the disease.

6.5.1 MPTP

The neurotoxin MPTP was inadvertently discovered as a major impurity from the synthesis of the opioid desmethylprodine (MPPP). The now infamous use of MPPP contaminated with MPTP by six individuals in the 1980s resulted in the discovery of MPTP as a potent and selective dopamine neuron toxin. Neurologist William Langston characterized the sudden onset of parkinsonism in the group of young adults who exhibited severe dopamine depletion from their nigrostriatal system [93]. This unique discovery has led to the widespread use of MPTP as a neurotoxin to produce a Parkinson-like syndrome in rodent and primate models of disease [35, 94].

MPTP readily crosses the blood–brain barrier where it is metabolized predominantly in the astrocyte by monoamine oxidase B (MAO-B) to 1-methyl-4-phenyl-1-2,3-dihydropyridinium ($MPDP^+$). It is postulated that spontaneous auto-oxidation causes further metabolism of MPDP to 1-methyl-4-phenylpyridinium (MPP^+), the final and most toxic metabolite. Due to its charge, MPP^+ requires active transportation across the membrane space with specific affinity for the dopamine transporter (DAT) [58]. Within the dopamine neuron, MPP^+ has been shown to interact with the vesicular monamine transporter (VMAT), which will uptake MPP^+ into synaptic vesicles, a process that helps mitigate its toxicity. Conversely, MPP^+ is most commonly linked to its accumulation within the mitochondria, where it reportedly binds and inhibits complex I at the same site as rotenone [58, 95]. The uncoupling of NADH–ubiquinone by MPP^+ leads to decreased ATP production as well as ROS generation within the mitochondria and cytoplasm [47, 58]. The resulting gross pathology is the precise and robust degeneration of the nigrostriatal system leading to motor deficits in neurobehavior [58, 96].

There is approximately a 20 % decrease in ATP levels from mouse midbrain and striatum following MPTP exposure [58]. Similarly, the leakage of superoxide from the electron transport chain caused by MPP^+ inhibition at complex I may have a synergistic effect with the production of NO by nitric oxide synthase (NOS) leading to protein damage and dysfunction from peroxynitrite ($ONOO^-$) adducts [58, 97]. In addition, DNA damage likely contributes to MPTP toxicity by forming adducts with nucleotides, as has been observed in postmortem PD midbrain tissue [98–100]. Not surprisingly, DNA damage following MPTP exposure does not appear to be limited to the nucleus; young and old mice (22 days and 12 months, respectively) treated with MPTP reported an age-dependent increase in mitochondrial DNA (mtDNA) damage within the SN [98]. These data also indicated that 12-month-old mice displayed an increase in mtDNA damage within the caudate–putamen and cerebellum; however, these lesions were not observed at the same rate in 22-day-old mice, suggesting that aged mice are more vulnerable to mtDNA damage produced by MPTP than their younger counterparts [98].

The molecular damage caused by MPTP in animal models of PD has revealed a broad spectrum of cellular pathologies. Readily apparent among these in brain tissue is the activation of glial cells to promote neuroinflammation. Following MPTP exposure in mouse and nonhuman primate models, a robust activation of microglia has been reported in the SN and ST, resulting in an increase in number of microglia within the ventral midbrain region [101]. In addition, it has been shown that MPTP administration also drives changes in microglial phenotype from a quiescent, resting state to an activated form associated with the increased expression of proinflammatory cytokines and chemokines [102, 103]. These results are consistent with the observed increased number of activated microglia and elevated cytokine levels in postmortem PD tissue, which would suggest that complex I inhibition by MPTP produces a similar neuroinflammatory response [101]. Microglial activation appears to be in response to neurotoxicity since direct activation of microglia by MPTP is ambiguous as microglia lack the MAO-B enzyme required for bioactivation [47]. This does not imply that microglial activation in MPTP models is not detrimental; indeed several studies indicate that microglia and astrocyte activation perpetuates dopamine neuron damage in chronic and low-dose scheduling, often remaining activated long after the cessation of MPTP treatment [104, 105]. In addition, treatments aimed at reducing the activation of microglia in MPTP models have shown to be neuroprotective [106–109], including the specific inhibition of NADPH oxidase [61].

The explicit inhibition of complex I in dopamine neurons by MPTP is an invaluable tool for modeling PD in mice and nonhuman primates, reproducing many features of the disease and providing both acute and chronic timelines by which therapies may be tested. Although nonhuman primate MPTP models have been shown to reproduce a similar Parkinsonian phenotype [94] 2, the merits of MPTP use in mice has been meticulously debated [35, 104, 110, 111] and is an important caveat within PD research, though at present, is outside the scope of this review. In addition, given its limited exposure in humans and synthetic nature, MPTP exposure represents a very restricted risk factor for the development of sporadic PD and instead remains a model for investigating the intrinsic link between complex I inhibition and dopamine neuron death.

6.5.2 Rotenone

The rotenoid family of chemical compounds is produced from the roots of the Fabaceae plant family, as well as the jicama plant, and is considered an organic broad-spectrum pesticide [112]. Rotenone is used primarily to control invasive fish populations in lakes; however, its use as a general pesticide has been phased out over the last decade, with limited use in the USA and removal from the EU market in 2007 [113]. Rotenone binds complex I at the ubiquinone site and inhibits electron transfer from NADH to ubiquinone, inhibiting mitochondrial respiration [114]. The inhibition of complex I induced by the presence of rotenone also causes electron leakage, driving the production of superoxide and oxidative stress [115]. Because of its lipophilic properties, rotenone readily crosses biological membranes, including the blood–brain barrier, without the need for a transporter and therefore functions as a system-wide inhibitor of complex I [116]. Despite its ability to produce systemic complex I inhibition, rotenone has shown selective toxicity for dopamine neurons and is able to recapitulate a parkinsonian phenotype in animal models of PD [117, 118].

The use of rotenone as a complex I inhibitor in PD models has greatly expanded the understanding of mitochondrial dysfunction within dopamine neurons. It is implicated that rotenone alters respiration in isolated rat brain mitochondria and induces toxicity through the resulting oxidative stress from complex I inhibition [119]. Not surprisingly, rotenone treatment yields abundant oxidative damage to cellular macromolecules including proteins, lipids, and DNA [11]. Indeed, treatment with antioxidants both in vitro and in vivo appears to mitigate the selective damage of rotenone to neurons, where antioxidant α-tocopherol protects dopamine neurons in midbrain slice cultures from oxidative damage, and conversely the depletion of glutathione enhances cellular toxicity [120]. In particular, mtDNA damage forms in the nature of abasic (apurinic or apyrimidinic) sites within dopamine neurons of the SN following rotenone treatment in rats, with specificity for the SN but not the cortex [121]. Moreover, consistent with the aforementioned findings, the accumulation of abasic sites were found in the SN from human PD tissue, indicating that mtDNA damage within the ventral midbrain is a pathology associated with PD etiology and/or complex I inhibition [121].

The inhibition of complex I by rotenone may also invoke Ca^{2+}-mediated cellular toxicity within dopamine neurons mediated through the NMDA receptor. Dopamine neurons maintain constant activation of the NMDA glutamate receptor, resulting in a high ATP requirement and the possibility for excitotoxicity [122, 123]. Such activity at the NMDA receptor leaves the dopamine neuron acutely sensitive to Ca^{2+} influx, and accumulation within the mitochondria leads to cytotoxicity [124]. Rotenone is able to dramatically increase the influx of Ca^{2+} via the NMDA receptor, indicating that when cellular energy is depleted by complex I inhibition, dysregulation of NMDA channels may lead to excitotoxicity [125, 126]. These data suggest that rotenone-induced toxicity within the dopamine neuron may have convergent mechanisms upon complex I inhibition that include both the production of ROS, as well as ATP depletion and subsequent calcium release and excitotoxicity.

Similar to MPTP, rotenone toxicity appears to include a neuroinflammatory component, including the activation of microglia and increase in proinflammatory cascades that lead to further dopamine neuron toxicity [127–129]. Unlike MPTP, however, rotenone does not require specific transport across membrane and therefore has the ability to inhibit complex I in non-neuronal cells, including the glia. Microglia may become directly activated following rotenone exposure, with evidence for both NF-kB and p38 MAPK pathway involvement leading to the upregulation of cytokines which include IL-1β and TNFα production [127, 129]. In addition, microglia have been reported to increase the sensitivity of dopamine neurons to rotenone in culture in a linear fashion, i.e., increasing microglial concentrations correlates with midbrain neuron toxicity [130]. These same studies indicated that 1 nM rotenone causes microglial activation prior to neuron death, with an increased amount of OX-42 positive microglia (activated) visible within neuron–glia cultures [130]. Given the ability for activated microglia to release cytokines with neurotoxic impact, it is not surprising that microglia would potentiate a synergistic toxicity of rotenone on neurons. Rotenone also causes microglial NADPH oxidase-mediated production of superoxide, increasing the amount of measurable oxidative stress in neuron–glia cultures when compared to neuronal cultures alone [130].

In addition to oxidative damage in the rotenone model of PD, α-synuclein accumulates in the SN in vivo; a phenotype rarely replicated in other complex I models of PD [117, 131]. Similar findings have been observed in vitro; rotenone treatment in a neuroblastoma cell line promotes α-synuclein aggregation [131]. Conversely, treatment with α-tocopherol mitigates the rotenone-induced accumulation of α-synuclein in neuroblastoma cells, indicating that the oxidative stress produced by complex I inhibition is tantamount to protein aggregation [132, 133]. It has been hypothesized that oxidative stress as a result of complex I inhibition may biochemically alter the α-synuclein protein and thus lead to its accumulation. The α-synuclein protein contains four tyrosine residues that serve as putative sites for protein nitration by peroxynitrite, the reactive nitrogen species produced by superoxide output from complex I inhibition in mitochondria as well as microglial-induced NADPH oxidase [51, 131, 134]. It has been shown that tyrosine nitration (Tyr-39) leads to elevated levels of α-synuclein oligomerization, a process that favors protein aggregation given that nitrated α-synuclein monomers and dimers accelerate the rate of fibril formation [134, 135]. In addition to nitration, dopamine modification of α-synuclein has been detected in brain and cultured cells following rotenone treatment but is not observed in non-dopaminergic cells [131, 135]. These data suggest that dopamine neurons of the SN are more vulnerable to α-synuclein toxicity due to dopamine modification of the protein. The oxidative deamination product of dopamine metabolism, 3,4-dihydroxyphenylacetaldehyde (DOPAL), was recently shown to cause dopaminergic neuron toxicity in the SN following injection [135, 136]. This degeneration within the SN occurred in conjunction with increased α-synuclein oligomers, a mechanism likely triggered by DOPAL-induced oxidation of methionine residues within the protein [135]. This relationship between an enriched environment of ROS and α-synuclein toxicity presents a partial mechanism for the unique susceptibility of dopamine neuron degeneration following complex I inhi-

bition. While the correlative data between these events is increasing, it will be pivotal to understand the molecular mechanisms underlying α-synuclein and ROS interaction.

PD prevalence is reported to be increased in individuals living in rural, agricultural locations, suggesting that pesticide exposure is a risk factor in the development of mitochondrial complex I dysfunction [23, 136, 137]. Recent analysis using rigorous case–control data over several decades of reported PD cases has revealed that pesticides which inhibit mitochondrial complex I, including rotenone, are correlated with an increased risk for developing the disease [137–139]. For example, those with environmental exposures to rotenone exhibited a 2.5-fold increase (95 % CI, 1.3–4.7) in developing PD than age-matched controls [137]. Of note, the mitochondrial toxin paraquat, which exhibits toxicity through the redox cycling that ultimately inhibits complex I, shows a similar increase in the risk of developing PD with a 2.5-fold increase (95 % CI, 1.4–4.7) [137]. Certainly no single causative agent has been reported to account for a large number of PD cases, and the intersection between genetic susceptibility and environmental exposures likely play a pivotal role in the etiology of the disease [23]. Likewise, the measurement of population variants in gene expression that are known to contribute to ROS toxicity in the dopamine neuron, such as α-synuclein, may successfully determine the predilection of PD development following exposure to complex I inhibitors.

6.5.3 Piericidins, Acetogenins, and Other Naturally Occurring Complex I Inhibitors

Several non-synthetic compounds found in the environment have been described to inhibit complex I, resulting in varying degrees of mitochondrial disease sequelae in human exposures. One particularly interesting case is the appearance of a large frequency of parkinsonism among a population on the island of Guadeloupe in the French West Indies [140, 141]. Given this atypical distribution of a Parkinson-like disease, an investigation into a probable exposure to an environmental toxin was undertaken. Using case–control studies, it was identified that the leaves of *Annona muricata*, which contain alkaloid toxins, were regularly consumed by the native population as part of herbal medicine [140]. Among these toxins are annonaceous acetogenins, a series of lipophilic compounds that exhibit a potent inhibition of complex I [142]. Indeed the major toxin within acetogenins, annonacin, has been shown to decrease ATP levels in the rat brain, leading to neurodegeneration, as well as induce glial activation in the basal ganglia [143, 144].

Plant derivatives of the vanilloids are another source for complex I inhibition and are perhaps the most commonly encountered of mitochondrial toxins [113]. Capsaicin, extracted from hot peppers, is a substance used in the food additive industry as well as treatment for neuropathic pain. Capsaicin has recently been shown to alter mitochondrial dynamics in sensory axons, inducing axonal degeneration;

however, the compound is a comparably weak complex I inhibitor, with reported complex IV inhibition as well [145, 146]. Despite this evidence, use of capsaicin as an analgesic remains, and an intrinsic link with the degeneration of dopamine neurons associated with PD has not been found.

Naturally occurring complex I inhibitors are not limited to plant extracts; the most potent inhibitor of complex I based on lipophilicity and decrease in ATP concentration are piericidins, a group of compounds produced by the bacterium strain *Streptomyces* [147]. Piericidins have been reported to induce neuronal death in models of sporadic tau pathology, with high toxicity attributable to its highly lipophilic nature and accumulation within the mitochondrial inner matrix [113, 147]. *Streptomyces* are among the most common bacteria found in soil; however, an association has yet to be made between exposure to naturally derived piericidins and the risk of developing PD and related neurodegenerative disorders.

In addition to *Streptomyces*, myxobacteria are also a source for soluble complex I inhibitors, producing the compounds myxothiazol and stigmatellin [113]. Unlike rotenone and other naturally available complex I inhibitors, the myxobacterial toxins do not appear to be specific for complex I but rather inhibit the cytochrome bc_1 site as well [148, 149]. In addition, myxobacteria are the source of several antibiotics with reported complex I inhibition, some with potency well above that of rotenone [113].

It is clear that many naturally occurring complex I inhibitors are present in the environment; less distinct is the risk factor that each of these compounds pose in contributing to the development PD or other neurodegenerative diseases. Exposures to a single toxin capable of producing a parkinsonian syndrome, such as the case with piericidins in the Guadeloupe population, are rare. It is possible, however, that unknown complex I inhibitors present in the environment may play a larger role in the pathogenesis of neuronal degeneration, a troubling idea as the list of toxins continues to expand (for review, see Hollerhage et al. [113]).

6.5.4 Occupational Exposures to Mitochondrial Toxins

Occupational exposures to chemical compounds exhibiting mitochondrial toxicity have been linked to dopaminergic degeneration in several instances, some of which act directly as complex I inhibitors [150]. One example of this is the documented industrial exposure of individuals to the solvent trichloroethylene (TCE) used in the degreasing step of a manufacturing process [151]. Based on the reports by Gash et al., 2008, three of the employees at a single manufacturing plant had developed PD as assessed by clinical criteria, and 14 others displayed symptoms of reduced motor abilities compared to age-matched controls. In addition, oral administration of TCE to Fisher 344 rats resulted in the degeneration of the nigrostriatal tract, with evidence of decreased complex I activity [152]. Accompanying the loss of dopamine neurons was evidence of oxidative stress in protein nitration (3-nitrotyrosine adducts), increased levels of protein carbonyls and 4-hydroxynonenal (4-HNE)

staining [152]. Further, neuroinflammation following TCE exposure with activated microglia and α-synuclein inclusions within the substantia nigra and the dorsal motor nucleus of vagus was observed [152].

The primary metabolism of TCE by cytochrome P450 (CYP2EI) leads to the formation of a highly reactive aldehyde, chloral, and subsequent production of 1-tr ichloromethyl-1,2,3,4-tetrahydro-*b*-carboline (TaClo) [153, 154]. Of note, TaClo is structurally similar to MPTP and has been shown to elicit complex I inhibition in rat brain homogenate with greater potency than MPP^+ [154, 155]. Given the extensive use and large quantities of the chemical reportedly released into the environment from industrial sources, including its prior use as a dry cleaning agent [156], TCE represents a considerable risk factor for environmental exposure to a complex I inhibitor. Indeed, it has been reported that TCE is released via vapor at degreasing operations at industrial plants, is detected in groundwater near disposal areas, and is commonly found in Superfund sites [150]. Twin studies investigating lifetime exposures to solvents have indicated that there is a significant increase in the risk of developing PD with exposure to TCE (odds ratio 6.1; 95 % CI) [157]. Polymorphisms for toxic metabolite production may be key to understanding the distribution of PD within certain exposure groups, as may be the case with TCE.

6.6 Conclusions

It is clear that complex I inhibition plays a key role in the etiopathogenesis of PD, whether from intrinsic dysfunction of the mitochondria or from exposure to an exogenous compound. The resulting mitochondrial dysfunction and oxidative damage appear to exert specific toxicity to dopamine neurons within the SN, a consequence that is multifactorial. First, dopamine neurons appear to be selectively vulnerable to disruptions in mitochondrial energy production and the high level of ROS generated by inhibition of complex I. In addition, the ventral midbrain contains a high number of microglia and neuroinflammatory conditions within the SN in both human postmortem tissue as well as in models using complex I inhibitors. Microglia have been shown to be directly activated by rotenone and may act in tandem with the complex I inhibitor generate damaging oxygen radicals that enhance dopamine neuron toxicity [128–130]. Furthermore, Lewy body pathologies associated with PD have been replicated in vivo and in vitro following complex I inhibition by rotenone, implying that oxidative damage produced within the dopamine neuron lays the framework for α-synuclein aggregation (illustrated in Fig. 6.2).

The overt toxicity within the SN demonstrated by complex I inhibition is coupled with distinct molecular changes; for example, mtDNA damage has been observed following both MPTP and rotenone models of PD, as well as in the SN of postmortem tissue, and may further impair the replication and assembly of complex I, contributing to a viscous cycle of ROS-mediated mitochondrial dysfunction (Fig. 6.1)

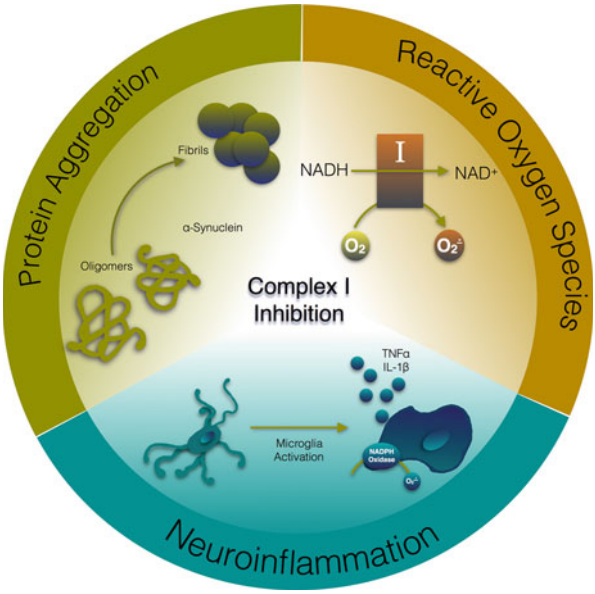

Fig. 6.2 Neurotoxic pathology of complex I inhibitors. Increased levels of reactive oxygen species within dopamine neurons occur following complex I toxin exposure, causing damage to cellular DNA and proteins. Damage from neuronal oxidative stress is mitigated by microglia, which become activated and may produce additional oxidative damage via NADPH oxidase and the release of proinflammatory cytokines such as interleukin (Il)-1β and tumor necrosis factor (TNF) α. Increased oxidative stress and the milieu of inflammatory protein release are associated with α-synuclein oligomerization and fibril formation, the toxic basis for Lewy pathology observed within dopamine neurons of the SN and other brain nuclei affected in PD

[98, 121, 158]. Such findings suggest that mtDNA damage may be a viable marker for complex I inhibition either from exposure to mitochondrial poisons (and other complex I inhibitors) or in cases of PD. Indeed, mtDNA damage can be detected in blood and skeletal muscle of rats following rotenone injection [33], suggesting that mtDNA damage from peripheral tissue may serve as an indicator to occupational or environmental exposure to complex I inhibitors, providing a benchmark for risk assessment and/or a feasible biomarker to assess PD progression and/or treatment.

It is remarkably challenging to estimate the effect that environmental exposures have on complex I and the development of sporadic PD. Recent epidemiological studies with rigorous case–control parameters have confirmed that an increased odds ratio exists for several of the solvent and pesticide complex I inhibitors [157, 159]. Genetic polymorphisms with low penetrance in the population likely increase individual susceptibility to complex I inhibitor exposure, as exemplified by the bioactivation of TCE to its ultimate toxicant TaClo [153]. Further investigation into gene–environment interactions will be pivotal to understanding the role that environmental exposure to complex I inhibitors plays in the development of sporadic PD.

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Chapter 7
Parkinson's Disease-Associated Mutations Affect Mitochondrial Function

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7.1 Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects 1 % of the population over 65 years of age. The motor symptoms of PD include tremor, bradykinesia, and impaired gait and balance [1]. Neuropathologically, PD is mainly characterized by the loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc), the subsequent depletion of DA levels in the striatum, and the presence of α-synuclein (α-Syn) protein aggregates (Lewy bodies (LB)) in affected brain regions [2, 3]. In addition, a variety of non-motor symptoms are observed, including neuropsychiatric symptoms (cognitive impairment, depression), pain, autonomic nervous system dysfunction, constipation, and sleep disturbance [4]. Although a large majority of PD cases are idiopathic, autosomal dominant and recessive familial forms have been identified. The exact etiology of most PD cases still remains unclear, and the detailed mechanisms that cause disease progression remain unknown. Alterations in numerous cellular processes have been implicated, including oxidative stress [5], the ubiquitin–proteasome system [6], the endolysosomal compartment [7], and calcium buffering of SNc neurons [8], all of which may come together to affect mitochondrial function.

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Many studies have described a mitochondrial complex 1 deficiency in the SNc of PD patients [9–12]. Moreover, we know that toxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or rotenone block respiratory chain complex 1 function and induce SNc degeneration. Additionally, many genes that have been linked to familial PD are increasingly being shown to affect mitochondrial function. Mitochondria are organelles that constitute the major source of adenosine triphosphate (ATP) production, the chemical energy of the cell. Mitochondrial dysfunction is mainly characterized by an increase in reactive oxygen species (ROS) levels, a decrease in mitochondrial respiratory transport chain enzyme activity, cytochrome *c* release, ATP depletion, and caspase 3 activation.

The first evidence linking mitochondrial dysfunction to PD arose in the late 1970s when unintentional exposure to MPTP, a derivate from the synthesis of 1-methyl-4-phenyl-4-propionoxy-piperidine, was found to cause parkinsonism and selective degeneration of DA-producing cells [13]. The mechanism by which MPTP crosses the blood–brain barrier and is oxidized to 1-methyl-4-phenylpyridinium (MPP^+) is well known [14]. MPP^+ accumulates in the mitochondria where it inhibits complex I in the mitochondrial electron transport chain, disrupting the flow of electrons [15]. Other pesticides that selectively inhibit complex I, such as rotenone, also cause parkinsonism and LB-like inclusions in animal models [16]. Another large piece of evidence for mitochondrial dysfunction related to PD comes from findings that mutations in genes encoding different proteins that have strong links to mitochondrial function are associated with familial forms of the disease. Mutations in the genes encoding the proteins PINK1 (encoded by *PARK6*), parkin (encoded by *PARK2*), ATP13A2 (encoded by *PARK9*), and DJ-1 (encoded by *PARK7*) all cause autosomal recessive PD, while mutations in a number of other PD-associated proteins primarily located in the cytosol, like LRRK2 (encoded by *PARK8*) or α-Syn (encoded by *PARK1*), cause autosomal dominant PD. The convergence of all of these proteins on mitochondrial dynamics reveals a common function in the mitochondrial stress response that might provide a possible physiological basis for the pathology of PD [17] (Fig. 7.1).

7.2 PINK1 and Parkin

Homozygous loss-of-function mutations in genes encoding the E3 ubiquitin ligase parkin and the serine/threonine kinase phosphotensin-induced kinase 1 (PINK1) account for about half of all cases of autosomal recessive PD and those before 21 years of age (young onset) and are the most common. Two subsequent chapters in this book are devoted to detailed discussion of efforts directed to understanding the mechanisms by which loss of parkin and PINK1 function causes neurodegeneration; therefore, this chapter will focus on what is known about the pathology of other PD-causing gene mutations.

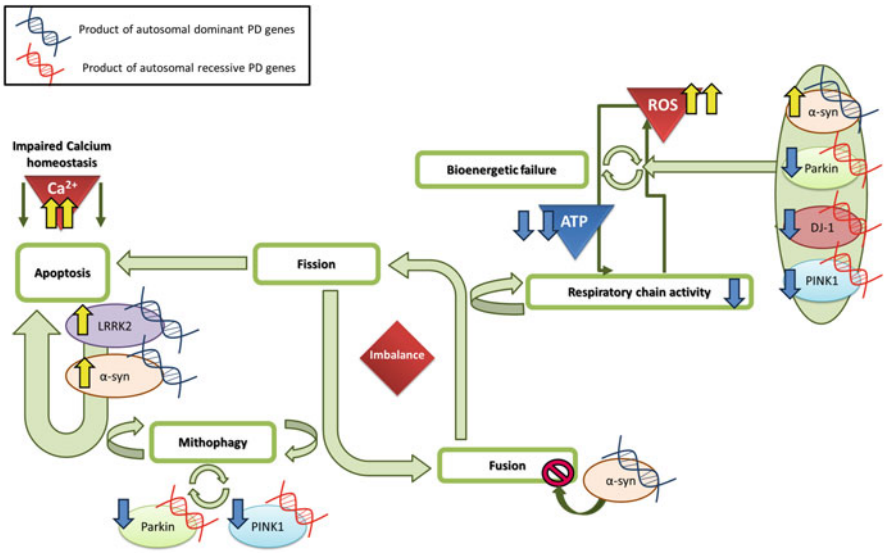

Fig. 7.1 Several PD-causing gene mutations are implicated in mitochondrial biology. Products of these genes affect an extensive range of mitochondrial processes, including bioenergetics, dynamic changes of morphology and connectivity (fusion, fission), and regulation of cell death pathways. Products of heterozygous (dominant) Parkinson's disease genes correspond to LRRK2 and α-synuclein. Products of homozygous genes correspond to DJ-1

7.3 DJ-1

DJ-1 (*PARK7*), the third gene associated with autosomal recessive PD [18], was firstly described as an oncogene [19]. DJ-1 is located in the cytoplasm, nucleus, and mitochondria in cells [20]. In normal conditions, DJ-1 can regulate the activity of complex I [21]. Under stress conditions, DJ-1 translocates to the mitochondria [22]. Briefly, DJ-1 is thought to be a neuroprotective antioxidant, since a deficiency of DJ-1 causes an increase in the level of ROS, which is believed to be due to perturbation of mitochondrial function because homeostatic ROS levels can be restored when parkin/PINK1 is expressed [23, 24]. DJ-1 may have a nonspecific antioxidant capacity in order to be able to affect the majority of ROS [25]. Signs of increased ROS can be detected in PD patients and in DJ-1 KO mice; these include a decrease in mitochondrial membrane potential, a drop in the complex I activity [10, 26], and mitochondrial fragmentation [23, 27].

When *Drosophila* genome paralogs DJ-1α and DJ-1β are eliminated, flies have decreased mitochondrial DNA, respiration, and ATP levels. This decrease in respiration is also observed in DJ-1 KO mouse embryonic fibroblasts and DA primary neurons [27]. Also, brain mitochondria from DJ-1 KO mice show increased respiration-dependent hydrogen peroxide consumption [28]. When DJ-1 is

overexpressed, it reverses the PINK1 mutant phenotypes, indicating an overlapping of PINK1 and DJ-1 functions [29] and highlighting the importance of DJ-1 in the maintenance of respiratory complex stability. Importantly, in SNc dopaminergic neurons, there is a basal mitochondrial oxidant stress [30], which is eliminated by DJ-1 [31].

It seems that in the mammalian system, DJ-1 may be particularly important for dopaminergic neurons, as DA release is affected in animals lacking DJ-1 [32, 33]. Interestingly, DJ-1 translocation to mitochondria relies on chaperones like C-terminus of HSC70-interacting protein (CHIP) and mortalin, both of which maintain mitochondrial homeostasis via the transport of nuclear-encoded proteins carrying the mitochondrial-targeting sequence, which is actually absent in DJ-1 [34]. Three amino acid exchanges in mortalin (R126W in the ATPase-binding domain and P509S and A476T in the substrate-binding domain) have been identified in PD patients [35]. In neuronal and nonneuronal human cell lines, all disease-associated mortalin variants cause increased ROS and reduced mitochondrial membrane potential, which are exacerbated upon proteolytic stress. These functional impairments correspond with characteristic alterations of the mitochondrial network in cells overexpressing mutant mortalin compared with wild-type (WT) mortalin [36].

Evidence suggests that DJ-1 may have roles other than that of an antioxidant. For instance, based on its analogy with other genes that share important characteristics, it has been suggested that it might be a chaperone or a protease, although the biology of these functions has not been described [37, 38]. Oxidization of C106 of DJ-1 triggers its translocation into the mitochondria where it has been shown to induce mitophagy [22], which seems to occur in parallel with parkin-/PINK1-mediated autophagy pathways [39]. DJ-1 C106 is particularly sensitive to oxidative modification; mutations that result in loss of oxidation here result in a failure to protect against ischemic damage, in both in vitro and in vivo studies [22, 40–43]. Oxidative modification of C106 has been proposed to allow DJ-1 to act as a sensor of cellular redox homeostasis and to participate in cytoprotective signaling pathways in the cell, although the exact mechanism by which oxidation of DJ-1 at C106 protects neurons from ROS stress is unclear [44].

7.4 SNCA

The first gene for which gain-of-function mutations were reported to cause autosomal dominant PD was *SNCA,* which encodes α-synuclein (α-Syn); disease-causing mutations include, gene duplications and triplications, and several point mutations [45, 46]. Symptomatically, patients with *SNCA* mutations usually have early-onset PD and present with dementia and cognitive deficits with rapid disease progression. The aggregation of α-Syn as a major component of LB is a histopathological hallmark of PD; LB spread through diverse regions like the SNc, locus coeruleus, hypothalamus, and cerebral cortex [47]. Alpha-Syn is located predominantly in the cytosol, but it can be localized in mitochondria [48–53]. Moreover, α-Syn phosphorylated at S129 is preferentially

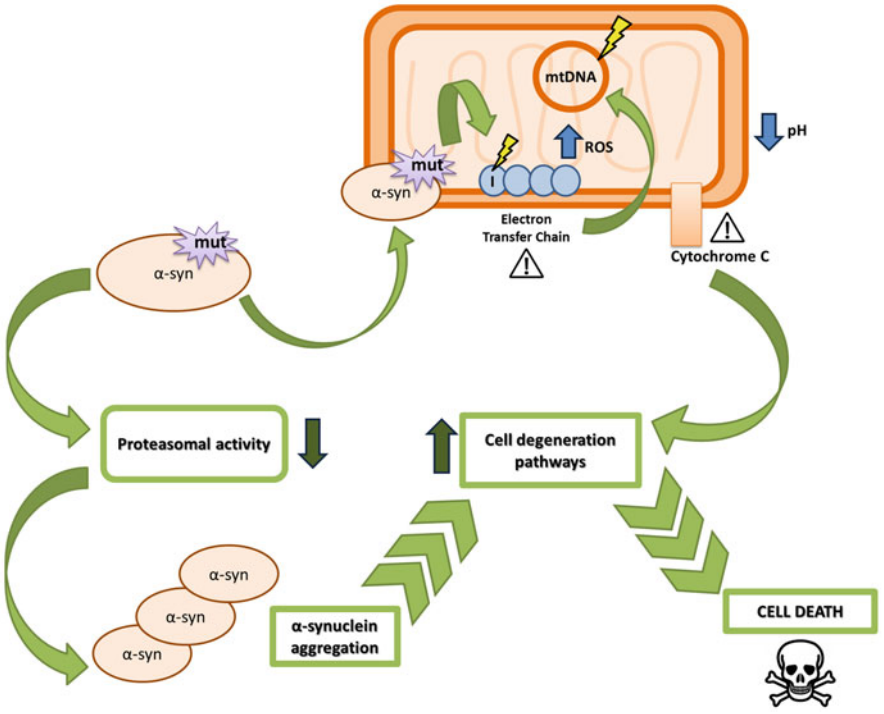

Fig. 7.2 Mutations or increased expression in the α-synuclein gene results in dysfunction of its protein product. Proteasome activity becomes compromised, leading the protein to aggregate. Mutated α-synuclein protein also causes complex I dysfunction. ATP production is consequently decreased and ROS levels increase so that mtDNA, and other local macromolecules, become oxidized, causing additional cell stress. The acidic cytosolic environment and metabolic impairment created by ROS result in the instigation of cell death mediators, such as cytochrome *c*. This process immediately activates the apoptotic pathways that finally produce the cell death

targeted to the mitochondria [54]. Different subcellular distribution patterns of α-Syn reflect the existence of various α-Syn conformations [54].

A large body of research has illustrated that mitochondria in cultured cells and in transgenic mice overexpressing WT and mutant forms of α-Syn have altered function and dynamics that are reminiscent of what has been observed in both sporadic and familial PD patients [55, 56] (Figs. 7.1 and 7.2). A common single nucleotide polymorphism (SNP) in the *SNCA* gene that is preferentially expressed in PD patients has been shown to increase α-Syn expression in vitro [57]. In mice, overexpression of WT or mutant *SNCA* leads to mitochondrial abnormalities, selective oxidation of mitochondria-associated metabolic proteins, degenerating mitochondria, and mitochondrial DNA damage [58]. Alpha-Syn accumulates in the mitochondria of PD patient's SNc and striatum, resulting in decreased complex I activity [49, 59], oxidative stress, and increased mitochondrial fragmentation [56]. Mutant α-Syn appears to disturb the transfer of calcium between ER and mitochondria [60, 61] at

mitochondria-associated ER membranes (MAMs) [62], which are subregions of the endoplasmic reticulum related to the control of mitochondrial division and dynamics [61, 63]. PD-associated α-Syn mutations result in reduced association of α-Syn with the MAM and reduced ER-mitochondrial association, concomitant with increased Drp1-independent fission [62]. Drp1 is critical for targeting mitochondria to the axon terminal, and a disruption in mitochondrial fission can contribute to the preferential death of nigrostriatal DA neurons [64]. By reestablishing the ER-mitochondria tethering, exogenous DJ-1 modulates α-Syn aggregation and improves p53 overexpression-induced impairment of mitochondrial matrix Ca^{2+} accumulation, mitochondrial morphology alteration, and reduction of ER-mitochondria contact, suggesting that α-Syn and DJ-1—and perhaps additional protein players—may be relevant not only to mitochondrial function but also to ER function [38, 65].

Alpha-Syn H50Q, A53P, A53T, E46K, and A30P are disease-causing variants that occur near the N terminus, which is essential for the regulation of mitochondrial membrane permeability and has been suggested to be a key factor in PD-associated neurodegeneration [66]. Overexpression of WT or mutant α-Syn can increase ROS levels in various cell lines [67, 68]. The H50Q variant induces a small number of pathological effects, including α-Syn oligomerization and aggregation in SH-SY5Y cells and increased mitochondrial fragmentation in hippocampal neurons [69]. Intracellular accumulation of α-Syn in the same cells following proteasomal inhibition leads to cell death and mitochondrial impairment, which can be prevented by knocking down *α-Syn* gene [70]. Expression of A53P mutant α-Syn decreases proteasome activity, increases sensitivity to mitochondria-dependent apoptosis, and induces loss of dopamine release and autophagic cell death [71, 72]. The presence of the A53T mutation results in the increased presence of fragmented mitochondria [73], as well as mtDNA damage, respiratory complex IV impairment, and increased sensitivity to MPTP and paraquat in mice [74]. Moreover, human A53T α-Syn overexpression in transgenic mice induces pervasive mitochondrial macroautophagy defects that precede DA neuron degeneration [75]. Mitochondria in neurons derived from α-Syn A53T patient-induced pluripotent cells (iPSCs) produce more ROS and reactive nitrogen species in response to stimulus compared to isogenic control lines, leading to increased oxidation and nitrosylation of the transcription factor myocyte enhancer factor 2C (MEF2C), resulting in decreased mitochondrial biogenesis via modifications of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [76].

Bacterial artificial chromosome (BAC) technology has allowed for the production of transgenic rodents with overexpression levels much closer to physiological levels. BACs contain large genomic DNA sequences that carry all the native genomic structures including promoters and regulatory elements of a gene [77]. BAC-transgenic mice expressing increased E46K α-Syn show a mild neurodegenerative phenotype and increased sensitivity to rotenone-induced cell death [78]. Using the same approach, overexpression of WT α-Syn leads to age-dependent loss of dopaminergic neurons and locomotor deficits [79].

Oligomerization or aggregation of α-Syn in response to oxidation or phosphorylation may well mediate its deleterious effects that include mitochondrial fragmentation and permeabilization of mitochondrial-like lipid vesicles [58, 80]. Overexpression of α-Syn induces complex I, II, IV, and V inhibition in the midbrain, while dysfunction in complex IV and V can be found in mouse striatum [58]. Oligomeric α-Syn aggregates, but not soluble monomers sensitize mitochondria to Ca^{2+}-induced dysfunction, leading to complex I dysfunction, altered membrane potential, disrupted Ca^{2+} homeostasis, and enhanced cytochrome *c* release [81]. Also, mitochondrial impairment and oxidative stress compromise autophagosomal degradation of α-Syn in oligodendroglial cells [82]. Finally, α-Syn inactivation phenocopies parkin overexpression and suppresses stress-induced mitochondria fission. The convergence of α-Syn, parkin, PINK1, and DJ-1 activity on mitochondrial dynamics and function may uncover an interplay between these genes in regard to the mitochondrial stress response [83]. Taken together, these results suggest that mitochondria are main targets of α-Syn and their defective autophagic clearance plays a significant role during α-Syn pathogenesis.

7.5 LRRK2

Leucine-rich repeat kinase 2 (LRRK2), encoded by the *LRRK2* (*PARK8*) gene, is a large, ubiquitously expressed dimer that contains different classes of phosphorylation sites, some of which can be autophosphorylated [84–86]. It is largely expressed in neurons but also observed to a lesser extent in astrocytes and microglia in response to inflammatory processes [87, 88]. LRRK2 mutations are present in some sporadic PD cases, but they are the leading cause of autosomal-dominant PD [89]. Observations made in DA neurons from PD patients with LRRK2 mutations indicate that LRRK2 is present in LB, and its toxicity is considered to be dependent on the presence of α-Syn [86]. Interestingly, patients carrying LRRK2 mutations show a differentiable PD phenotype, indicating that it can play disparate roles in various forms of the disease [90–92]. There are at least seven disease-causing LRRK2 variants: G2019S, R1441C, R1441G, R1441H, N1437H, Y1699C, and I2020T [93]. LRRK2 is a member of the ROCO protein family, in which all members contain a C-terminal of ROC (COR) region, as well as GTPase and kinase domains. Via unknown mechanisms, certain LRRK2 mutations seem to cause inefficient dimerization [94], which occurs in the ROC-COR region and is required for kinase activity and for localization to cell membranes [95]. Activity of the Ras-related GTPase and the ROC domain of LRRK2 can prevent the GTP binding and inactivate the kinase function [93, 96, 97]. Intriguingly, while the GTPase activity of the LRRK2 dimer seems to occur freely [98], its kinase activity depends on a functional GTPase domain. Nevertheless, LRRK2 dimerization seems to regulate GTPase activity by recruiting cellular proteins, indicating that the ROC-COR kinase domain is a crucial modulating region.

So far, almost all substrates of LRRK2 kinase activity have been described using cellular or animal models. These substrates include but are not limited to LRRK2 autophosphorylation sites (T1343, T1503) [99, 100], α-Syn [101], and members of the mitogen-activated protein (MAP) kinase family [84] and the ezrin/radixin/moesin (ERM) protein family [102, 103]. Several studies highlight the importance of LRRK2 in the maintenance of neurite length, branching, and outgrowth via phosphorylation of ERM proteins [103–105]. Autophosphorylation seems to be a regulatory key involved in the modulation of kinase activity occurring adjacent to the ROC GTPase domain [99, 100]. Pathogenic LRRK2 mutations are found in functional domains, and they perturb enzyme activity, indicating that aberrant GTPase and kinase activity have crucial roles in the LRRK2-mediated degenerative process. Studies using *C. elegans* have demonstrated the presence of LRRK2 in mitochondria and that its overexpression increases survival when animals are exposed to mitochondrial toxins [106]. Interestingly, mitochondrial membrane potential is decreased, intracellular ATP levels are lowered, and mitochondria are elongated and interconnected in immortalized neuroblastoma cultures as well as in fibroblasts of patients harboring LRRK2 G2019S [107, 108]. Overexpression of either wild-type or LRRK2 G2019S in SN4741 cells (dopaminergic neurons derived from the SNc of transgenic mouse embryos) leads to cell death and elevated ROS [109, 110]. Enhanced mitochondrial proton leakage and dysfunctional mitochondrial mobility have been observed in iPSC fibroblasts from LRRK2 G2019S and R1441C carriers [111]. Additionally, imbalances in calcium homeostasis and mitochondrial degradation can be alleviated by L-type calcium channel inhibition in mouse cortical motor neurons expressing LRRK2 mutations [112]. In summary, like other gene products associated with PD, LRRK2 affects an array of functional and morphological features of mitochondria, indicating that mitochondria could be a major component of LRRK2-mediated PD pathobiology. Strategies aimed at modulating its enzymatic activity could offer promising therapeutic approaches.

7.6 ATP13A2

ATP13A2 (*PARK9*) mutations are normally linked to an autosomal recessive juvenile-onset form of PD known as Kufor–Rakeb syndrome (KRS). Studies in fibroblast cells extracted from PD patients with ATP13A2 mutations have shown that ATP13A2 has an important role in lysosomal function and, consequently, the prevention of α-Syn aggregation in neurons [113]. ATP13A2 contains a unique N-terminal hydrophobic extension that lies on the cytosolic membrane surface of the lysosome. Interactions with signaling lipids that are recognized by the N-terminal hydrophobic domain of ATP13A2 enhance resistance to rotenone-induced mitochondrial stress in a cellular model of PD [114].

Lack of ATP13A2 causes lysosomal dysfunction and α-Syn accumulation, whereas its overexpression suppresses α-Syn toxicity [115]. Surviving nigral DA neurons in PD patients express *ATP13A2* mRNA at five- to tenfold higher levels

than controls [116], although ATP13A2 protein levels show a more modest increase in these neurons [117]. In contrast, a separate study found ATP13A2 levels to be reduced in nigral DA neurons relative to controls [113]. Recently, it has been shown that ATP13A2 protein levels are reduced in brain tissue of Lewy body disease cases [118]. Mitochondria from KRS patient fibroblasts and cell cultures lacking ATP13A2 have lower membrane potential and lower levels of autophagy, which lead to increased levels of ROS and concurrent oxidative stress and disrupted zinc homeostasis [119–121]. Overexpression of wild-type ATP13A2 can rescue decreases in respiration caused by lack of ATP13A2 [122]. Knockdown of ATP13A2 can increase in mitochondrial mass in primary mouse cortical neurons and in SH-SY5Y cells forced into mitochondrial dependence [120]. Mitochondria in both of these knockdown cell types exhibited increased fragmentation and increased production of ROS. Moreover, ATP13A2 KO mice develop age-related motor dysfunction that is preceded by neuropathological changes, including gliosis, accumulation of ubiquitinated protein aggregates, lipofuscinosis, and endolysosomal abnormalities [123]. Interestingly, this mouse study demonstrates that these phenotypes are α-Syn independent, in contrast to what has been shown in vitro [123]. Thus, evidence supports a convergence on impaired mitochondrial quality control as a central pathogenic mechanism among various genetic causes of PD-related syndromes, including ATP13A2.

7.7 Other Parkinson's Disease-Causing Mutations

In addition to the genes discussed above, a large number of additional genes can cause PD when mutated. These include *FBXO7* (*PARK15*), *VPS35*, *OMI/HTRA2* (*PARK13*), and *GBA*. Few PD cases are caused by mutations in these genes, and although most of their protein products can be linked to mitochondria, the precise mechanism by which their dysfunction causes PD is not known.

7.7.1 FBXO7

Fbxo7 is a member of the F-box domain-containing protein family. The F-box is a protein motif of approximately 50 amino acids that functions as a site of protein–protein interaction. F-box proteins were first characterized as components of Skp, cullin, F-box-containing complex (SCF) ubiquitin–ligase complexes that bind substrates for ubiquitin-mediated proteolysis [124]. Loss-of-function mutations in *Fbxo7* (*PARK15*) are associated with severe forms of autosomal recessive early-onset PD [125]. It seems quite probable that Fbxo7 facilitates the autophagic clearance of damaged mitochondria through a direct interaction with parkin and PINK1 during parkin-mediated autophagy [126]. Cells with reduced Fbxo7 expression show deficiencies in translocation of parkin to mitochondria, lesser ubiquitination

of mitofusin 1, and inhibition of mitophagy [127]. On the other hand, overexpression of Fbxo7 leads to protein aggregation and DA neuron degeneration in transgenic *Drosophila* [128]. When human dopaminergic cells are challenged with DA, rotenone or H_2O_2, Fbxo7 is concentrated in mitochondria where it forms aggregates that become toxic by promoting further protein aggregation in mitochondria and inhibiting mitophagy [128]. These data further implicate changes in levels of mitophagy in PD pathogenesis, and suggest that therapeutic strategies targeting the mitophagy pathway may be of potential relevance for PD patients harboring *Fbxo7* mutations.

7.7.2 VPS35

Together with vacuolar protein sorting-29 (VPS29) and VPS26, VPS35 forms a core component of the retromer complex that mediates the retrieval of membrane proteins from endosomes back to the Golgi or to the plasma membrane [129]. Loss-of-function mutations of this gene have been linked to late-onset PD and result in loss of DA and accumulation of α-Syn [130].

Typically, VPS35 is present in autosomal dominant PD patients, but its expression is decreased in the SNc [131–133]. In recent years, VPS35, together with parkin, has been associated with the process of mitochondria-derived vesicle (MDV) formation and the control of mitochondria quality mediated by the degradation of proteins [134]. PD-associated VPS35 mutations cause mitochondrial fragmentation and cell death in fibroblasts from a PD patient with the VPS35 D620N mutation. Notably, these deficits can be prevented by inhibition of mitochondrial fission [135]. VPS35 dysfunction also impairs lysosomal degradation of α-Syn and exacerbates neurotoxicity in a *Drosophila* model of PD [136]. Mutation or deficiency of VPS35 in cultured DA neurons, neuroblastoma cell lines, and the ventral midbrain of mice causes loss of mitochondrial fusion [130, 135, 137]. VPS35 regulates mitochondrial fusion by promoting MUL1 (a mitochondrial associated E3 ubiquitin ligase) degradation. Deletion of the VPS35 gene in DA neurons results in PD-like deficits, including loss of DA neurons and accumulation of α-Syn, increased MUL1, Mfn2 degradation, and mitochondrial fragmentation and dysfunction, all of which can be reversed by the expression of WT VPS35. These results suggest that VPS35 has a crucial regulatory role for MUL1 traffic and degradation, which balances the expression of Mfn2 and mediates mitochondrial fusion and DA neuron subsistence [130].

7.7.3 HTRA2

HtrA2 (also known as Omi) is a serine protease located in the mitochondrial intermembrane space. It was initially studied because of its role in the apoptosis processes [138–140]. Loss-of-function HtrA2 mutations cause mitochondrial dysfunction,

leading to a neurodegenerative disorder with parkinsonian features. Recently, the G399S variant was identified in sporadic PD patients [141]. Nevertheless, this relationship has been questioned after the publication of two human genetic studies reporting no association of *HTRA2* mutations and PD [142, 143]. HtrA2 interacts with PINK1 and both are components of the same stress-sensing pathway. Thus, it has been demonstrated that phosphorylation (activation) of HtrA2 by PINK1 protects neurons from stress-induced mitochondrial dysfunction and stress-induced apoptosis [144].

7.7.4 GBA

Homozygous *GBA* gene mutations cause Gaucher disease, but both homozygous and heterozygous mutations pose a risk of sporadic PD [145]. Glucocerebrosidase (GCase) is a lysosomal enzyme encoded by *GBA*, and mutations of *GBA* are associated with a lysosomal storage disorder [146]. PD patients carrying *GBA* mutations typically show more frequent signs of dementia and autonomic impairment [147]. Studies in cultivated human brain cells have revealed that loss of GCase activity leads to reduced mitochondrial membrane potential [148], which may result from impairment of the autophagy–lysosome pathway and changes in lipid metabolism [149–152], neuroinflammation [153–155], altered calcium homeostasis [156–158], and degraded mitophagy [148, 149, 152, 159]. Additionally, GCase deficiency causes an increase of α-Syn accumulation in human and mice SNc and striatum mitochondria [49, 160]. These studies implicate defective GCase function as risk factor for mitochondrial dysfunction and the multi-proteinopathies.

7.8 Concluding Remarks

As summarized in this chapter, protein products of several genes associated with familial forms of PD can be linked to mitochondrial function and morphology, including mitophagy, transport, and biogenesis. Defects in both complex I and complex IV of the respiratory chain are observed in SNc mitochondria of PD patients, and mitochondrial-related toxins like MPTP or rotenone can cause selective SNc degeneration in both humans and other animal models of PD, suggesting that mitochondria may play a central role in all forms of PD. As mitochondria are essential to the regulation of different functions such as energy production, calcium homeostasis, and apoptosis, maintaining their functions is critical for cell survival. Better understanding of the mechanisms of mitochondrial functions and how pathogenic PD mutations disturb them will increase our understanding of PD. Research into the function and dysfunction of PD-associated mutations and identifying novel therapeutic approaches are imperative to this endeavor.

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Chapter 8
PARKIN/PINK1 Pathway for the Selective Isolation and Degradation of Impaired Mitochondria

Derek P. Narendra

8.1 Clinical Features of Parkinsonism Due to PARKIN and PINK1 Mutations, the Human Phenotype

Parkinson's disease (PD) is a progressive movement disorder affecting 1 % of the population over 60 years of age [1]. It is characterized clinically by slowness of movement (bradykinesia), tremor at rest, rigidity, and postural instability. These signs are due to loss of the substantia nigra neurons supplying dopamine to the basal ganglia, which are critical for motor control. Early in the disease course, treatment with the metabolic precursor to dopamine, L-DOPA, relieves many of these motor symptoms and reduces mortality from PD complications [2]. However, the disease remains disabling in its advanced stages with excess mortality compared to the general population [3]. A principal motivation for studying genetic forms of PD is the hope that novel targets will be uncovered to slow or halt disease progression.

Mutations in the gene *PARK2* (hereafter *PARKIN*) coding for the PARKIN protein and mutations in *PARK6* (hereafter *PINK1*) coding for the PINK1 protein are the most common genetic causes of parkinsonism with onset before the age of 45 [4, 5]. A recent systematic review estimates that *PARKIN* mutations are responsible for 8.6 % of early onset PD cases (and about 0.3 % of PD cases overall), whereas *PINK1* mutations account for 3.7 % of early onset cases [6]. *PARKIN* and *PINK1* mutations have a recessive pattern of inheritance. Additionally, causative mutations have been identified in virtually every domain of PARKIN and PINK1, consistent with the proposed loss of function mechanism of disease pathogenesis.

Clinically, the parkinsonian motor symptoms due to *PARKIN* and *PINK1* mutations resemble early onset sporadic PD. Other neurologic systems spared in sporadic PD

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are also spared in *PARKIN* and *PINK1*-related parkinsonism, and, in this sense, *PARKIN* and *PINK1* mutations cause a relatively pure parkinsonian phenotype. This is in contrast to other causes of recessive parkinsonism such as mutations in *ATP1A3*, in which early dementia and pyramidal signs are often pronounced [7].

Although motor symptoms are characteristic, non-motor symptoms that are common in sporadic PD may be less common in parkinsonism due to *PARKIN* or *PINK1* mutations. Unlike most patients with sporadic PD, for instance, patients with *PARKIN* and *PINK1* mutations probably have a normal sense of smell [8]. Additionally, a recent report found that unlike most patients with sporadic PD, patients with PARKIN-related parkinsonism do not exhibit loss of the sympathetic sudomotor ("sweat motor") and piloerector ("hair-raising") nerve fibers in skin biopsies. The ALPHA-SYNUCLEIN (ASYN) deposits detected in these autonomic fibers in sporadic PD cases are likewise absent in cases of parkinsonism due to *PARKIN* mutations [9]. Additionally, cognitive dysfunction and dementia, which develop almost universally in sporadic PD at a late stage, may be less common in PARKIN disease [10]. Together these findings suggest that the disease process initiated by *PARKIN* (and perhaps *PINK1*) mutations may affect only a subset of the neurons typically affected in sporadic PD.

More than a dozen cases of PARKIN disease and one case of PINK1 disease have come to autopsy and suggest a pathobiology that is related to but distinct from sporadic PD. In a retrospective series of PARKIN autopsy cases, the same general pattern of neuronal loss and gliosis was seen in PARKIN-related and sporadic PD cases, although neuronal loss and gliosis in areas outside the substantia nigra such as the locus ceruleus and the dorsal motor nucleus of the vagus nerve were generally less pronounced [11]. ASYN deposits called Lewy bodies and Lewy neurites are pathognomonic for sporadic PD. In at least five cases of parkinsonism due to PARKIN mutations, Lewy pathology was observed, albeit the degree of Lewy pathology was minor in comparison to the extensive loss of neurons in the substantia nigra in most cases examined (reviewed in [11, 12]). In many other cases (at least nine), Lewy pathology was absent altogether. In general those cases of PARKIN-related parkinsonism with Lewy pathology had a later age of onset than those cases without Lewy pathology, suggesting that duration of disease may contribute to ASYN pathology [11]. Only one case of PD due to two mutated *PINK1* alleles has come to autopsy, and in that case, typical Lewy pathology was observed. Together the autopsy material suggests that ASYN aggregation can occur in PARKIN and PINK1-related parkinsonism. It also suggests that ASYN aggregation may not be a strong driving force of PARKIN-related (and perhaps PINK1-related) pathogenesis.

8.2 PARKIN and PINK1 Function in a Common Pathway in *Drosophila*

PARKIN, identified early as a cytosolic E3 ubiquitin ligase [13], was initially proposed to have a variety of functions in different cellular compartments. However, the recognition that the mitochondrial kinase PINK1 has a strong genetic interaction

with PARKIN suggested that the primary function of PARKIN in the pathogenesis of parkinsonism likely relates to its effects on mitochondria.

The first strong evidence linking PARKIN and PINK1 in a common pathway important for maintenance of mitochondrial integrity was the observation that knockout of their orthologs in *Drosophila* (*dParkin* and *dPink1*) results in a highly characteristic and largely indistinguishable phenotype [14–18]. The *Drosophila* lose dopaminergic neurons, their indirect flight muscles waste, and the males have disrupted spermatogenesis. Pronounced abnormalities in mitochondrial morphology are evident in each affected tissue consistent with loss of mitochondrial quality control. Loss of both dParkin and dPink1 is no worse than loss of either dParkin or dPink1 alone, suggesting that each protein has a sequential function in a common pathway. dParkin overexpression rescues dPink1 loss, and dPink1 overexpression fails to rescue dParkin loss, suggesting that dParkin functions downstream of dPink1 in the common pathway. Together these genetic findings strongly suggested that dParkin and dPink1 function together to support mitochondrial integrity and that Pink1 acts upstream of dParkin in that pathway.

8.3 PINK1 Activates PARKIN in Mammalian Cells

In mammalian cells, several lines of evidence have converged to suggest that PARKIN and PINK1 function in a novel mitochondrial quality control pathway similar to what was observed first in *Drosophila* (Fig. 8.1). PINK1, the bioenergetic sensor of the pathway, is first stabilized on the outer mitochondrial membrane (OMM) in response to mitochondrial damage [19, 20]. Subsequently, PINK1 activates the ubiquitin ligase activity of PARKIN both by phosphorylation of ubiquitin (UB) on OMM proteins and direct phosphorylation of PARKIN on its homologous UB-like domain (UBL) [21–27]. The phospho-ubiquitin signal on OMM proteins, initiated by PINK1, is amplified by activated PARKIN to promote the selective isolation and degradation of damaged mitochondria [28].

In mammalian cells with healthy mitochondria, the PARKIN/PINK1 pathway is quiescent. The E3 ligase activity of PARKIN is autoinhibited [29, 30]. PINK1 is imported to the inner mitochondrial membrane (IMM) where it is cleaved and its

Fig. 8.1 Overview of the PINK1/PARKIN mitochondrial quality control pathway. In response to mitochondrial damage, PINK1 is selectively stabilized on the surface of the damaged mitochondria. The accumulation of PINK1 activates PARKIN, both by direct phosphorylation on the UBIQUITIN-like domain of PARKIN and by exposure of PARKIN to phospho-ubiquitin. Upon activation, PARKIN ubiquitinates proteins on the surface of mitochondria. Phospho-ubiquitination of proteins on the surface of mitochondria leads to (*1*) their isolation through the degradation of GTPases necessary for mitochondrial fusion and transport and (*2*) their elimination in lysosomes through the macroautophagy pathway

remnant rapidly degraded in the cytosol (discussed further below). PINK1 expression on the surface of healthy mitochondria, where it could interact with PARKIN, is, thus, kept low.

When a damaged mitochondrion loses its ability to import PINK1 to the inner membrane, the PINK1/Parkin pathway is activated. PINK1, no longer imported to the inner membrane to be cleaved and degraded, instead accumulates selectively on the OMM of the damaged mitochondrion where its expression is stable [19, 20]. The accumulation of PINK1 on the OMM is both necessary and sufficient for PARKIN activation [19, 20, 31, 32]. On the OMM with its serine/threonine kinase domain facing the cytosol [33], it phosphorylates the UBL domain of PARKIN on serine 65 [21, 22]. Additionally, PINK1 phosphorylates ubiquitin (UB), also on serine 65, forming phospho-UB (pUB) [23–27]. The E3 ubiquitin ligase activity of PARKIN is robustly activated independently by phosphorylation on its UBL and by exposure to pUB conjugated to outer mitochondrial membrane (OMM) proteins (with activity increased more than 1000-fold in response to either stimulus in a sensitive assay) [26].

Once activated, the ubiquitin ligase activity of PARKIN is confined to the OMM by its affinity for pUB-conjugated OMM proteins. This affinity likely accounts for the robust translocation of PARKIN from the cytosol to the mitochondria observed following collapse of the mitochondrial inner membrane potential [26–30].

Thus, upon exposure to the cytosol face of the OMM, PINK1 sets in motion a feedforward cycle of PARKIN activation, OMM protein ubiquitination, and UB phosphorylation (Fig. 8.2). This activity is confined to the OMM cytosolic surface by the integration of PINK1 into the OMM membrane and the affinity of activated Parkin for pUB accumulating on OMM membrane proteins.

8.4 Substrate Specificity of PARKIN Conferred by Local Activation Rather Than Intrinsic Activity

The first structures of autoinhibited PARKIN show the HECT/RING hybrid ubiquitin ligase to be folded over itself like a pocketknife [34–36]. The PARKIN UNIQUE DOMAIN blocks the active site (C431) onto which the E2 loads UB [37]. Additionally, the linker region C-terminal to the RING-Between-RING (RBR) domain blocks the predicted binding site of the E2-conjugating enzyme. The activity of PARKIN increases greatly upon exposure to PINK1 both in cells and in vitro by allowing the pocketknife-like structure to unclasp, thereby freeing both the E2-binding site and the active site for UB loading onto C431 by the E2 ubiquitin-conjugating enzyme (Fig. 8.3). Specifically, binding of pUB causes a helix in RING1 to straighten, freeing the UBL domain and driving the conformational change. The active structure of PARKIN is further stabilized by phosphorylation of the freed UBL domain [38].

Once activated, PARKIN appears to be promiscuous with respect to its substrates. MITOFUSIN1/MITOFUSIN2 and MIRO1/MIRO2 (discussed below) may be among

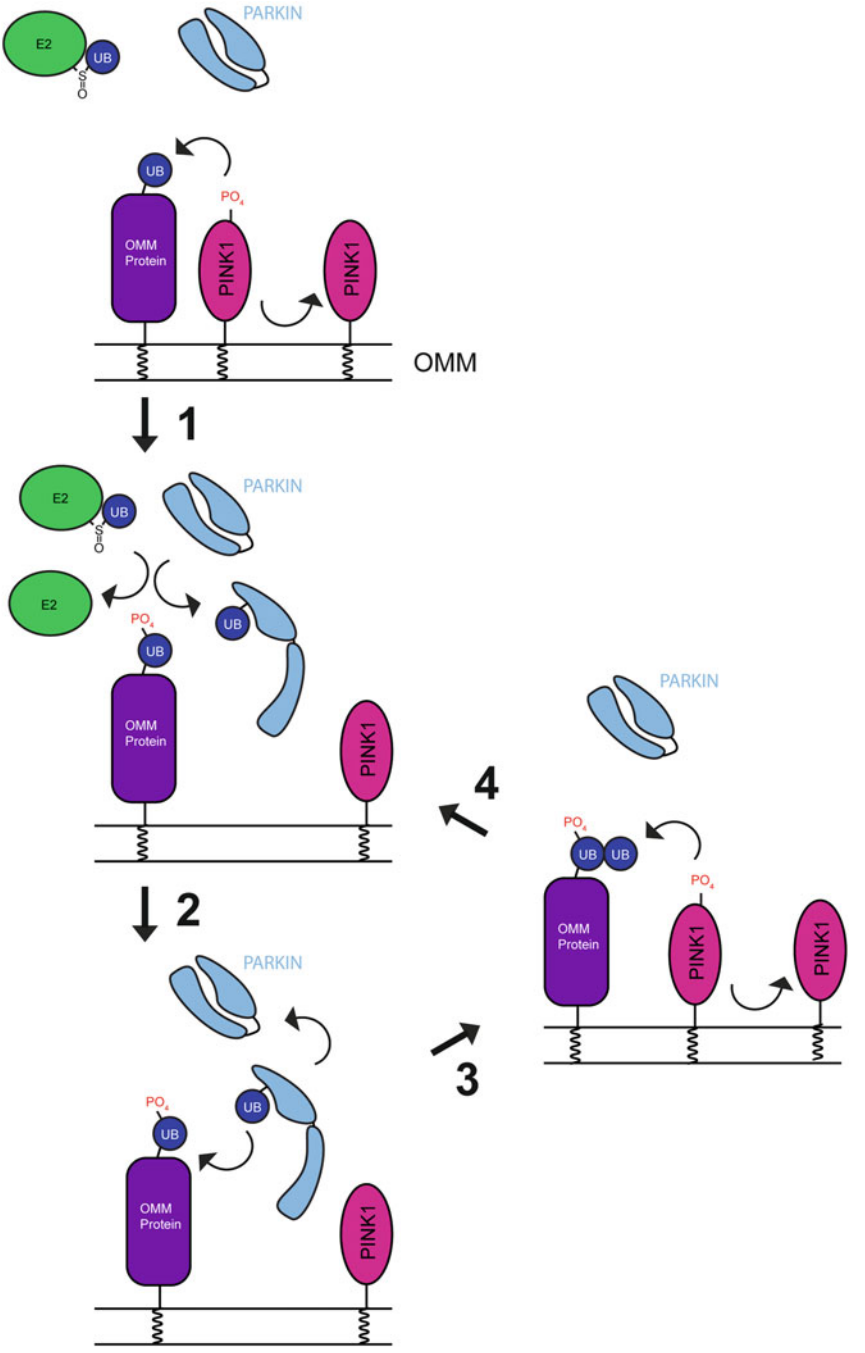

Fig. 8.2 The PINK1/PARKIN phospho-ubiquitin cycle. The accumulation of PINK1 on the outer mitochondrial membrane (OMM), resulting from failed import, is a signal of mitochondrial dysfunction. This signal is amplified through the feedforward PINK1/PARKIN phospho-UB cycle. OMM proteins that already carry UB are phosphorylated on serine 65 by PINK1 (*Step 1*). PARKIN, usually inactive in the cytosol, is activated both by ubiquitination on its UBL and by exposure to phospho-UB conjugated to OMM proteins. This conformational change allows PARKIN to be loaded with UB on its active site by the E2-conjugating enzyme (*Step 2*). Activated PARKIN, which has an affinity for phospho-ubiquitin, transfers UB to a solvent-exposed lysine local to phospho-UB conjugated to OMM proteins (*Step 3*). The addition of unmodified UB to OMM proteins provides additional substrate for PINK1 phosphorylation (*Step 4*), allowing the cycle to repeat

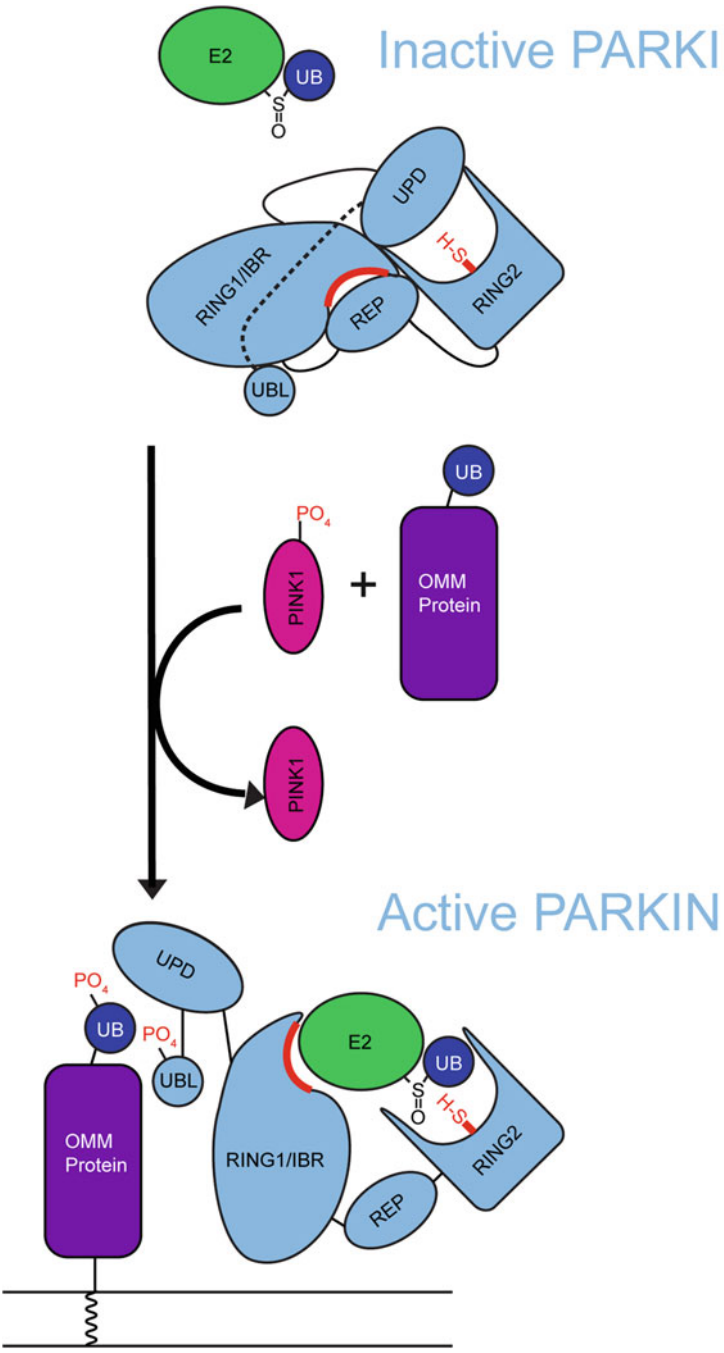

Fig. 8.3 Activation of PARKIN by PINK1. Under basal conditions, PARKIN (*light blue*) is in an autoinhibited form in the cytosol (*top* of figure). Structures of autoinhibited PARKIN show its active site in RING2 (*red* in the figure) to be blocked by the UNIQUE PARKIN DOMAIN (UPD).

the proteins most robustly ubiquitinated by PARKIN but at least 36 OMM proteins demonstrate a PARKIN-dependent increase in ubiquitination in response to loss of the mitochondrial membrane potential [39–41]. Analysis of UB sites targeted by PARKIN fails to reveal a common motif [41]. Thus, selectivity of ubiquitination appears to be determined chiefly by the localization of activated PARKIN, which depends in turn on the membrane in which PINK1 is integrated. Indeed, if PINK1 is targeted to the cytosolic face of peroxisomes instead of mitochondria, it will activate PARKIN on the surface of peroxisomes, and peroxisomal membrane proteins facing the cytosol will be ubiquitinated by PARKIN instead of OMM proteins [42]. Thus, the presence of PINK1 on the cytosolic face of a membrane marks the spot of PARKIN ubiquitination. This mechanism allows an individual impaired mitochondrion to be ubiquitinated on its surface by PARKIN while sparing healthy mitochondria in the cell.

As a corollary to its lack of substrate specificity, PARKIN does not seem to favor a single polyUB chain topology. PARKIN most abundantly forms K48- and K63-linked polyUB chains in cells but a mix of topologies is present on OMM proteins ubiquitinated by PARKIN (including K6, K11, and K33) [26]. Thus, the specific response to the ubiquitination of OMM proteins by PARKIN may reflect less the specific UB topology and more the abundance of polyUB (or the abundance of phosphorylated UB) attached to the OMM proteins. Indeed, when K63 polyUB chains are eliminated from PARKIN's repertory (by knockout of the E2-conjugating enzyme required for K63 polyUB formation), no effect is seen on the efficiency of mitophagy induced by the activation of PARKIN [43].

8.5 Bioenergetic (and Biogenetic) Control of the PINK1/PARKIN Pathway

As the activity of PARKIN strictly depends on PINK1 exposure to the OMM [19, 20, 31, 32], primary control over PINK1/PARKIN pathway activity is exerted through modulation of PINK1 protein abundance on the surface of individual mitochondria (Fig. 8.4). In turn, PINK1 abundance on the outer membrane is inversely related to the efficiency of its import to the inner membrane [19, 20, 44]. Healthy mitochondria import and cleave PINK1; damaged mitochondria accumulate PINK1 on their surface thus marking them for isolation and destruction by PARKIN.

Fig. 8.3 (continued) Additionally, the predicted E2-binding site in RING1 (also, *red*) is blocked by an α-helix (named Repressor of PARKIN [REP]) in the linker region between the In-Between-RING (IBR) domain and RING2. Activation of PARKIN by exposure to phosphorylated UB conjugated to outer mitochondrial membrane (OMM) proteins and/or by direct phosphorylation of the UB-like domain (UBL) of PARKIN is predicted to lead to a rearrangement of the structure of PARKIN (*bottom* of figure). This rearrangement is predicted to permit binding of the E2 ubiquitin-conjugating enzyme to RING1, thereby facilitating transfer of UB to the thiol group of cysteine 431 in the active site of RING2. PARKIN with UB loaded on its active site is predicted to act as a hybrid RING/HECT E3 ubiquitin ligase. UB is subsequently transferred from the active site of PARKIN to a lysine on its protein substrate (not pictured)

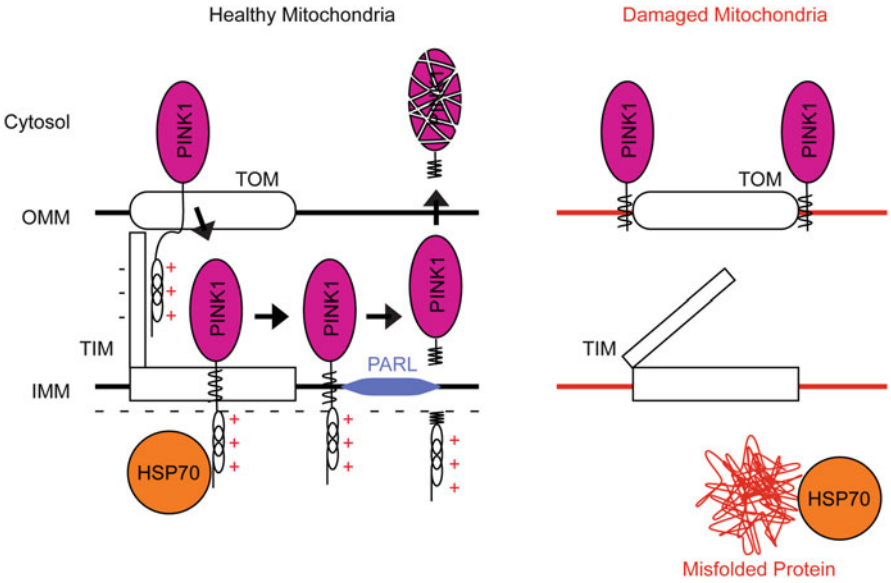

Fig. 8.4 PINK1 is a sensor of mitochondrial impairment. In healthy mitochondria (*left side*), PINK1 is imported to the inner mitochondrial membrane by the TOM and TIM complexes, on the outer and inner membranes, respectively. Import requires the negative potential across in the inner mitochondrial membrane and the action of the matrix chaperone protein mtHSP70 (*orange*). Shortly after transport to the inner membrane, PINK1 is cleaved by PARL and other proteases leaving a soluble form of the kinase. Cleaved PINK1 is rapidly degraded in the cytosol by the proteasome due to a destabilizing amino acid at its N-terminus. Following mitochondrial impairment sufficient to inhibit mitochondrial import (e.g., loss of the inner mitochondrial membrane potential or excess misfolded protein in the matrix), PINK1 is stabilized on the outer mitochondrial membrane where it associates with the TOM complex (*right side*). PINK1 is comparatively long-lived on the outer membrane allowing it to accumulate. This mechanism allows the selective accumulation of PINK1 on the surface of impaired mitochondria. Thus, PINK1 is able to flag impaired mitochondria for isolation and degradation (not pictured). PARKIN amplifies the signal

In healthy mitochondria, PINK1 is imported to the inner mitochondrial membrane, driven in part by the electrochemical potential across the inner membrane [45]. Import is signaled by a canonical N-terminal mitochondrial targeting sequence as well as a transmembrane domain between residues 94 and 110, which stops transfer in the IMM [33]. Once imported to the IMM, a small pool of PINK1 may mature at inner membrane, where PINK1 may have a function independent of PARKIN (e.g., regulation of complex I [46]). The bulk of imported PINK1, however, is cleaved sequentially by the mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) [47–52]. Cleavage by PARL (between residues 103 and 104) results in a destabilizing phenylalanine residue at its N-terminus [49, 53]. Once cleaved, PINK1 is retrotranslocated to the cytosol, where it is rapidly degraded by the N-end degradation pathway [53]. Before degradation, cleaved PINK1 in the cytosol may also bind and inhibit PARKIN, helping form a surround to the center of PARKIN activation at the OMM surface [54].

The processing of PINK1 by healthy mitochondria accounts for its short half-life and very low abundance in healthy mitochondria [44]. PINK1 is virtually absent from the OMM and so is kept from activating PARKIN.

In the event that a mitochondrion becomes bioenergetically impaired, the driving force for the import of PINK1 to the inner mitochondrial membrane is lost. PINK1 expression on the OMM is comparatively stable, which allows it to accumulate selectively on the OMM of the impaired mitochondrion [19, 20]. With its kinase domain facing the cytosol, PINK1 is able to activate PARKIN specifically at the surface of the impaired mitochondrion, as discussed above.

Stabilization of PINK1 on the OMM and the resultant activation of PARKIN have been demonstrated following a number pharmacological and genetic manipulations that have in common loss of the inner mitochondrial membrane potential, maintained by the proton-pumping action of the respiratory chain complexes. These manipulations include mitochondrial uncoupling drugs carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and valinomycin, agents that increase mitochondrial reactive oxygen species production such as paraquat or the mitochondria-targeted photosensitizing protein Killer Red, inhibitors of the respiratory chain such as antimycin (which increases reactive oxygen species) or antimycin with oligomycin (which collapses membrane potential), mtDNA mutations or mtDNA depletion, and overexpression of proteins that uncouple mitochondria [19, 26, 31, 55–58]. Although early protocols used chemicals such as CCCP that were too toxic to achieve robust Parkin activation and recruitment in neurons, gentler protocols using antimycin or overexpression of proteins that induce uncoupling have now conclusively demonstrated that PARKIN activation and recruitment is triggered in cultured neurons following loss of the inner mitochondrial membrane potential [57, 58].

In addition to bioenergetic failure, mitochondrial import is sensitive to other forms of stress, such as the accumulation of misfolded proteins in the mitochondrial matrix. Accumulation of misfolded proteins in the matrix may disrupt mitochondrial import by sequestering chaperones necessary for mitochondrial import. Such a stress would also be predicted to activate the PINK1/PARKIN pathway, as it would prevent the import of PINK1 to the inner membrane, allowing it to accumulate on the outer mitochondrial membrane. Consistent with this prediction, a recent study found that PINK1 is stabilized on the OMM following overexpression of an aggregating mitochondrial matrix protein, in the absence of membrane potential loss [59]. Accumulated PINK1 activated PARKIN to induce the mitophagy of bioenergetically competent mitochondria. A recent study in *Drosophila* suggests that this proteostatic stress may have relevance for PINK1/PARKIN activation in vivo [60]. Overexpression of the aggregating mitochondrial matrix protein was found to phenocopy *Drosophila* with *dParkin* or *dPink1* mutations. Additionally, the authors found that dParkin overexpression compensated for excess mitochondrial matrix protein aggregation in an autophagy-dependent manner. Thus, proteostatic stress in the mitochondrial matrix can activate the PINK1/PARKIN pathway even in the absence of outright bioenergetic failure, presumably by inhibiting mitochondrial import (and thus biogenesis).

Control of the PINK1/Parkin pathway is enhanced by an “off switch,” which stops PARKIN accumulation in the event that a biogenetically impaired mitochondrion recovers. On the surface of impaired mitochondria, PINK1 associates with the

TOMM40-containing outer mitochondrial membrane import complex [42]. The association with the import complex allows PINK1 to be rapidly removed from the outer mitochondrial membrane if mitochondrial import is subsequently restored. As the recruitment of PARKIN to mitochondria lags behind membrane potential collapse and the start of PINK1 accumulation by tens of minutes, there is a window during which PINK1 accumulation can be reversed before the exponential phase of Parkin recruitment [19].

An additional "off switch" may be provided by the deubiquitinating enzyme ubiquitin-specific protease 30 (USP30), which is resident to the OMM [61]. USP30 opposes the action of Parkin by deubiquitinating OMM proteins that are ubiquitinated by PARKIN. Knockdown of USP30 or overexpression of the catalytically inactive mutant substantially increases ubiquitination of OMM in response to PARKIN activation as well as its downstream effects. Having an active deubiquitinating process at the OMM membrane would be predicted to lead to a faster off rate of PINK1/PARKIN activity should PINK1 levels decrease following restoration of mitochondrial import. Additionally, the presence of USP30 would be predicted to help constrain the activity of PINK1/PARKIN to the membrane around the individual TOM complex with which PINK1 has assembled, which may have consequences for involvement of PARKIN/PINK1 in a more local mitochondrial quality control pathway involving mitochondria vesicles targeted to lysosomes, discussed below [62].

8.6 Mitochondrial Pathways Regulated by PARKIN Ubiquitination in the PINK1/PARKIN Pathway

The consequences of local activation of PARKIN/PINK1 on impaired mitochondria are several: (1) mitochondrial fusion is inhibited through the degradation of MITOFUSIN1/MITOFUSIN2 (MFN1/MFN2), (2) mitochondrial transport is blocked through the degradation of MIRO1/MIRO2, and (3) mitochondria are degraded wholesale by mitophagy (a mitochondria-specific form of macroautophagy) or in part by autophagy-independent mechanisms (e.g., mitochondrial vesicles targeted to the lysosome) (Fig. 8.5). These processes are likely interconnected and serve a common purpose: isolation and elimination of mitochondria with a critical failure in their capacity for mitochondrial import (and thus biogenesis) from the mitochondrial network.

8.6.1 Regulation of Mitochondria Fusion by the PARKIN/PINK1 Pathway

Mitochondria in cells continually undergo cycles of fusion and fission (for a recent review, see [63]). These dynamics are not only critical for the division of growing mitochondria during biogenesis, they serve a quality control function and help size mitochondria for specialized jobs in cells such as neurons.

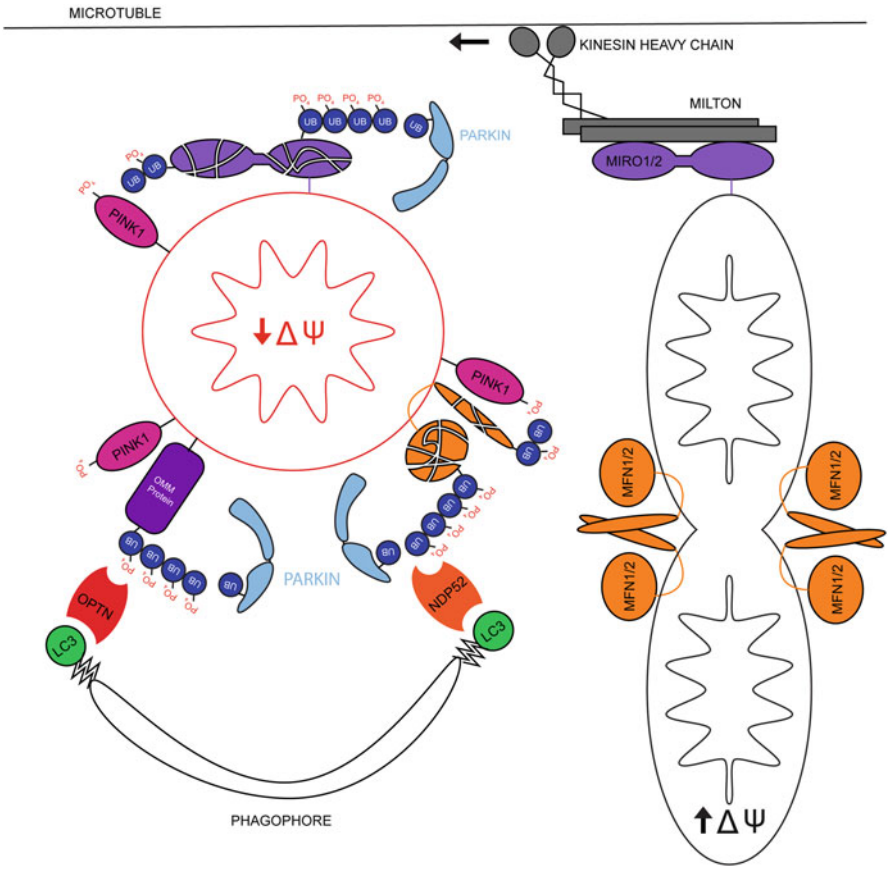

Fig. 8.5 Ubiquitination of surface mitochondrial proteins by the PINK1/PARKIN pathway mediates mitochondrial isolation and degradation by mitophagy. Bioenergetically healthy mitochondria (*right*) maintain a strong electrochemical potential (Δψ) across the inner mitochondrial membrane by proton pumping. This potential is essential for ATP production and calcium buffering by mitochondria (not pictured). On healthy mitochondria, MFN1/MFN2 (*orange*) mediate outer mitochondrial membrane fusion with other healthy mitochondria displaying MFN1/MFN2. MIRO1/MIRO2 (*purple*) mediate anterograde transport along microtubules through a complex with MILTON and KINESIN HEAVY CHAIN (*gray*) as well as retrograde transport with a complex containing DYNEIN (not pictured). Following the loss of Δψ, the mitochondrion becomes dysfunctional and is deficient in ATP production, calcium buffering, and mitochondrial import (*left*). PINK1 (*pink*) accumulates on the surface and phosphorylates UB conjugated to outer mitochondrial proteins (OMM) and activates PARKIN by direct phosphorylation (not pictured). Activated PARKIN, recruited from the cytosol, ubiquitinates (*dark blue*) outer mitochondrial membrane proteins. Proteins such as MFN1/MFN2 and MIRO1/MIRO2 are degraded following ubiquitination, causing inhibition of mitochondrial fusion and arrest of transport. Phospho-UB recruits NDP52 and OPTINEURIN (OPTN) to the impaired mitochondria. In turn, NDP52 and OPTN (*orange* and *red*, respectively) recruit both early autophagy machinery (including ULK1, DFCP1, and WIPI1 [not pictured]) and LC3. A phagophore is expanded around the ubiquitinated mitochondrion with the aid of the autophagy protein LC3 (*green*). The phagophore envelops the impaired mitochondrion and fuses to form a double-membrane-containing structure called an autophagosome (not pictured). The outer membrane of the autophagosome subsequently fuses with a lysosome allowing degradation of the impaired mitochondrion (not pictured)

Disruption of fusion–fission cycles leads to accumulation of oxidatively damaged macromolecules, increased mtDNA mutations, and mtDNA depletion within the mitochondrial network, consistent with its having a quality control function [64–67]. Quality control may be achieved through the continual mixing of macromolecules in cycles of fusion and fission that stochastically concentrate damaged macromolecules within a subset of mitochondria in the network. Those mitochondria that reach a threshold level of damage may be isolated from the mitochondrial network by losing their competence for fusion with other mitochondria (and may ultimately be degraded by autophagy), leading to a net reduction of damaged macromolecules within the mitochondrial network. The selective isolation and degradation of critically impaired mitochondria (measured as a substantial decrease in their inner membrane potential) have been demonstrated experimentally, giving this model feasibility [65]. More recent data suggest that in addition to this stochastic mechanism, damaged macromolecules may be actively segregated within the mitochondrial network to allow selective degradation of the damaged components [52, 62, 68–70]. The mechanism(s) mediating this segregation has yet to be defined.

Mitochondrial fission and fusion are regulated chiefly by three sets of GTPases: the homologous MFN1 and MFN2 (MITOFUSIN1 and MITOFUSIN2), OPA1 (OPTIC ATROPHY 1), and DRP1 (DYNAMIN-RELATED PROTEIN 1). MFN1/MFN2 promotes outer mitochondrial membrane fusion likely through tethering of two MFN1/MFN2 molecules on apposed mitochondrial membranes by forming a coiled coil structure with their C-terminal domain [71, 72]. OPA1 promotes inner membrane fusion in a manner that is regulated by the balance of short and long isoforms of the protein generated by alternative splicing and proteolysis [73–76]. Finally, the GTPase DRP1 promotes mitochondrial fission by forming a polymeric spiral around the outer diameter of the mitochondrion at ER–mitochondria contact sites, following its recruitment to the OMM membrane from the cytosol by MITOCHONDRIAL FISSION FACTOR [77–81].

Notably, MFN2, OPA1, and DRP1 have all been linked to neurogenetic disorders highlighting the importance of mitochondrial dynamics for neuronal integrity. Mutations in MFN2 are a common autosomal dominant cause of Charcot–Marie–Tooth syndrome, leading to an axonal sensorimotor polyneuropathy [82]. Mutations in OPA1 are the chief cause of autosomal dominant optic atrophy and have been additionally linked to multiple mtDNA deletion syndromes such as progressive external ophthalmoplegia [83–85]. A DRP1 mutation was found to be the cause of a lethal syndrome of microcephaly, abnormal brain development, optic atrophy, and persistent lactic acidemia in a newborn girl [86].

The PINK1/PARKIN pathway was first identified as a critical regulator of mitochondrial dynamics in *Drosophila*. Knockout of *dPink1* or *dParkin* results in arrest of spermatogenesis at a stage that depends on the coordinated fusion of mitochondria into two extended leaflike structures that wrap around the axoneme of the *Drosophila* spermatid. Instead of two fused mitochondria, *dPink1* and *dParkin*

knockout *Drosophila* have only one hyperfused mitochondrion [14, 16–18]. The phenotype of dParkin and dPink1 null *Drosophila* is rescued by overexpression of the mitochondrial fission protein Drp1 but is unchanged following knockout of the testis-specific MFN1/MFN2 ortholog *Fuzzy Onion* (*Fzo*), providing genetic evidence that dParkin and dPink1 promote mitochondrial fission [87, 88]. Similarly, in indirect flight muscles, overexpression of Drp1 or knockdown of the Mfn partially rescues the mitochondrial morphology defect resulting from loss of dParkin or dPink1; and knockdown of dParkin or dPink1 in cultured *Drosophila* S2 cells results in dramatic elongation of mitochondria. dParkin likely regulates mitochondrial dynamics by directly ubiquitinating Mfn. Mfn, which is substantially ubiquitinated under basal conditions in *Drosophila*, is largely unmodified in *dParkin* or *dPink1* knockout *Drosophila*, consistent with the constitutive ubiquitination of Marf by dParkin [89, 90].

In mammalian cells the orthologous proteins MFN1/MFN2 are also degraded by PARKIN but only in response to mitochondrial stress. Knockdown of *PARKIN* or *PINK1* in human cells or knockout of *Parkin* or *Pink1* in mice has not been consistently observed to alter mitochondrial morphology (see, for instance, [91]). Similarly, MFN1/MFN2 abundance and ubiquitination are unaltered following knockdown of *PARKIN* or *PINK1* in mammalian cells under basal conditions [92, 93]. However, upon uncoupling of the inner mitochondrial membrane potential, MFN1/MFN2 are robustly ubiquitinated by PARKIN. Subsequently, they are removed from the OMM by VCP/p97 and are degraded by the proteasome [93–95]. The mitochondrial network becomes fragmented. The impaired mitochondria denuded of MFN1/MFN2 are incompetent to refuse with healthy mitochondria and are, thus, kept from "poisoning" the mitochondrial network with damaged macromolecules [93].

The observation that MFN1/MFN2 are ubiquitinated in a PARKIN- and PINK1-dependent manner following mitochondrial uncoupling may be useful clinically as a biomarker for PARKIN/PINK1 pathway function [96]. In fibroblasts from healthy controls, MFN1/MFN2 are ubiquitinated by endogenous PARKIN following treatment with a mitochondrial uncoupling agent. By contrast, patients with homozygous or compound heterozygous mutations in PARKIN or PINK1 fail to ubiquitinate MFN1/MFN2 following mitochondrial uncoupling. An independent group recently used this biochemical assay to characterize individuals in a new pedigree [97]. In fibroblasts from the proband's father, which had one wild-type allele and one mutated allele, MFN2 was efficiently ubiquitinated after mitochondrial uncoupling similar to fibroblasts from a healthy control. By contrast, the proband and her mother, both of whom had two mutated alleles, failed to ubiquitinate MFN2 upon uncoupling, providing biochemical support for loss of PARKIN function in fibroblasts from the proband and her mother. This assay could potentially clarify the effect of mutations of uncertain pathogenicity as well as test the biological effect of therapies that are proposed to restore PINK1/PARKIN pathway activity.

8.6.2 Regulation of Mitochondrial Transport by the PINK1/PARKIN Pathway

Similar to the PINK1/PARKIN pathway blocking mitochondrial fusion by stripping the mitochondria of OMM fusion proteins, the PINK1/PARKIN pathway halts mitochondrial transport by degrading the GTPases needed for transport, the homologous MITOCHONDRIAL RHO FAMILY 1 and 2, or MIRO1 and MIRO2 [98, 99]. Also similar to the degradation of MFN1/MFN2 by the PINK1/PARKIN pathway, the degradation of MIRO1/MIRO2 appears to be constitutive in *Drosophila* and a conditional response to mitochondrial damage in mammalian cells.

Mitochondrial transport plays an essential role in the proper distribution of mitochondria and is particularly critical in large, specialized cells such as neurons (reviewed in [100]). In neurons, failure of mitochondrial transport results in clustering of mitochondria in the cell soma instead of near the synapse and the nodes of Ranvier along the axon. Consequently, mitochondria are absent where the need for energy and calcium buffering capacity is greatest.

In mammalian cells including axons of neurons, mitochondria travel along microtubules, which are oriented with their minus end near the nuclear envelope and their plus end in the cell periphery. For anterograde transport from the soma down the axon, mitochondria are connected to the microtubules through a complex of three proteins: (1) the motor-containing KINESIN HEAVY CHAIN (KHC), which directly binds microtubules; (2) the homologous GTPases MIRO1 and MIRO2 (MIRO1/MIRO2), which are tail anchored in the OMM; and (3) MILTON, which serves as an adaptor between KHC and MIRO1/MIRO2 [101, 102]. Retrograde transport from the axon toward the cell soma depends on a complex formed by the motor-containing protein DYNEIN and MILTON and MIRO1/MIRO2 [103].

In normal axons, approximately 20 % of mitochondria move anterograde, 20 % move retrograde, and 60 % are stationary at any given time. Knockdown of dParkin or dPink1 in *Drosophila* axons leads to a dramatic increase in both anterograde and retrograde movement of mitochondria, demonstrating that the dParkin/dPink1 pathway negatively regulates mitochondrial transport under basal conditions in *Drosophila* [98, 99]. Overexpression of dParkin or dPink1 has the converse effect, leading to increased pausing of mitochondria. This pausing is likely due to degradation of Miro by activated dParkin. Mitochondria targeted by the dPink1/dParkin pathway are in a sense taken off the microtubule tracks by Miro degradation leaving them in the stationary phase.

In mammalian cells, the PARKIN/PINK1 pathway does not regulate mitochondrial transport under basal conditions but does lead to decreased mitochondrial transport in response to mitochondrial damage [98, 99]. Following mitochondrial damage signaled by loss of the inner mitochondrial membrane potential, MIRO1/MIRO2 is degraded, and mitochondria become stationary in axons of cultured neurons, in a manner that depends on PINK1 and PARKIN expression. In the absence of mitochondrial damage, overexpression of PINK1 is sufficient to degrade MIRO1/MIRO2 and halt

mitochondria in neuronal axons, demonstrating that mitochondrial damage activates the PINK1/PARKIN pathway likely through the stabilization of PINK1 on mitochondria. Mitochondria with higher velocities tend to be those that fuse with other mitochondria, and MFN2 has been shown to affect mitochondrial transport supporting coordination between transport and fusion [104, 105]. Slowing down mitochondria by degrading MIRO1/MIRO2 has the effect of preventing their circulation throughout the neuron and (along with degradation of the OMM fusion proteins MFN1/MFN2) contributes to their isolation from the mitochondrial network, before they can be degraded by mitophagy.

8.6.3 Regulation of Mitophagy by the PINK1/PARKIN Pathway

In addition to isolating damaged mitochondria by preventing their fusion with healthy mitochondria and halting their transport, the PINK1/PARKIN pathway promotes the selective degradation of damaged mitochondria by macroautophagy. In contrast to inhibition of fusion and transport, which depend on the degradation of GTPases mediating these processes, the phospho-ubiquitination of OMM proteins provides a positive "eat-me" signal to promote mitophagy. Phospho-UB recruits the autophagy adaptor proteins OPTINEURIN and NDP52, which initiate the selective degradation of the dysfunctional mitochondria [106–108].

The ability of PARKIN to promote mitophagy in response to mitochondrial damage was first demonstrated dramatically in HeLa cells exogenously expressing PARKIN following treatment with the mitochondrial uncoupler CCCP [28]. Evidence of a robust increase in mitophagy could be detected within 1 h of treatment. Within 24 h all mitochondria were eliminated from most of the cells. The promotion of mitophagy by PARKIN in response to mitochondrial uncoupling was shown to be strictly dependent on PINK1 expression on the OMM, consistent with a model in which the induction of mitophagy by PARKIN is mediated by the stabilization of PINK1 in response to mitochondrial damage [19, 20, 31, 32].

The PARKIN/PINK1 mitophagy pathway is capable of mediating long-term surveillance of dysfunctional mitochondria—at least in cultured cells. PARKIN was overexpressed in cybrid cells containing a stable mix of wild-type and mutant mitochondrial DNA (mtDNA) genomes [55]. Over the course of months, cells stably overexpressing PARKIN selectively cleared mtDNA containing the deleterious mutation, leaving the cells with only wild-type copies of the mitochondrial genome. Thus, over time the PINK1/PARKIN pathway is capable of selectively identifying and degrading impaired mitochondria, thereby cleansing the mitochondrial network.

Although early reports focused on mitophagy in established cell lines, more recent studies have confirmed that mitophagy is also induced by mitochondrial damage in primary cells. In an elegant experiment, cultured mouse and rat neurons were grown in a microfluidic chamber which allowed the authors to selectively

apply pharmacological agents to only a segment of axon [57]. The application of the complex III inhibitor antimycin, which increases superoxide production and stochastically leads to mitochondrial uncoupling, caused a marked increase in the colocalization between LIGHT CHAIN 3 (LC3), a marker of autophagosomes, and mitochondria within the axonal segment exposed to the drug. The recruitment of autophagosomes to impaired mitochondria in the neuronal axons was strictly dependent on endogenous expression of Parkin and Pink1, as it was not observed in neurons from *Parkin* knockout mice or *Pink1* knockout rats. This study provides the clearest demonstration that PINK1/PARKIN-dependent mitophagy of damaged mitochondria occurs in axons.

Recent in vivo studies in mice suggest that PINK1/PARKIN-induced mitophagy may also be activated within the muscle damaged by denervation or ischemia. Denervation-induced muscle atrophy upregulates autophagy in mice. In mice lacking the essential autophagy gene *Atg7*, this stress leads to the accumulation of dysfunctional mitochondria, presumably due to a block in the autophagy pathway—a phenotype that Parkin knockout mice share [109]. Consistent with Parkin functioning upstream of Atg7 following this stress, Parkin accumulates on the damaged mitochondria that fail to clear in the $Atg7^{-/-}$ mice. Together these findings suggest that Parkin-induced mitophagy may be upregulated in muscle undergoing atrophy and may prevent the accumulation of dysfunctional mitochondria. Similar to denervation-induced injury of skeletal muscle, a Parkin-dependent pathway appears to protect against myocardial infarction. Parkin protein levels dramatically increase in the borderzone of the infarct. Additionally, borderzone mitochondria become ubiquitinated, and the autophagosome-associated LC3-II protein increases in wild-type mice but not in *Parkin* knockout mice, consistent with a role of Parkin in clearing dysfunctional mitochondria from the vulnerable borderzone [110]. Perhaps as a result of decreased mitophagy, larger infarcts result from the ischemic insult in $Parkin^{-/-}$ mice compared to $Parkin^{+/+}$ mice. A similar phenotype with an increased infarct size following an ischemic insult has been reported for the $Pink1^{-/-}$ mouse, suggesting that clearance of damaged mitochondria from the borderzone may be similarly affected in $Pink1^{-/-}$ mice [111].

In contrast to its effect on basal mitochondrial dynamics, the PINK1/PARKIN pathway appears to be critical for maintaining a high basal rate of mitophagy in mammalian cells. Studies of the basal rate of mitophagy have benefited greatly from the development of sensitive methods for measuring mitophagic flux. One such tool is the mitochondrial targeted pH-sensitive fluorescent protein mt-Keima. mt-Keima, which has differential emission spectra at the pH of the mitochondrial matrix (~7.8) and the pH of lysosomes (~4.8), ratiometrically reports on the proportion of mitochondria-targeted protein contained in the acidic lysosomal compartment. A block in the delivery of mitochondria to lysosomes would be predicted to decrease the pool of mt-Keima in the lysosomal compartment, as is observed in mouse embryonic fibroblasts (MEFs) lacking the essential autophagy gene *Atg5* [112]. When introduced into *Parkin* knockout MEFs, the ratio of lysosomal to mitochondrial mt-Keima was found to be reduced by about 40 % compared to wild-type MEFs reflecting a substantial decrease in the delivery of mitochondrial protein to

lysosomes [113]. Using the same mt-Keima probe, an independent study demonstrated that knockdown of Pink1 and Parkin in rat cortical neurons increases lysosomal mitochondria, consistent with the necessity of Pink1 and Parkin for efficient mitophagy under basal conditions in neurons [61].

The dependence of basal mitochondrial turnover on dParkin and dPink1 was demonstrated by a different method in *Drosophila. Drosophila* were fed food containing deuterium-labeled leucine, and the half-lives of mitochondrial proteins were calculated based on the percent incorporation of deuterium into mitochondrial proteins identified by mass spectrometry [69]. Compared to wild-type *Drosophila*, a significant increase in mitochondrial protein half-life (about 50 %) was observed in *Drosophila* lacking the essential autophagy gene *Atg7*, consistent with autophagy being a major pathway for mitochondrial protein turnover under basal conditions. *Drosophila* lacking dParkin similarly had increased mitochondrial protein half-lives compared to wild type (about 30 % increased), consistent with a requirement of dParkin to support efficient mitophagy under basal conditions in vivo.

Somewhat surprisingly, in this study a subset of mitochondrial proteins—enriched for membrane-bound respiratory chain proteins—had a turnover rate that was substantially greater than other mitochondrial proteins in *dParkin* knockout *Drosophila*. The *dPink1* knockout *Drosophila* closely resembled the *dParkin* knockout with a somewhat milder phenotype. This selective increase in the half-life of some mitochondrial proteins more than others was in contrast to the *Atg7* null flies, which showed a more uniform increase in mitochondrial half-lives. These findings suggest that under basal conditions, dParkin may support the turnover of a subset of mitochondrial proteins preferentially, consistent with a sorting mechanism allowing some mitochondrial proteins to be retained within mitochondria, whereas others are degraded in a dPink1/dParkin-dependent manner.

The finding that dPink1/dParkin supports selective mitochondrial protein turnover is interesting in light of a recent study suggesting that the PINK1/PARKIN pathway promotes the budding of vesicles from mitochondria that target to lysosome in response to mild mitochondrial stressors in mammalian cells [62]. This autophagy-independent pathway may allow for the degradation of proteins sensitive to damage without sacrificing the whole mitochondrion through mitophagy. A possibly related phenomenon was observed in neurons derived from iPSC from a patient harboring a deleterious mtDNA mutation. PARKIN was recruited to vesicles containing the mutated protein but not to other mitochondrial proteins, consistent with a mechanism for the selective degradation of damaged mitochondrial proteins in these cells, which involves PARKIN [70]. Although there is little direct evidence for the mechanism allowing the selective turnover of damaged mitochondrial proteins by the PINK1/PARKIN pathway, one attractive proposal theorizes that chaperones may amass damaged matrix proteins around a mitochondrial TIM/TOM transport complex. This may locally block PINK1 import (which depends on mitochondrial chaperones that have dual functions in import and quality control), thereby leading to local PINK1 OMM accumulation, PARKIN activation, and OMM protein ubiquitination in the vicinity of the blocked transport complex [114]. The mitochondrial import complexes (TOM and TIM) bridge the IMM and OMM

and thus may connect the UB signal on OMM proteins associated with TOM complex and damaged, misfolded matrix proteins associated with the TIM complex. The blocked TOM/TIM pore and its associated damaged proteins may then bleb off and traffic to the lysosome where the damaged contents can be degraded, leaving the mitochondrion with a decreased burden of oxidatively damaged proteins.

8.6.4 Molecular Basis for PINK1/PARKIN-Induced Mitophagy

The molecular mechanism by which PINK1 and PARKIN promote mitophagy has recently been defined at least in cell culture. In an elegant study, five of the six known autophagy adaptor proteins (p62, NBR1, OPTINEURIN, NDP52, and TAX1BP1) were knocked out of HeLa cells in different combinations, using CRISPR-mediated genome editing [107]. Double knockout of NDP52 and OPTINEURIN led to substantial inhibition of mitophagy that could be rescued by exogenous expression of either NDP52 or OPTINEURIN—a finding that was independently reported by a second group [108]. This suggests that NDP52 and OPTINEURIN have necessary but redundant functions in the PINK1/PARKIN mitophagy pathway and is partially consistent with an earlier report supporting a key role for OPTINEURIN in the PINK1/PARKIN mitophagy pathway [106]. TAX1BP1 was also found to have a more limited role in the PINK1/PARKIN pathway [107]. Consistent with prior studies using p62 knockout mouse embryonic fibroblasts (and in contrast to some earlier reports), p62 was found to be dispensable for mitophagy in this study [32, 115, 116]. Additionally, p62 was not sufficient for mitophagy in the absence of OPTINEURIN and NDP52, as exogenous expression of p62 in cells lacking OPTINEURIN and NDP52 could not be rescued. As was reported earlier, p62 was necessary and sufficient for aggregation of mitochondria downstream of PARKIN activation [115, 116].

Surprisingly, this study found that PINK1 was able to recruit NDP52 and OPTINEURIN (but not p62) even in the absence of PARKIN. In turn, NDP52 and OPTINEURIN were found to recruit LC3, which is resident to the developing autophagosome, and, independently, key components of the upstream autophagy machinery, ULK1, DFCP1, and WIPI1. This is consistent with an earlier report that LC3 and the early autophagy machinery are recruited independently in PARKIN/PINK1-mediated mitophagy [117].

Using a sensitive marker of mitochondria delivered to lysosomes (mt-Keima, described above), the study found that PINK1 accumulation on the outer mitochondrial membrane was sufficient to support mitophagy in the absence of PARKIN, albeit at a low level. In the presence of PARKIN, the mitophagic flux induced by PINK1 was greatly amplified, as would be expected given PARKIN amplifies the phospho-UB signal as reviewed above. NDP52 and OPTINEURIN (but not p62) were found to preferentially bind UB phosphorylated by PINK1, suggesting the mechanism for their recruitment by PINK1 and the subsequent induction of mitophagy.

Together these findings support a model in which phospho-ubiquitin, resulting from PINK1 phosphorylation and amplified by activated PARKIN, marks dysfunctional mitochondria for selective autophagic degradation, mediated by autophagy adaptors NDP52 and OPTINEURIN.

8.7 Summary

The beauty of the PINK1/PARKIN pathway is its specificity: its ability to pick out impaired mitochondria, isolate them from the mitochondrial network, and eliminate them by lysosomal degradation.

The details of this elegant pathway have been the subject of number of studies in the last decade and a coherent model has emerged. (1) PINK1 is stabilized on the surface of mitochondria that have lost their capacity for mitochondrial import. (2) On the surface of mitochondria, PINK1 phosphorylates UB as well as the UB-like domain (UBL) of PARKIN, both of which independently activate PARKIN. (3) Activated PARKIN, in turn, ubiquitinates additional mitochondrial proteins, providing further substrate for PINK1 phosphorylation in a feedforward cycle. The phospho-UB cycle is confined to the membrane containing PINK1 by the affinity of PARKIN for phospho-UB attached to proteins in the same membrane as PINK1. (4) Ubiquitination by PARKIN isolates the damaged mitochondria, through the degradation of GTPases necessary for mitochondrial fusion and transport, MFN1/MFN2 and MIRO1/MIRO2. (5) Finally, phospho-ubiquitination, initiated by PINK1 and amplified by PARKIN, induces the degradation of the impaired mitochondria in lysosomes through the recruitment of the autophagy adaptors OPTINEURIN and NDP52.

An attractive (but as of yet unproven) hypothesis is that loss of the PINK1/PARKIN mitochondrial quality control pathway in patients with *PARKIN* or *PINK1* mutations causes parkinsonism by allowing damaged mitochondria to accumulate in the highly oxidative environment of substantia nigra neurons [28]. Additional in vivo work, particularly in mammalian models, will help assess the importance of the PINK1/PARKIN mitochondrial quality control pathway in Parkinson's disease. The identification of druggable targets that modify this pathway, such as USP30, holds promise for the development of novel therapies for PD and will likely be a major focus of future research in academia and industry [61].

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Chapter 9
Mitochondrial Therapeutic Approaches in Parkinson's Disease

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Abbreviations

6-OHDA	6-Hydroxydopamine
ASYN	alpha-Synuclein
CoQ10	Coenzyme Q10
Cybrid	Cytoplasmic hybrid
DA	Dopamine
ETC	Electron transport chain
HDAC6	Histone deacetylase 6
LBs	Lewy bodies
MitoQ	Mitoquinone
MPP^+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,4-tetrahydropyridine
mtDNA	Mitochondrial DNA
PARL	Presenilin-associated rhomboid-like
PD	Parkinson's disease
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PINK1	PTEN-induced kinase 1
PPAR-γ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SNpc	Substantia nigra pars compacta
SOD	Superoxide dismutase
TPP	Triphenyl phosphonium

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9.1 Introduction: Mitochondrial Function Overview

Mitochondria are essential organelles of eukaryotic cells, composed of a double membrane, and are thought to have evolved from a symbiotic relationship between prokaryotic cells, in order to create a more favorable bioenergetic state [1, 2]. Mitochondrial shape and number differ in the various tissues and measure about 0.5–1 μm in diameter and up to 7 μm long. Mitochondria comprise some vital functions such as energy synthesis, in the form of ATP, from the breakdown of carbohydrates, fatty acids, and proteins that form the cofactors $FADH_2$ and NADH, and supply electrons to the electron transport chain (ETC). The transfer of electrons through ETC creates a gradient of protons (H^+) from the matrix to the intermembrane space, and the resultant energy is used by ATP synthase to phosphorylate ADP to ATP, in a reaction known as oxidative phosphorylation [3]. The production of ATP, through oxidative phosphorylation, is particularly important in the brain since it consumes a very high percentage of the resting energy production of our body [4, 5]. In fact, mitochondrial oxidative phosphorylation accounts for 87 % of the total ATP production [6], highlighting the importance of neuronal energetics in brain function and its potential involvement in brain pathology [7]. Due to the incomplete efficiency of the ETC, the release of free electrons results in the production of oxygen radicals, namely, superoxide anion ($O_2^{-\bullet}$), commonly referred to as reactive oxygen species (ROS). Superoxide anion is readily metabolized by mitochondrial antioxidant defenses, including superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD), glutathione, and glutathione peroxidase [8]. The constant metabolization of oxygen by mitochondria continuously produces ROS as a by-product [9], and despite their potential deleterious effects, evidence suggests that ROS are important signaling molecules, regulating intracellular signaling pathways including redox homeostasis and signaling transduction [10, 11]. The problem arises when ROS production is no longer neutralized by the antioxidant defenses, a condition known as oxidative stress. Unrestrained, excessive ROS production causes the damage of biomolecules including lipids, proteins, and DNA [12]. In the brain, mitochondrial dysfunction and depolarization are believed to be the cause of increased ROS associated with NMDA excitotoxicity, especially in neurons exposed to glutamate [8, 13]. Glutamate-induced neurotoxicity, through NMDA receptor activation, has been associated with increased intracellular calcium levels [14, 15]. Substantial accumulation of Ca^{2+} activates cascades that reach a no-return point into cell death by activating proteases such as calpains, initiating signal cascades that lead to the activation of caspases or lipases and nucleases [16]. In superoxide dismutase 1 (SOD1) transgenic mice, a model of amyotrophic lateral sclerosis, a fatal neurodegenerative disease, a significant decrease in Ca^{2+}-buffering capacity of mitochondria in spinal cord cells has been reported to occur presymptomatically [17]. In another study using the same transgenic mice, the enhancement of mitochondrial capacity of Ca^{2+} buffering rescued motor neurons from apoptosis [18].

In fact, the capacity of mitochondria to accumulate Ca^{2+} was first documented in the 1950s [19] and is controlled by a number of ion channels, pumps, and exchangers that drive the fluxes of Ca^{2+} across the mitochondrial membrane [20]. Mitochondrial Ca^{2+} uptake has a role of shaping cellular Ca^{2+} signals, for example, in excitable cells, such as neurons, mitochondria are localized nearby voltage-operated Ca^{2+} channels (VOCs) at the plasma membrane, and the uptake of Ca^{2+} controls the exocytosis of secretory vesicles during synapse [19, 21]. The activation of mitochondrial-driven cell death pathways occurs beyond Ca^{2+} overload and consequent excitotoxicity. Age-related ROS production is an early event in apoptosis initiation since mitochondrial ROS may cause the opening of permeability transition pore, causing cytochrome *c* and other proapoptotic factors to be released [22]. The implication of mitochondria in apoptosis regulation and the activation of apoptosis during aging, age-related mitochondrial ROS production, may contribute to the aging process [23], where mitochondrial dysfunction is a striking feature. Supporting this rationale, a number of studies have demonstrated that mitochondrial integrity declines with age [24–26]. Age-dependent mitochondrial abnormalities lead to the decline of mitochondrial function, namely, decreased oxidative phosphorylation and ATP synthesis mainly due to an increase in superoxide anion production and accumulation of mitochondrial DNA (mtDNA) mutations [27–29]. Data obtained with platelets from Alzheimer's disease (AD) patients, age-matched controls, and young control subjects showed that mitochondrial membrane potential was higher in young controls than in AD patients and aged control subjects [27]. These observations point to mitochondrial dysfunction occurring during aging and that can predispose elderly individuals to age-related disorders such as AD and Parkinson's disease (PD).

9.2 Mitochondrial Role in Parkinson's Disease

PD is a common progressive movement neurodegenerative disease, characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and by the presence of the histopathological hallmarks known as Lewy bodies (LBs), which contain ubiquitinated alpha-synuclein (ASYN) in surviving neurons [30, 31]. Accumulating evidence that is shared by both sporadic and familial PD posits mitochondrial impairment as a trigger of PD pathophysiology establishing mitochondria as an exciting therapeutic target [32]. Over the last decades, our understanding of the molecular etiology of PD has greatly increased, and a number of studies have placed mitochondria at the center of the PD pathology [32]. Mitochondria play a key role inside cells, as energy suppliers, intracellular calcium-buffering agents, endogenous ROS producers, cellular metabolism regulators via NAD^+/NADH ratio, and cell death controllers [33]. Therefore, a proper function of mitochondria is a major requirement.

9.2.1 Mitochondrial Dysfunction as the Trigger of Sporadic Parkinson's Disease

The first evidence of dysfunctional mitochondria in PD came from the discovery that accidental exposure to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), which is an inhibitor of the mitochondrial ETC complex I (NADH/ubiquinone oxidoreductase), caused PD-like symptoms in humans [34]. Later, a connection between MPTP toxicity and idiopathic PD was established by evidence of a deficiency in the complex I activity in SNpc of *postmortem* PD brains [35, 36]. In addition, complex I deficits were also found in peripheral cell models, such as lymphoblasts, fibroblasts, platelets, and muscle [37–42]. Moreover, *postmortem* SNpc of PD brains shows an increase of some oxidative stress markers [43] and a decrease in glutathione levels [44]. Recently, mitochondria were also linked to the high frequency of atypical parkinsonism in the French island of Guadeloupe [45–47].

In order to address these observations, King and Attardi [48] developed cytoplasmic hybrid (cybrid) cell line, which contains the same nuclear background but mtDNA from sporadic PD (sPD) patients [49]. Studies using this cell line—sPD cybrids—reported a complex I deficit, decreased ATP levels [49–54], a mitochondrial membrane depolarization, a less mitochondrial cytochrome *c* [50], an increased ROS production [51], and an alteration in the mitochondrial metabolism [55]. In line with these observations, mouse models with mtDNA gene defects demonstrate respiratory chain deficiencies [56–58]. For instance, MitoPark mice with a dopamine (DA) neuron-specific knockout of the gene for mitochondrial transcription factor A (TFAM) was designed to directly test the mitochondrial dysfunction hypothesis. This model was shown to develop slow progressive parkinsonism phenotype in DA neurons, mimicking the key features of PD [59]. TFAM is a key activator of mitochondrial transcription as well as a participant in mitochondrial genome replication. Loss of TFAM in the midbrain dopaminergic neurons of these mice results in mtDNA depletion and abolishes mtDNA expression, causing severe respiratory chain deficiency.

Similarly, the expression of the mitochondrially targeted PstI restriction enzyme, in DA neurons, results in deficits in the mtDNA, with consequent progressive neuronal degeneration and striatal DA decrease [60]. Consistently, defects in mtDNA have been found in SNpc dopaminergic neurons of PD patients [61–64].

Further evidence of the mitochondrial involvement in PD pathology arose from studies using rotenone and paraquat pesticides that appear to reproduce some of the PD features in animal models [65, 66]. Moreover, mitochondrial-mediated microtubule disorganization has also been shown to occur in PD [67]. In fact, mitochondrial toxins, such as 1-methyl-4-phenylpyridinium (MPP^+) (toxic metabolite of MPTP), 6-hydroxydopamine (6-OHDA), and rotenone, have been found to decrease mitochondrial mobility [68–71] and impair the microtubule dynamics [72–74]. Mitochondrial motility is essential for transport to areas within the cell with increased energy or calcium-buffering requirements [67]. Impaired microtubule

dynamics were observed in sPD cybrids [75], and mitochondrial calcium buffering was decreased [32]. In addition, sPD cybrids also show an accumulation of autophagic vacuoles and ASYN [53, 75]. Autophagosome accumulation was equally found in Rho0 cells (cells without mtDNA), primary cortical neurons treated with mitochondrial toxins, differentiated sPD cybrids, and PD patient lymphocytes [53]. At last, SNpc neurons of PD patients also have an abnormal increase of autophagosomes [76]. The mitochondrial fission and fusion process is also well known to be imbalance in PD [77–80]. In particular, sPD cybrids display an impaired mitochondrial fission [81], which is required to dilute damaged macromolecules and to prevent mitochondria from excessive enlargement that decreases its ability to be degraded by mitophagy [82]. Taking into account the existing data, we proposed the mitochondrial cascade hypothesis to explain the etiology and pathogenesis of sporadic PD [32].

9.2.2 Mitochondrial Involvement in Familial Parkinson's Disease

Despite that the majority of PD cases are sporadic with a complex multifactorial onset, 10 % of the cases are due to inherited single-gene mutations [83, 84]. Mitochondrial involvement in PD gained renewed importance with the discovery and characterization of mutations resulting in loss of function of PTEN-induced kinase 1 (PINK1), Parkin, and DJ-1 [85, 86]. It is noteworthy that the clinical phenotypes arising from mutations in *parkin*, *pink1*, and *dj-1* are indistinguishable [87]. This fact is likely to occur due to the role of the referred proteins in mitochondrial quality control [88]. Mitophagy is a mitochondrial quality control that insures healthy mitochondrial pool maintenance. The failure in this mechanism has been linked to the pathogenesis of several neurodegenerative disorders, including familial and sporadic PD [89]. Mutations in *parkin* gene were the first mutations identified as causing PD and are the most common [87]. Parkin is a RING finger protein acting as an ubiquitin E3 ligase [90] that induces mitophagy by ubiquitination of dysfunctional mitochondria. Histone deacetylase 6 (HDAC6) and p62 are recruited to assemble autophagy machinery and eliminate dysfunctional mitochondria, which is impaired in parkin mutants [91]. Parkin function in the cell has been investigated in a variety of models: deficient cells show decreased mitochondrial biogenesis and parkin-null mice show reduced mitochondrial respiratory capacity along with nigrostriatal abnormalities [92, 93]. Further *parkin* mutant *Drosophila* larvae have evident locomotion impairment, with reduced synaptic potentials and decreased ATP production and oxygen consumption [94]. Parkin does not act alone on deciding mitochondrial fate through mitophagy. *Pink1* gene mutations are another cause for autosomal recessive familial PD and are the second most common mutation causing early-onset PD [95]. It was shown that full-length PINK1 accumulates in the outer membrane of dysfunctional mitochondria, dependent on

loss of mitochondrial membrane potential [96], whereas in healthy mitochondria, with normal membrane potential, PINK1 levels are very low as it is cleaved by presenilin-associated rhomboid-like (PARL) and consequently degraded by proteasome in the cytosol [97, 98]. Parkin is then recruited to mitochondria, ubiquitinating mitochondrial substrates initiating mitophagy [96, 98]. During mitochondrial insult, PINK1, which has a serine/threonine kinase activity [99], is targeted to mitochondria, and when mutated, the impaired phosphorylation of its substrates causes mitochondrial cristae fragmentation and increased susceptibility to oxidative stress [95]. It is hypothesized that PINK1 phosphorylation of mitochondrial proteins protects against mitochondrial dysfunction, in response to an insult [95]. In human dermal fibroblasts after an insult with valinomycin, Parkin translocation to mitochondria occurs only in healthy controls in opposition to *pink1* mutation carriers [100]. Moreover, morphological abnormalities and loss of mitochondrial membrane potential were described in human-derived cell lines transfected with PINK1 RNAi [101]. Another study using patient fibroblasts found decreased mitochondrial membrane potential as well as decreased ATP content when compared with age-matched controls, with a prominent deficiency on complex I, which could be rescued by phosphomimetic NdufA10 [102]. Recently, it was described that PARL, a mitochondrial protease, is responsible for PINK1 cleavage [103]. The role of *PARL* gene for PD was accessed and a novel missense mutation in PARL's N-terminus was found only in PD patients [103]. Although no validation as a disease-causing variant has occurred, it was demonstrated that the amino acid substitution affects PARL's biological activity and ultimately impairs Parkin recruitment [103] and the elimination of damaged mitochondria. In agreement to what was observed in cells with *parkin* and *pink1* mutations, *dj-1* mutations give rise to an autosomal recessive early-onset parkinsonism [104]. DJ-1 mutant models and patient fibroblasts show mitochondrial abnormalities, namely, increased fragmentation and impaired mitochondrial dynamics [105–107]. In primary cortical neurons, *dj-1* mutant overexpression induces mitochondrial fragmentation accompanied by bioenergetic failure and increased susceptibility to H_2O_2 or MPP^+ exposure [106]. A number of studies have shown the protective role of DJ-1, namely, in DJ-1-deficient mice challenged with MPP^+ that show exacerbated dopaminergic neuron loss and are more susceptible to oxidative stress insults [108]. Despite that the exact function of DJ-1 has not yet been disclosed, it is hypothesized that it may be involved in cellular responses to oxidative stress [104]. DJ-1 has been implicated in neuroprotection against oxidative stress by promoting ERK1/ERK2 signaling pathway [109]. Recently, DJ-1 was described to interact with paraoxonase-2 (PON2) [110], a protein ubiquitously expressed in a wide variety of tissues [108] involved in preventing oxidative stress [111]. DJ-1 interaction with PON2 promotes its activity against oxidative damage promoting cell survival [108].

9.3 Targeting Mitochondria as a Therapeutic Strategy

Current therapies for PD have been proven ineffective over time and efficacy decays as the clinical symptoms of the disease progress. Overall, the availably of these therapies does not stop the degeneration that underlies PD pathology. The quest for therapies that help restore mitochondrial function is ongoing, and the ultimate goal is to slow or arrest the progression of this disabling disorder.

For quite some time, antioxidants have been suggested to be an interesting therapy for the treatment of PD. Many of these strategies include the addition of antioxidant supplements to the diet. Creatine, for instance, is a nitrogenous compound that is generated endogenously in muscle and nerve cells or acquired exogenously through the diet. Intracellular phosphorylation of creatine by creatine kinase generates phosphocreatine, which can be used to generate ATP and has antioxidant properties. In fact, oral supplementation of creatine protects against neuronal striatal DA depletion and loss of SNpc tyrosine hydroxylase immunoreactive in an MPTP mouse model of PD [112, 113]. Creatine treatment also provides neuroprotection in ventral mesencephalic neurons exposed to MPP^+ [114]. Based on these results, the National Institutes of Health selected creatine for an efficacy trial for PD. Patients with early-stage symptomatic PD are given daily administration of creatine (10 g day^{-1}) and evaluated for 5 years [115].

Polyphenols are low-molecular-weight secondary plant metabolites that are consumed by humans in the forms of fruits, vegetables, and beverages such as tea, coffee, and red wine. These metabolites show antioxidant, ROS scavenging, and metal-chelating activities [116]. Not surprisingly, habitual intake of polyphenol-rich foods reduces PD risk [117]. Several polyphenols have been shown to protect against MPTP toxicity by preserving tyrosine hydroxylase-positive neurons in the SNpc, by increasing antioxidant activity and DA levels in the striatum, and by improving motor performance [118–121]. In transgenic *Drosophila* expressing human ASYN, polyphenol-rich extract from whole grape improved their climbing ability [122]. Moreover, a polyphenol component of red wine decreased ROS production protecting dopaminergic neuronal cells against MPP^+ [123]. More interestingly, resveratrol, a plant-derived polyphenol present in red grapes, giant knotweed rhizome, and peanuts, has been shown to alleviate 6-OHDA-induced mitochondrial dysfunction in dopaminergic neurons of rat and to protect cells from mutated ASYN toxicity [124, 125]. Additionally, Blanchet and colleagues revealed that a diet rich in resveratrol can counteract the toxic effects of the neurotoxin MPTP [126]. Remarkably, besides its antioxidant and anti-inflammatory properties, resveratrol stimulates the activity of sirtuin-1, an NAD^+-dependent deacetylase that regulates mitochondrial function, cellular metabolism, energy metabolism, cell survival, and aging [127]. Moreover, the activation of sirtuin-1 by resveratrol can promote activation of various transcription factors and coactivators including peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), the master regulator of mitochondrial biogenesis [128]. In diseases like PD, where impaired mitochondria are implicated in pathology, resveratrol-induced mitochondrial biogenesis and

antioxidant effects would theoretically improve mitochondrial function. Nevertheless, resveratrol is not specific and activates other sirtuins; therefore, these results should be analyzed with caution. Likewise, quercetin is found in many fruits, vegetables, leaves, and grains, and it is also a sirtuin-1 activator [129]. This sirtuin-1 activator significantly increases levels of DA, glutathione, and SOD and preserves the number of striatal neurons after 6-OHDA treatment [130].

Another dietary supplement that shows neuroprotective effects is nicotinamide which is an NAD^+ precursor and sirtuin-1 inhibitor. In two MPTP mouse models of PD, nicotinamide prevented cell loss and striatal DA depletion [131]. Furthermore, NAD was used as medication in 885 parkinsonian patients in an open label trial and 80 % of the patients showed improvement in symptoms [132]. Nevertheless, a larger trial would be necessary to establish effectiveness of this treatment strategy.

Vitamin E, an antioxidant that shows neuroprotective potential, protected against iron and MPTP-induced neurodegeneration in mice [133] and attenuated oxidative stress after the administration of MPTP [134]. In 6-OHDA lesioned rats, vitamin E administration exerts a prompt protective effect on the nigrostriatal dopaminergic neurons [135]. Moreover, exposure of dopaminergic neurons to vitamin E blocks rotenone-induced oxidative damage and loss of tyrosine hydroxylase-positive neurons and ameliorates motor functions [136, 137]. Vitamin E also blocks paraquat-induced apoptosis in cerebellar granule neurons [138]. The protective effect of vitamin E administration also has been observed in the locus coeruleus in an early model of PD [139]. Additionally, the formation of ASYN inclusions and mitochondrial alterations can be attenuated by pretreatment with vitamin E [140]. In 2002 a large cohort study demonstrated that high dietary vitamin E intake reduced the risk of PD [141]. However, double-blind, randomized controlled trials have shown no benefits in PD [142, 143].

Coenzyme Q10 (CoQ10) is found in mitochondrial membranes, is an electron transporter in the mitochondrial respiratory chain, and may additionally function as an antioxidant in mitochondria and lipid membranes [144]. CoQ10 has been reported to prevent the loss of dopaminergic neurons and to slow the progression of PD in nonhuman primates and aged mice exposed to MPTP [145–147]. Furthermore, CoQ10 remarkably reduced the selective death of dopaminergic neurons and mitochondrial depolarization in rat mesencephalic primary neurons exposed to rotenone [148]. A more recent study reported that CoQ10, given orally as Ubisol-Q10 in drinking solution, was effective in blocking the progression of neurodegeneration induced by paraquat [149]. However a phase III randomized, placebo-controlled, double-blind clinical trial demonstrated that CoQ10 was safe and well tolerated, but that it showed no evidence of clinical benefit [150].

Other nutritional strategies showing neuroprotective effects include a ketogenic diet that is a high-fat diet in which carbohydrates are almost eliminated from the regimen [151]. Indeed, infusion of the ketone body D-*beta*-hydroxybutyrate in mice protects dopaminergic neurons and improves motor deficits induced by MPTP and 6-OHDA toxicity [152, 153]. These neuroprotective effects are believed to result from an increase in energy storage (which can ameliorate the ability of neurons to adapt and resist to metabolic challenges), as well as their antioxidant effects [154].

Furthermore, ketone bodies are used preferentially by the mitochondrial respiration bypassing the glycolytic pathway, which would allow the selection of cells with wild-type mtDNA molecules [155].

Potential reasons for the failure of these trials may include the inability of the treatment molecule to cross the mitochondrial membrane, since many of these therapeutics do not have the structural components necessary to cross it; also, the concentration that reaches the brain may not be enough to interfere with the disease process. Although these compounds can cross the blood–brain barrier, they often show poor intestinal absorption and limited bioavailability even with new formulations. Indeed, one of the major limitations of these compounds is that only a small fraction is taken up by mitochondria having access to the cytosol with little impact on mitochondria. As a result great effort has been made to develop antioxidant therapies targeted to mitochondria and so becoming a hot topic in the field [156]. Overall, this strategy consists of encapsulating drugs or DNA into a carrier that is targeted to mitochondria, such as lipophilic cations or mitochondrial-targeted peptides.

Some of these examples are mitoquinone (MitoQ), MitoVitE, and Mito TEMPO. These mitoconjugates are drug molecules that are covalently bound to mitochondriotropic moieties, enabling their selective delivery to the mitochondria, such as the lipophilic triphenyl phosphonium (TPP). These cations can move through membranes and accumulate in the mitochondrial matrix in response to the negative mitochondrial membrane potential. One example, MitoQ, consists of a TPP cation covalently bound to a ubiquinone moiety of CoQ10 that accumulates selectively in the mitochondria. Treatment with MitoQ prevents mitochondrial fragmentation, oxidative stress, mitochondria-induced apoptosis, and loss of dopaminergic neurons and terminals in the nigrostriatum and reverses the loss of DA and its metabolites; it also improves locomotor ability in MPTP and 6-OHDA models of PD [157, 158]. However, MitoQ shows a narrow window between antioxidant and prooxidant properties that should be taken into account [159]. In fact, a double-blind clinical trial failed to prove that MitoQ could halt the clinical progression of PD [160]. MitoTEMPO and MitoVitE are also a TPP cation coupled to the redox-cycling nitroxide TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) or to vitamin E, respectively. Mitochondrial targeting of quercetin and resveratrol has also been described. In this case these compounds were conjugated with a butyl-TPP [161]. Unfortunately, their efficacy and therapeutic potential to PD have not yet been evaluated.

Differently than these mitoconjugates, the Szeto–Schiller (SS) peptides are cell-permeable, positively charged, and aromatic–cationic peptides directed to the inner mitochondrial membrane [162]. However, despite its positive charge, it seems that SS peptides accumulate into mitochondria regardless of the membrane potential and currently the mechanism is not known [162]. These peptides have free radical scavenging activity, which is likely to originate from their tyrosine residues, and have been shown to reduce mitochondrial ROS production and prevent mitochondrial depolarization [163]. They have been described to protect against MPTP neurotoxicity in mice and to prevent MPP^+-induced inhibition of mitochondrial respiration in isolated mitochondria [164].

In addition to these, other peptides named XJB have been formulated. These peptides are composed of an electron and ROS scavenger (4-NH2-TEMPO) conjugated to the Leu-^{D}Phe-Pro-Val-Orn fragment of gramicidin S as the mitochondrial targeting portion, which has SOD mimetic activity [165, 166]. Overall, the gramicidin peptide acts as the targeting moiety, and the conjugated free radical acts as the ROS scavenging drug.

To counteract the previously described problem with MitoQ, another alternative termed "SkQs" has been developed by Skulachev and coworkers. In this case, plastoquinone is used to replace the ubiquinone moiety of MitoQ because it is a more effective antioxidant and it has less prooxidant activity [167]. These compounds have been shown to be mitochondrial-targeted antioxidants of very high efficiency and specificity that can be used to prevent ROS-induced oxidation of lipids and proteins in the inner mitochondrial membrane in vivo [168]. Interestingly, another study demonstrated that SkQs prolong lifespan and slow the development of senescence traits [169].

Another approach to targeting mitochondria with small bioactive molecules is through polymer-based nano-carriers that are capable of encapsulating biologic molecules of interest for drug delivery [170]. One example involves biodegradable poly-lactide-co-glycolide (PLGA) nanoparticles, which include, for example, the PLGA-CoQ10 nanoparticles [171]. However, the biological efficacies of these CoQ10 nanoparticles remain to be investigated.

The mitochondrial cascade hypothesis posits that mitochondrial dysfunction starts PD pathologic changes observed in both late-onset and early-onset PD, as a result of inherited polymorphic mtDNA variation, in the case of late-onset PD or due to mutation in genes that regulate mitochondrial function and dynamics in the case of early-onset PD. In both cases mitochondrial function declines over time, and when a certain threshold is overcome, PD-related neurodegeneration arises [32]. Taking this into account, mitochondrial gene therapy strategies that involve the delivery of wild-type mtDNA into the mitochondrial matrix or the selective inhibition of the mutant mtDNA replication are promising. Indeed, inhibition of mutated mtDNA replication by gene therapy has been attempted by Taylor et al. [172].

Mitochondriotropic nanotechnology, in which nanovesicles such as DQAsomes (DeQuAlinium-based liposome-like vesicles) for mitochondrial targeting are used, has gained more attention [173]. This technology has been proving their efficacy in delivering both drug and DNA to the mitochondria. For instance, DQAsomes are cationic bola-lipid vesicles with a unique mitochondrial-targeted drug carrier formed of a dicationic compound "dequalinium" that accumulates inside mitochondria of living cells in response to the mitochondrial membrane potential [174]. Other examples are MITO-Porter which is a liposomal-based nano-carrier that selectively delivers cargos into mitochondria via a membrane fusion mechanism and multifunctional envelope-type nano-device (MEND), a gene delivery system that allows packaging of macromolecules [175].

A recent improvement in MITO-Porter has been developed that incorporates mitochondrial fusogenic inner and endosome-fusogenic outer envelopes that encapsulate a nanoparticle of cargo; it has a much higher mitochondrial delivery than the conventional MITO-Porter [176]. These systems are under investigation for anticancer chemotherapy, but they may also be applied to neurodegenerative disorders such as PD, where mitochondrial impairment has a pivotal role.

Another therapeutic target prospect in PD is the peroxisome proliferator-activated receptor gamma (PPAR-γ). PPAR-γ is a member of nuclear receptor superfamily that is known to regulate several functions including mitochondrial function [177]. Because PPAR-γ has extensive cellular distribution and diverse biologic functions, it is difficult to define a unique cellular target for its neuroprotective activity. Recently, PPAR-γ agonists, such as pioglitazone and rosiglitazone, have been described as possible targets for PD treatment, since there is evidence that genes responsible for controlling cellular bioenergetics and that are expressed in response to PGC1α are downregulated in dopaminergic neurons of PD patients [178]. PGC1α activates a set of metabolic programs in different tissues and regulates mitochondrial biogenesis, respiration, and the metabolic status of cells [179]. Importantly, the regulation of mitochondrial biogenesis holds a crucial role in the pathophysiology of mitochondrial dysfunction [128]. The PPAR-γ agonist pioglitazone augments mtDNA content, oxygen consumption, expression of factors involved in mitochondrial biogenesis (such as PGC1α and mitochondrial transcription factor A, TFAM), glucose metabolism, and mitochondrial function in several tissues and cell lines [180–182], whereas rosiglitazone induces mitochondrial biogenesis and glucose utilization in mouse brain [183]. Pioglitazone was reported to prevent nigral degeneration, DA loss in the striatum, and cognitive and motor impairment in acute and subacute models of PD in mice, rat, and nonhuman primates [184–188]. Pioglitazone is, in fact, an FDA-approved agent for the treatment of type II diabetes and acts to reduce insulin resistance [189]. Hence, pioglitazone entered in a clinical trial that was finalized in February 2015 (NCT01280123); however, the results are still not available. Similarly, rosiglitazone has been shown to protect from nigrostriatal damage and prevent olfactory and motor deficits in MPTP models of PD [190–192]. Notably, this drug treatment was able to halt neurodegeneration during the progressive degenerative process, resembling what happens in human PD, suggesting that PPAR-γ agonists could be a putative disease-modifying benefit in PD [192]. However, rosiglitazone has been linked to an increased cardiovascular risk in diabetic patients by a meta-analysis study [193]. Two other PPAR-γ agonists L-165041 and GW-501516 demonstrate neuroprotective efficacy in in vitro and in vivo PD models [194]. Furthermore, another PPAR-γ agonist named fenofibrate prevents dopaminergic cell death in an MPTP mouse model of PD [195].

Until now, none of these therapeutics have proven to have an unequivocal disease-modifying effect in PD trials. But there are still many promising untested therapeutics that may alter PD progression.

9.4 Concluding Remarks

PD is the second most common neurodegenerative disorder that is strongly associated with aging, increasing exponentially in incidence above the age of 65 [196]. The incidence of PD is expected to rise dramatically worldwide in the next 25 years with the extension of life expectancy by improved health care. Although there are signs of distributed neuropathology (as judged by LB formation) [197], the motor symptoms of PD, including bradykinesia, rigidity, and resting tremor, are clearly associated to the degeneration and death of SNpc dopaminergic neurons [198]. The determination that those cardinal manifestations are primarily due to a profound depletion of DA in the striatum led to the development of rational therapies aimed at correcting this deficiency [199–201]. However, despite several breakthrough discoveries in symptomatic PD therapy, the current therapies are palliative at best and just provide effective control of symptoms, particularly in the early stages of the disease [202]. Motor complications such as the wearing-off phenomenon (the return of PD symptoms too soon after a given levodopa dose), the presence of involuntary abnormal movements (dyskinesias and dystonia), and treatment-resistant symptoms such as gait impairment, cognitive decline, autonomic dysfunction, and medication-induced psychosis occur in the middle and late stages of disease [203]. Clearly, the current symptomatic therapies cannot completely improve later-stage symptoms and fail to halt the degeneration process in the dopaminergic and nondopaminergic systems. This indeed emphasizes the urgency of developing a more effective therapeutic for PD patients. However, a major hurdle for the development of neuroprotective therapies is the restricted understanding of disease causes and mechanisms leading to death of dopaminergic neurons.

While the etiological factors involved in the development of PD are still uncertain, a combination of genetic susceptibilities and environmental factors seems to play a critical role. Nevertheless, over the course of the past decade, remarkable advances have been made in the identification of genes associated with familial forms of PD. Although familial PD is relatively rare compared with idiopathic disease, the associated genes provide an opportunity to gain important insights into molecular pathways that lead to parkinsonism and that may be important in sporadic PD. Recent evidence seems to converge on mitochondria as a primary target in the process of dopaminergic neuronal loss observed in PD. Mitochondrial metabolism and energy production impairment are certainly two important causes, and the study of the protein products involved in genetic forms of PD deeply contributes to extension of our knowledge on this topic. It seems evident that mitochondrial function is a major determinant of cellular physiology. In addition, mitochondrial fusion and fission are not isolated in the cell, and other intrinsic cellular alterations, such as impaired vesicular trafficking and axonal transport, intracellular degradation systems, and mitochondrial metabolism, have also been described in PD; any of these could further alter mitochondrial biogenesis, turnover, and maintenance.

The mitochondrial cascade hypothesis [32] may explain the pathophysiology of familial and sporadic PD. In idiopathic PD cases, a complex I defect may lead to

alterations in mitochondrial-dependent metabolism (reduced ATP levels and decrease in mitochondrial membrane potential). A bioenergetic failure seems to play a role in microtubule network breakdown leading to microtubule disassembly, which undermines anterograde and retrograde flux along the axon, leading to defects in the supply and clearance of mitochondria. In familial PD cases, mutations in proteins that regulate mitochondrial quality control will forfeit the clearance of dysfunctional mitochondria with the obvious consequences to the cell (having a dysfunctional mitochondrial pool). Our premise implies that mitochondrial demise may be the initial event in sporadic PD, although it has a prominent role in some of PD familial forms.

While it may be difficult to decipher the individual contribution of the different molecular pathways affected by mitochondrial dysfunction, our findings reinforce the concept that restoration of its function is indeed able to provide neuroprotection against PD-related dopaminergic neurodegeneration and may thus represent a potentially beneficial therapeutic strategy for PD.

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Chapter 10
Altering Mitochondrial Fusion and Fission Protein Levels Rescues Parkin and PINK1 Loss-of-Function Phenotypes

Lori M. Buhlman

10.1 Mechanisms of Mitochondrial Fission

Vital functions performed by mitochondria for cells include ATP production and delivery, Ca^{2+} buffering, and apoptosis induction. Thus, the effects of functionally relevant mutations in nuclear or mitochondrial DNA that affect mitochondrial proteins tend to manifest in the nervous system, as it constantly requires disproportionately high levels of ATP and generally does not replace lost neurons or does so at a relatively slow rate. Mitochondria continually and efficiently produce ATP and balance Ca^{2+} levels by maintaining optimal levels of functional proteins and by eliminating damaged, oxidized proteins, lipids, and mitochondrial DNA. Mitochondrial fusion and fission facilitate the dynamic morphological changes required for optimal function. Mitochondrial fission is necessary during cell division and for maintaining optimal levels of mitochondrial motility, which is required for delivery of ATP and Ca^{2+} buffering capabilities to regions of cells that can be functionally distinct and up to 1 m from the soma, as in some lower motor neurons. Fission is also an integral step in mitophagy, a quality control mechanism by which dysfunctional, burdensome regions of a mitochondrion are separated from the organelle and directed to the lysosome by autophagosomes. The mechanisms of mitochondrial membrane fission are relatively simpler than those for fusion, and directly participating proteins are fewer. The mammalian homologue of the dynamin-like fission-promoting GTPase is cytosolic dynamin-related protein 1 (Drp1), which exists in at least six isoforms that have tissue-specific expression. Drp1 is ubiquitously expressed, with particularly high levels observed in the brain, skeletal muscle, heart, and kidney [1]. Drp1 is the only known protein involved in the mechanochemical separation of mitochondria; thus, it is not surprising that loss of just one functional

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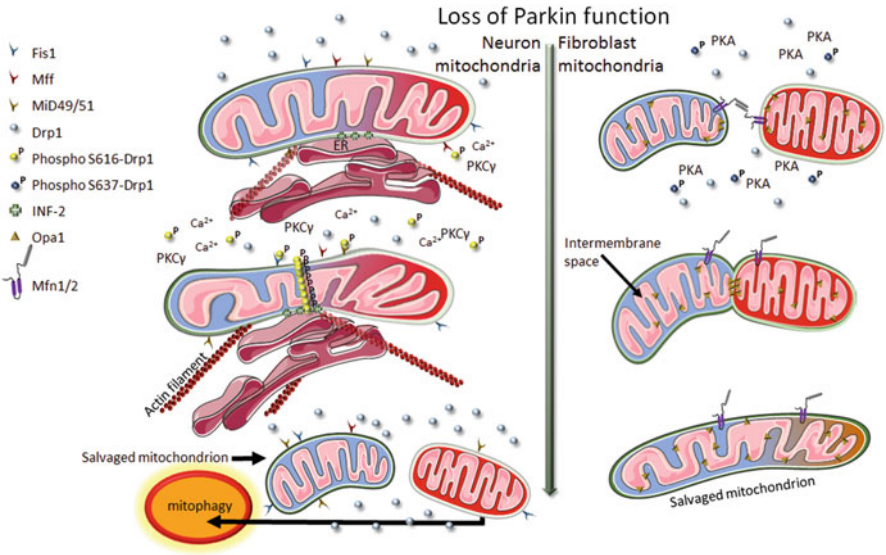

Fig. 10.1 Effects of Parkin loss-of-function on mitochondrial network dynamics may depend on the cell's energy demands and morphology. Loss of Parkin expression causes disruptions in mitochondrial function, increased outer membrane permeability, and loss of inner membrane potential, as indicated by the blue (healthy) to red (unhealthy) gradient. Parkin is expressed in many different cell types; however, its loss of function may selectively affect neurons. High ATP requirements and extensive aborizations may make neurons exceptionally sensitive to Parkin loss-of-function, which may manifest in increased Drp1 activation, recruitment to mitochondria, mitochondrial fission, mitophagy, and subsequent apoptosis. In contrast, for the less ATP-consuming fibroblast, the effects of loss of Parkin function may be surmountable if damaged mitochondria fuse with healthy mitochondria, mixing contents and thus "buffering" the increase in macromolecule oxidation. In the former case, mitochondria/endoplasmic reticulum associations with actin and INF-2 precede Drp1 recruitment and binding to Fis1, Mff, and MiD49 or MiD52 mitochondrial receptors. Drp1 recruitment and receptor binding can be enhanced by PKCγ-mediated phosphorylation at S616. In the latter case, outer mitochondrial membrane mitofusins 1 and 2 (Mfn1/Mfn2) interact to promote outer membrane fusion. Inner membrane fusion protein Opa1 facilitates fusion of the inner mitochondrial membrane, facilitating efficient mixing of mitochondrial contents and dilution of oxidized macromolecules. During fusion, Drp1 may be made less active by PKA-mediated S637 phosphorylation

Drp1 allele causes lethality during development [2]. Drp1 is recruited to the outer membrane by various signals including membrane depolarization to promote fission in a manner that has been described in detail for the yeast homologue, dynamin 1 (Dmn1) [3] (Fig. 10.1). In general, upon recruitment to mitochondria, Dmn1 self-oligomerizes, wraps around mitochondria perimeters, and contracts, separating outer and inner membranes to create two daughter organelles. Contact between mitochondria and the endoplasmic reticulum, as well as constriction of mitochondria by formin-like protein inverted formin 2 (INF-2) at fission sites prior to Drp1 recruitment, also has been described, implicating a role for actin in orchestration and initiation of mitochondrial fission events [4]. Upon recruitment, Drp1 is thought

to bind to a host of putative outer membrane-bound receptors, including fission protein 1 (Fis1), mitochondrial fission factor (Mff), mitochondrial dynamics proteins of 49/51 kDa (MiD49/MiD51), and ganglioside-induced differentiation-associated protein 1 (GDAP1) [5–10]. While these receptors seem to confer partially redundant functions, recent evidence suggests that they may mediate fission initiated by specific signals under particular circumstances such as initiation of movement or mitophagy [11]. Activity of Drp1 can be regulated by several serine–threonine kinases, and the effect of phosphorylation on activity seems to depend on the Drp1 variant and the target serine residue. Phosphorylation of Drp1 isoform 1 at Ser616 by protein kinase C δ [12] has been shown to induce fission activity, implicating Ca^{2+} as a pro-fission signaling molecule. Phosphorylation by CDK1/cyclin B on Ser616 leads to increased fragmentation in mitotic cells [13], but not in cultured HeLa cells [14]. Phosphorylation at Ser637 by protein kinase A (PKA) seems to decrease fission events by reducing the affinity of Drp1 for its outer membrane-bound receptors [14]; cycles of PKA and calcineurin phosphatase activity at Ser367 may contribute to Ca^{2+}-mediated regulation of fission rates [15]. Interestingly, CaMK-Iα phosphorylation of human Drp1 isoform 3 and ROCK kinase phosphorylation of mouse isoform b at serine residues that correspond to Ser637 of human Drp1 isoform 1 promote fission [16, 17] by increasing Drp1 recruitment to mitochondria [17]. Phosphorylation at Ser693 by GSK3β also has been shown to decrease Drp1-mediated fission [18]. Other forms of regulatory posttranslational modification of Drp1 have been reported and warrant further investigation.

10.2 Mechanisms of Mitochondrial Fusion

Many intracellular reactive oxygen species (ROS) are products of oxidative phosphorylation, and under normal conditions, local antioxidants like manganese superoxide dismutase (MnSOD) and reduced glutathione (GSH) maintain homeostatic levels of mitochondrial ROS. However, mitochondrial respiratory chain enzyme function can become compromised by mutation, oxidation or other posttranslational modifications, and/or interactions with other proteins, causing an increase in ROS production (reviewed in [19]). If ROS levels exceed homeostatic levels, proximal proteins, lipids, and mitochondrial DNA can incur oxidative damage, causing them to function improperly, potentially exacerbating the imbalance of ROS. One mechanism by which mitochondria buffer the effects of molecule damage is by fusing together and "sharing" properly functioning molecules (Fig. 10.1). Fusion events, mediated by conserved GTPases, can continually occur at an average rate of 0.023 and 0.045 fusions/mitochondria/min in cultured rat hippocampal neurons and cerebellar granule cells, respectively [20]; the process is so efficient that the contents of matrices in an entire mitochondrial network can be changed within 12 h in cultured HeLa cells [21]. The fusion process initiates when outer membrane-bound GTPases mitofusins 1 and 2 (Mfn1, Mfn2) interact with one another to tether organelles to one another, ultimately forcing adjacent outer membranes to fuse. Subsequently, inner

membrane-associated Optic atrophy 1 (Opa1, a GTPase) facilitates fusion of the inner membrane, and soluble matrix molecules are exchanged (Fig. 10.1). Mitofusins 1 and 2 require ATP to confer redundant and independent functions that are important for embryonic development [21, 22]. Under stress conditions in which Drp1-mediated fragmentation occurs in order to promote mitophagy, mitochondrial fusion seems to be downregulated; evidence suggests that Mfn1 and Mfn2 are directed to the proteasome after ubiquitination by E3 ligases including Parkin [23–26]. Opa1 exists in eight isoforms that can be generated by alternative splicing following induction of presenilin-associated rhomboid-like (PARL) and paraplegin, which incidentally causes hereditary spastic paraplegia when normal function is disrupted [27–29]. Isoforms 3–8 can be further processed to S-Opa1 (Short-Opa1) by intermembrane space AAA-protease Yme1L [30, 31]; Opa1 isoforms 1 and 2 lack Yme1L cleavage sites and thus exist as L-Opa1 (Long-Opa1). Upon mitochondrial membrane depolarization or decreased ATP production, all Opa1 isoforms can be cleaved into non-fusion-promoting forms by Zn-protease Oma1 [32, 33]. Loss-of-function mutations in Mfn2 and Opa1 cause dominantly inherited but phenotypically unrelated neurological disorders Charcot–Marie–Tooth 2A disease and dominant optic atrophy, respectively [34, 35]. Differences in phenotypes of patients carrying loss-of-function mutations in different fusion proteins likely result, at least in part, from loss of non-fusion-promoting Opa1 functions, which include structural organization of mitochondrial cristae [36, 37]. Both Charcot–Marie–Tooth 2A disease and dominant optic atrophy are progressive neurodegenerative diseases whose symptoms present as early as the first decade of life.

10.3 Mitochondrial Morphology and Respiration Are Intricately Linked

The devastating effects of loss of mitochondrial fusion and fission protein function seem to involve changes in mitochondrial respiratory chain function and apoptosis induction. Homeostatic degrees of mitochondrial network connectivity among cell types vary, likely reflecting the energy demands and size/shape of the cell (reviewed in [38]). In general, variations in homeostatic mitochondrial morphology and network connectivity reflect changes in fusion/fission events and changes in respiration [39, 40]. Under relatively mild stress conditions (e.g., nutrient deprivation) that increase ATP requirements, mitochondrial networks respond by becoming more interconnected, presumably in order to “ration” respiratory chain complex activity and ATP [41–43]. For cases in which increasing mitochondrial interconnectivity may be incapable of salvaging organelles that have sustained extensive macromolecular damage and membrane permeabilization, Drp1-mediated fission separates poorly functioning regions of the mitochondrial network for mitophagy [11, 44]. Toxins that directly or indirectly disrupt oxidative phosphorylation efficiently induce mitochondrial network fragmentation, which occurs, at least in part, when

membrane depolarization (permeabilization) triggers Drp1 recruitment [11] and induces cleavage of Opa1 into its inactive form; increased rates of mitochondrial fusion or decreases in fission events can slow or prevent apoptosis and maintain membrane potential [39, 45].

10.4 Disruptions in Mitochondrial Function Are Implicated in Parkinson's Disease

Disruptions in cellular respiration were first implicated in Parkinson's disease (PD) pathology in the mid-1980s [46, 47]. Shortly thereafter, it was determined that 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of parkinsonism-causing compound 1-methyl-4-phenyl-1,2,3,6-tetrahyrdropyridine (MPTP), increases ROS production [48] and decreases respiratory chain complex I (NADH/ubiquinone oxidoreductase) activity by preventing the passage of electrons to ubiquinone [49, 50]. Further, decreases in NADH/ubiquinone-mediated electron transport and evidence of oxidative damage have been observed in post-mortem PD brain samples [51]. Later studies indicate that, like other mitochondrial toxins including membrane permeabilizing ionophores, MPTP exposure induces Drp1-dependent mitochondrial fragmentation and mitophagy [52, 53], which can be partially prevented by decreasing Drp1 activity [54] or overexpressing Opa1 [55].

The search for mechanisms by which gain- or loss-of-function genetic mutations cause parkinsonism has important implications for determining the pathology of the much more common idiopathic form of PD, as many of the hallmark phenotypes of patients with idiopathic and genetic forms of PD are similar and are generally thought to be caused by selective loss of dopaminergic projection neurons in the substantia nigra *pars compacta*. The most common causes of autosomal recessive juvenile parkinsonism (AR-JP) are loss-of-function mutations in genes encoding E3 ubiquitin ligase Parkin and phosphotensin-induced kinase I (PINK1). While loss of Parkin or PINK1 function does not cause nigral dopaminergic cell loss in mice, abnormal respiratory chain function has been documented in both loss-of-function models [56–60] and in cultured fibroblasts from patients harboring Parkin loss-of-function mutations [61]. Interestingly, loss of PINK1 function in rats causes progressive motor deficits and significant dopamine neuron degeneration by 8 months, while Parkin knock out rats have no apparent phenotype [62]. Time will tell whether loss of Parkin or PINK1 function in rats causes abnormal mitochondrial morphology or function. Parkin or PINK1 mutant *Drosophila* have aberrant mitochondrial morphology and function, fewer dopaminergic neurons, decreased life span, impaired motor and olfactory function, and male infertility [57, 63–67]. Evidence from mammalian cell culture and *Drosophila melanogaster* studies indicates that PINK1 functions as an upstream activator

of Parkin [66, 68, 69], and when overexpressed, both translocate from the cytosol to depolarized mitochondria and promote Drp1-mediated fission [11] and subsequent mitophagy [70]. PINK1 knockdown can increase mitochondrial network connectivity and decrease the interaction of exogenous Parkin and Drp1 at mitochondria when mitochondria are depolarized by protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) treatment. Similarly, Parkin knockdown can increase network connectivity and all but abolish the interaction of exogenous PINK1 and Drp1. Further, when levels of Drp1 receptors MiD49 and MiD51 are reduced in CCCP-challenged cells, the Drp1-Parkin interaction and the Drp1-PINK1 interaction are eliminated, and the number of cells with fragmented mitochondrial networks decreases [11]. While numerous studies from independent teams corroborate that Parkin and PINK1 promote mitophagy, more recent studies in neuronal and patient fibroblast cultures indicate that neither Parkin nor PINK1 may be required [71–74]. Additionally, it has been observed that Parkin and PINK1 can promote mitochondrial *fusion* or suppress fission [75, 76] and that downregulation of Parkin or PINK1 induces oxidative stress [59] and mitophagy [74]. Studies in *Drosophila* show that loss of Parkin or PINK1 causes a decrease in the turnover of respiratory chain proteins [77] and Parkin has been shown to promote the turnover mitochondrial import proteins including TOM20, TOM40, TOM70, and Omp25 [78]. Perhaps aberrant mitochondrial function observed in Parkin and PINK1 loss-of-function models is directly or indirectly caused by an increase in ROS levels produced when loss of Parkin-mediated homeostatic proteasomal degradation of poorly functioning import and respiratory chain enzymes is impaired. Indeed, evidence suggests that antioxidant demand is increased in Parkin and PINK1 mutant *Drosophila* and that these phenotypes can be ameliorated with antioxidant treatment [67, 79–82]. Decreases in NADH/ubiquinone oxidoreductase function as observed in PINK1 patients and loss-of-function models may be due to loss of mitochondrially targeted PINK1 phosphorylation of the DnufA10 subunit, which is required for ubiquinone reduction [56]. Still, evidence from cultured neurons indicates that endogenous Parkin and PINK1 are required for mitophagy [83]; however, when induced by several cell stressors like iron and ionophores, mitophagy can occur independently of Parkin and PINK1, suggesting that mitophagy may continue, perhaps even at a higher rate in patients with Parkin or PINK1 mutations [71–74]. Thus, an alternative interpretation of studies implying a Parkin-PINK1 mitophagy-promoting pathway could be that mitophagy-promoting properties of Parkin and PINK1 may be artifacts of their overproduction. Other recent reports suggest that loss of Parkin function may cause cell death due to decreased proteasomal degradation of Fbw7 (an F-box protein also known as human Cdc4), which, like other F-box proteins, functions as E3 ubiquitinase substrate-binding adaptors. One study proposes that the loss of Fbw7 degradation results in increased ubiquitination and subsequent degradation of myeloid cell leukemia 1 (MCL-1), which has been shown to translocate to the mitochondrial matrix where it stabilizes inner membrane morphology and respiratory chain enzyme activity [45].

10.5 Altering Levels of Mitochondrial Fusion/Fission Events Can Restore Mitochondrial Function and Rescue Phenotypes

Overexpression of Drp1 or knockdown of Opa1 or Mfn1/Mfn2 can ameliorate the *Parkin* or *PINK1 Drosophila* mutant phenotype [68, 84, 85], suggesting that Parkin and PINK1 affect mitochondrial morphology and/or function and that restoring an optimal balance of mitochondrial fusion and fission events may be sufficient to protect against the phenotype. While altering levels of fusion and fission proteins has been shown to be protective in various types of cultured cells and in *Drosophila* studies, it appears that the beneficial direction toward which the balance of fusion/fission protein levels should be tipped depends on the model organism and the experimental conditions. In *Drosophila*, improvements in the mutant phenotypes are observed when fission is increased or fusion is decreased [84, 85]; however, loss of Parkin or PINK1 seems to cause mitochondrial fragmentation in many cultured cell models, including SHSY-5Y, human dopaminergic M17 cells, and rat dopaminergic $1RB_3AN_{27}$ (N27) neuronal cells; replacing or overexpressing Parkin or PINK1 in these models attenuates this fragmentation [74, 86–88]. Not all results of these types of cell culture studies are in accord, however; decrease or loss of PINK1 function in cultured rat hippocampal neurons and *Drosophila* S2 cells and loss of Parkin in human patient fibroblasts result in more interconnected mitochondrial networks [61, 89, 90]. While few would dispute that Parkin and PINK1 can work together to affect mitochondrial function, it could be that their loss most severely affects neurons because these proteins play different roles in different cell types. What is probably more plausible is that they affect mitochondrial function in all cells in a similar way, but the severity of the effects of their loss is more dependent on the cell's morphology, ATP requirements, and primary site of ATP production [91]. Thus, altering expression of mitochondrial fusion and fission proteins in Parkin or PINK1 loss- or gain- of-function paradigms can improve mitochondrial function and/or prevent apoptosis depending on the extent to which mitochondrial networks are challenged (Fig. 10.1). Perhaps the direction toward which the balance of fusion/fission events is tipped in order to observe improvement depends on the cell's requirement for mitochondrial mobility and/or its energy demands. Inhibiting mitochondrial fission is protective in both idiopathic and PINK1 loss-of-function mice, indicating that altering fusion and fission rates is probably improving mitochondrial function in a manner that is not specific to PINK1 loss [54]. For cells like fibroblasts that have relatively low ATP requirements and/or sufficient capacity to balance ROS levels, decreased mitochondrial function caused by loss of Parkin or PINK1 may have only minor effects on cell function and survival. Conversely, for neurons, which have high energy demands, concurrent increases in ROS production, extensive mitochondrial mobility requirements, and an inability to store glucose, increasing mitochondrial network interconnectivity, may be sufficient for cell survival. Thus, decreases in mitochondrial function caused by loss of Parkin or PINK1 in neurons may cause more extensive damage and outer membrane permeabilization so that promotion of fission is more beneficial as it causes mitochondria that are damaged beyond recovery to be directed toward the lysosome for digestion.

10.6 Conclusion

This chapter has reviewed current evidence suggesting that loss of Parkin or PINK1 function causes aberrant mitochondrial respiratory enzyme function via decreased ubiquitination of respiratory enzyme subunits or by compromising inner membrane structural integrity. Improvements are observed when employing strategies that alter fusion and fission events, introduce antioxidants, or utilize gene rescue in various models, likely as a result of directly or indirectly correcting mitochondrial structural integrity and/or respiratory function, which are inextricably linked. Perhaps the largest obstacle in effective, practical therapeutic approaches is targeted delivery in patients.

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Chapter 11
Early Nicotine Exposure Is Protective in Familial and Idiopathic Models of Parkinson's Disease

Lori M. Buhlman and Jun Hu

11.1 Tobacco Use and Decreased Incidence of Parkinson's Disease

Results of epidemiological investigations in different countries and including different races, genders, and experimental designs have highlighted a negative correlation between tobacco use and the incidence of Parkinson's disease (PD). The studies referenced here include only idiopathic PD subjects, or no distinction was made between idiopathic and familial PD. By some estimates, the incidence of PD in smokers is about half of that of nonsmokers, and the strength of such protective effects is proportional to the amount of cigarettes consumed [1–7] (Table 11.1). A meta-analysis of 54 epidemiological studies concluded that the PD risk for current smokers was 63 % lower than that for nonsmokers and the risk of former smokers was 41 % lower than that of nonsmokers [8]. For former smokers, the longer the time she/he has been smoke-free, the weaker the protective effect [2, 4]. Additionally, the risk of idiopathic PD in passive smokers may be lower than that of nonsmokers [9], and a dose-dependent reduction of PD risk has been associated with other types of tobacco use [5]. While other reports of the effects of smoking on PD incidence are inconclusive or do not support that smoking tobacco is neuroprotective [10, 11], about 75 % of large-scale case–control and prospective studies summarized by Morens et al. suggest a protective effect of cigarette smoking [3]. Obvious alternative explanations for the results of these epidemiological studies lie in subject selection bias. More specifically, subjects who are or have been tobacco smokers and who

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Table 11.1 Stratified analyses of smoking and Parkinson's disease (from Li et al. 2015)

Subgroup	No. of studies	RR	95 % CI	I^2 (%)	Analysis model
Source of controls					
Population based	30	0.61	0.57–0.66	26	Fixed effect model
Hospital based	22	0.56	0.50–0.62	0	Fixed effect model
Gender					
Male	12	0.59	0.52–0.68	43	Fixed effect model
Female	9	0.69	0.59–0.81	0	Fixed effect model
Dose response					
≤30 pack-years	3	0.66	0.49–0.88	0	Fixed effect model
>30 pack-years	4	0.39	0.29–0.53	0	Fixed effect model
Year of publication					
Before 1990	12	0.60	0.52–0.69	0	Fixed effect model
1990–2000	23	0.61	0.55–0.69	28	Fixed effect model
2000–2010	22	0.58	0.54–0.62	30	Fixed effect model
2010–now	5	0.55	0.45–0.67	56	Random effect model

reach late adulthood may have more robust health that allows them not only to overcome the health risks of smoking behavior but also to withstand environmental factors that lead to PD. On the other hand, smokers, compared with nonsmokers, are more likely to be afflicted by other diseases, leading to higher rates of mortality at earlier ages. These caveats are particularly important when considering that the average age of onset for idiopathic PD is 65 years. Attempts to address potential selection biases that may confound the outcomes of these studies include a 29-year follow-up study reporting that the increased mortality rate among the older smoking population was not sufficient to account for the effect of smoking on idiopathic PD incidence; indeed, a protective effect of smoking was observed in subjects as young as 50 years of age [12]. In fact, smokers diagnosed with PD may present symptoms earlier than nonsmoking patients [13, 14]. Studies of PD incidence among mono- and dizygotic twin smokers further support that the protective effect of smoking is not due to selection bias [13, 15]. Finally, smoking behavior is not protective against other late-onset neurodegenerative diseases like Alzheimer's disease and cerebrovascular dementia; in fact, tobacco smoking is a risk factor for these diseases/disorders [16–21]. While tobacco cigarettes contain thousands of different molecules, the search for the potentially protective component(s) began with nicotine, because of its well-characterized modulation of neuronal nicotinic acetylcholine receptors (nAChRs).

11.2 Nicotine Is Neuroprotective in Animal Models of Parkinson's Disease

Administration of 6-hydroxydopamine (6-OHDA) or toxins that inhibit electron transport from NADH/ubiquinone oxidoreductase (mitochondrial respiratory complex I) to ubiquinone causes selective degeneration of dopaminergic neurons in the

substantia nigra *pars compacta* and induces parkinsonism in rodents and primates; thus, much of what is understood about the mechanisms by which nicotine is neuroprotective has been realized in rodent and nonhuman primate models generated by treatment with these compounds. Several studies support that nicotine administration prior to toxin-mediated induction of parkinsonism can decrease dopaminergic cell death, mitigate dopamine depletion, reduce parkinsonism, and/or inhibit activation of microglia [22–29], and that this protection can be dose dependent [30, 31]. Interestingly, there may be a damage threshold beyond which nicotine administration is ineffective [23], which suggests that nicotine exposure may only be protective in earlier stages of PD when degeneration of substantia nigra dopaminergic neurons is less severe. Alternative hypotheses for the mechanisms of protection mediated by tobacco smoking suggest that nicotine may slow the conversion of NADH/ubiquinone oxidoreductase inhibitor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+) [32]. Indeed, levels of monoamine oxidase B (MAOB), the enzyme that converts dopamine (DA) to dihydroxyphenylacetaldehyde (DOPAL) and MPTP to MPP^+ [33], are decreased in the brains of tobacco smokers [34, 35]. Thus, it may be that by decreasing brain MAOB levels, nicotine could increase brain DA levels and decrease H_2O_2, an important reactive oxygen species. Reduced MAOB levels also may lower the generation of potentially toxic environmental compounds (interactions of MAOB and substrates are reviewed in [36]). This mechanism of protection, however, may be offered by 2,3,6-trimethyl-1,4-naphthoquinone (TMN), another component of tobacco smoke, rather than by nicotine [37].

11.3 Reports of the Effects of Nicotine Administration in PD Clinical Trials Are Less Encouraging

In the early twentieth century, observations of the potentially protective effects of nicotine administration began in patients with PD secondary to encephalitis [38]. Acute intravenous drip administration of nicotine can improve central processing speed and decrease reaction time and tracking error in mild to moderate PD patients; when intravenous administration was followed by subsequent transdermal patch application, subjects' motion operation test scores improved, as did their extrapyramidal function for up to 1 month following treatment [39]. A case study describing symptoms following nicotine gum and transdermal patch administration reports that tremor and confusion were decreased in one patient and bradykinesia decreased in the other [40]. A pilot study suggests that transdermally delivered nicotine may improve motor scores and slow degeneration of dopaminergic neuron terminals [41]. The follow-up study (ClinicalTrials.gov ID NCT00873392) is complete, but published results were not presently available. Nevertheless, several trials in which patients were given nicotine gum or patches have failed to demonstrate the beneficial effects of smoking on PD symptoms [42–44]. Factors including inconsistency in nicotine administration routes, doses, time intervals, duration, and lack of

statistical power prevent consensus in the scientific community on the efficacy of nicotine in PD patients, warranting further scientific and systemic clinical study. Furthermore, whether route of administration affects efficacy is yet to be determined, and achieving brain peak and fluctuating concentrations of nicotine that mimic those of tobacco users is, for now, impossible to confirm. A clinical trial that is currently underway is assessing scores for parts I, II, III of the Unified Parkinson's Disease Rating Scale (UPDRS) in subjects after 52 weeks of continual transdermal patch administration and again 2 months after patches are removed (ClinicalTrials.gov study ID NCT01560754). An additional, potentially limiting factor in previously published nicotine trials could be that an effective regimen of nicotine was not achieved. A 2007 pilot study indicates that high doses of nicotine delivered via transdermal patch (105 mg day^{-1}, exceeding the dose of most trials) for 17 weeks can be tolerable; subjects in this trial had improved motor scores and DA replacement therapy was reduced [45]. Further, with the possible exception of patients who inherit PD-causing mutations, patients are diagnosed based on the onset of motor symptoms, which is believed to occur years after nigral dopaminergic neurons degenerate. As imaging technology improves and as promising PD biomarkers become available, PD patients will be diagnosed in earlier stages of the disease, perhaps increasing the opportunity to discover a therapeutic window.

The vast majority of patients with PD have the idiopathic form; only about 10 % inherit PD-causing mutations [46]. Familial PD has incomplete euchromosomal dominant or recessive heredity, with onset in multiple generations, multiple family members, and different clinical manifestations [47]. Symptoms more associated with familial PD include ataxia, pyramidal signs, early onset, and short duration. While there are important pathological differences between idiopathic and familial PD, including the presence or absence of intraneuronal α-Synuclein-rich protein aggregates (Lewy bodies), understanding how genetic mutations result in parkinsonism will likely provide insight into the pathology of the more common idiopathic form. Currently, at least 15 PD pathogenic gene loci have been observed, among which 11 causative genes have been identified (*SNCA*, *PARK2*, *PINK1*, *UCH-L1*, *DJ-1*, *LRRK2*, *atp13a2*, *GIGYF2*, *Htra2*, *fbxo7*, *and PLA2G6*) [48]. While most mouse global loss-/gain-of-function models of inherited PD do not have strong phenotypes or loss of dopaminergic neurons, *Drosophila* models of PD caused by mutations in Parkin, PINK1, α-Synuclein, and DJ-1 have relatively stronger phenotypes and, in some cases, loss of dopaminergic neurons [49–52]. Further, heterozygous Parkin loss-of-function *Drosophila* raised on nicotine-containing food live longer and climb better than untreated controls, and nicotine prevents decreases in flight and olfaction caused by the mutation [53]. Relatively fewer clinical studies involve patients with rare-inherited forms of PD. Results of one small clinical study in which early onset familial PD patients were given nicotine chewing gum showed that patients who were tobacco smokers and received nicotine gum performed better on the UPDRS. No significant change in UPDRS scores was demonstrated among nonsmoking patients, suggesting that nicotine may only be protective when administered in earlier disease stages or before disease onset in familial PD as well [54].

11.4 Putative Mechanisms of Nicotine Protection

11.4.1 Nicotinic Acetylcholine Receptor Modulation

Nicotinic acetylcholine receptors (nAChRs) were the first identified and are the best described member of Cys-loop family of ligand-gated ion channels. In the mammalian nervous system, combinations of gene products from nine alpha-subunit genes (*CHRNA 2–10*) and three beta-subunit genes (*CHRNB 2–4*) come together to form a variety of somewhat predictable alpha-subunit-containing homopentamer and alpha- and beta-subunit-containing heteropentamer cation channel subtypes, each with unique ligand affinities, receptor channel kinetics, and expression patterns that include dopaminergic brainstem regions and the basal ganglia [55] (also reviewed in [56, 57]). Acutely, nicotine is a nAChR agonist; however, several different nAChR subtypes can be desensitized by chronic nicotine exposure (reviewed in [58, 59]). Since mammalian nAChR subunit sequences and assembly are highly conserved, as are the functions, projections, and inputs of the substantia nigra pars compacta and the basal ganglia, much attention has been devoted to elucidating the nAChR subtypes responsible for nicotine-mediated protection against dopaminergic neuron loss in various PD models. Nicotine may directly or indirectly affect nigral DA cell survival via modulation of pre- or postsynaptic nAChR expressed on DA neurons [60–64], nigral inhibitory interneurons [65], and afferent glutamatergic projection neuron terminals [66]. Several nAChR subtypes have been implicated in nicotine-mediated protection, particularly those containing α7 and α6β2 subunits. Reports in rodents and monkeys corroborate that nigral DA neurons expressing α6*-nAChR seem to be more sensitive to insult than those expressing α4β2 subunits [29, 67, 68]; however, to date, no α6*-nAChR knockout mice or α6*-nAChR-selective antagonist studies have demonstrated that α6*-nAChR participate in nicotine-mediated neuroprotection. Reports of PD pathology indicate that a decrease in nigral DA terminals precedes cell death [69, 70] and that α6*-nAChRs are preferentially expressed on DA neuron terminals, while α4*-nAChRs are preferentially expressed on DA neuron soma [71]. As such, it may be that the selective decrease in α6*-nAChR expression is principally due to a decrease in nigral DA terminals of neurons that express both α4*- and α6*-nAChR rather than to the selective vulnerability of α6*-nAChR-expressing neurons. Still, there is evidence indicating that nicotine does not offer protection against 6-OHDA-induced nigral degeneration in mice lacking α4*-nAChR [30]. Nicotine also may protect against neurodegeneration caused by inflammation, which is implicated in PD pathology. Selective blockade of Ca^{2+}-permeable α7 subunit-containing nAChR (α7*-nAChR) in microglial-mesencephalic neuronal cocultures prevents nicotine-mediated protection against lipopolysaccharide (LPS)-induced microglia activation, DA cell loss, and Tumor necrosis factor alpha (TNF-α) production [72]. The α7*-nAChR also is implicated in nicotine-mediated prevention of cultured astrocytes activation by MPP^+ or LPS [31]. Other studies suggest that nicotine may preserve DA neurons in PD models as a result of α7*-nAChR-mediated activation of cell survival pathways.

Activity of α7*-nAChR expressed in the substantia nigra *pars compacta* initiates the calmodulin and phosphatidylinositol survival pathway via T-type voltage-gated Ca^{2+} channel activation [73]. Astrocyte α7*-nAChR activation may alleviate mitochondrial membrane potential loss, stabilize Bax/Bcl-2 balance, and inhibit cleaved caspase-9 activity in astrocytes [28], which have been implicated in PD-associated immunoreactivity. Further, α7*-nAChR modulation can promote survival via the Janus kinase 2/phosphatidylinositol-3 kinase (JAK2/PI-3K) pathway [74], and it also can increase Bcl-2, NF-kB, AP-1, and STAT1/STAT3 levels and prevent cytochrome *c* release from mitochondria [75]. Intriguingly, α7*-nAChR expression has been observed on outer mitochondrial membranes, where it mediates intramitochondrial Ca^{2+} concentration and release of cytochrome *c* [76]. Studies implicating other nAChR subunits are fewer. An allele encoding a variant of the α5 nAChR subunit may be implicated in nicotine-mediated protection, as the presence of the minor allele (rs588765) delays PD onset only in patients with a history of tobacco smoking [77]. Finally, chronic nicotine can induce changes in mouse nigral neuron gene transcription [78], and in rat prefrontal cortex and medial habenula, chronic intermittent nicotine administration can decrease expression of genes involved in the ubiquitin–proteasome system [79], which is implicated in both familial and idiopathic PD. Whether these changes in gene expression are mediated via nAChR modulation, independently of nAChR modulation, or both, remains unclear.

11.4.2 Nicotinic Acetylcholine Receptor-Independent Targets of Nicotine

Nicotine has good aqueous and lipid solubility and, as such, can penetrate the blood–brain barrier and the plasma membrane freely [80], allowing it to trigger responses throughout the central nervous system. Nicotine can stabilize the soluble form of α-Synuclein, prevent Lewy body formation, destabilize α-Synuclein fibrils, and inhibit fibril formation in a dose-dependent and nAChR-independent manner [81, 82]. Although it can generate oxidative stress in the periphery and in the central nervous system, nicotine also has been reported to have antioxidant properties as it can interfere with the Fenton reaction [83, 84]. Nicotine has hydroxyl radical and superoxide free radical scavenging capabilities that exceed those of vitamin C [85], but its effects on lipid peroxidation are inconsistent [86]. Additionally, high- and low-affinity nicotine binding sites have been identified at the NADH and ubiquinone binding sites (respectively) of NADH/ubiquinone oxidoreductase [87]. In isolated rat brain mitochondria, acute nicotine exposure can decrease superoxide anion generation by about 15 % [87], and chronic exposure can modestly protect respiratory control ratio under anoxia–reperfusion conditions [88]. When simultaneously administered, nicotine also can mitigate the effect of rotenone on respiratory control ratio, superoxide anion generation, and increased mitochondrial membrane anisotropy for up to 14 days after beginning chronic drug administration. Nicotine can decrease cytochrome *c* release in isolated rat brain mitochondria treated with MPP^+

or Ca^{2+} and phosphate, and this protection seems to be mediated via interaction with NADH/ubiquinone oxidoreductase [89]. The same study also reported that nicotine reduces MPP^+-mediated increases in superoxide and H_2O_2 generated by NADH/ubiquinone oxidoreductase activity in a concentration-dependent manner.

11.5 Conclusion

While the majority of PD cases are idiopathic, and the mechanisms by which genetic mutations cause PD are elusive, mounting evidence implicates mitochondrial pathology in the etiology of both forms. Modulation of α7*-, α6β2-, and perhaps α4β2*-containing nAChR subtypes expressed in the substantia nigra *pars compacta* and striatum may offer protection against mitochondrial toxins that induce parkinsonism by affecting DA transmission, cell survival signaling pathways, and modulating inflammatory responses. Additionally, nicotine may confer neuroprotection independently of nAChR modulation, as it can easily traverse the plasma membrane where it may act as an intracellular antioxidant via interruption of the Fenton reaction and/or by interfering with the activity of NADH/ubiquinone oxidoreductase. Despite mounting evidence for and proposed mechanisms of nicotine-mediated neuroprotection, clinical trials have failed to simulate results obtained in animal model studies, perhaps because clinical trial participants are inherently too far along in regard to disease progression, and/or study paradigms have failed to accurately mimic therapeutic nicotine exposure to the brain. Well over half of dopaminergic neurons in the substantia nigra *pas compacta* are lost when hallmark motor symptoms of PD present, yet epidemiological and model studies suggest that nicotine should be administered much earlier in the disease process, perhaps even before onset. While this creates a major obstacle in therapeutic development, as imaging technology improves and more reliable biomarkers are identified, clinicians will be able to identify Parkinson's disease patients in earlier disease stages, thus, improving the chances of targeting a potentially therapeutic window for nicotinic compounds.

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Chapter 12
Transcription Modulation of Mitochondrial Function and Related Pathways as a Therapeutic Opportunity in Parkinson's Disease

Audrey S. Dickey and Albert R. La Spada

12.1 Models of PD Featuring Mitochondrial Dysfunction

Due to its central role in the disease, one approach to modeling PD involves the use of α-synuclein [1]. An alternative approach involving mitochondrial-based toxin models based on involvement in "sporadic" PD is very useful to mimic aspects of PD [2, 3]. Evidence of specific involvement of mitochondrial dysfunction in the pathogenesis of PD was brought to the forefront following accidental exposure to the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which causes parkinsonism by selective inhibition of mitochondrial complex-I of the electron transport chain [2, 4]. Other complex-I inhibitors such as rotenone, pyridaben, fenazaquin, tebufenpyrad, trichloroethylene, and fenpyroximate cause degeneration of dopaminergic neurons in *Drosophila*, rodents, and humans, implicating mitochondrial dysfunction in PD [5]. These mitochondrial toxins inhibit the mitochondrial electron transport complex activity [6], increase the mitochondrial permeability transition, reduce mitochondrial movement [7], and increase mitochondrial nitric oxide synthase (NOS) activity, suggesting involvement of mitochondrial complex-I in PD pathogenesis. More direct evidence for involvement of mitochondrial dysfunction

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in PD pathogenesis comes from studies of complex-I activity in PD patients. There is an impairment of mitochondrial complex-I activity in the substantia nigra (SN), platelets, and skeletal muscle of PD patients [5, 8, 9].

In the cytoplasmic hybrid ("cybrid") model of sporadic PD, PD donor mitochondrial DNAs (mtDNAs) are expressed in neural tumor cells with identical nuclear genetic and environmental backgrounds [10]. These PD cybrids demonstrate many abnormalities including increased oxidative stress driving downstream antioxidant response and activation of cell death signaling pathways. Extensive literature has documented mitochondrial oxidative phosphorylation pathway dysfunction in PD patients and mammalian cell lines [11–13]. The PD cybrids regulate mitochondrial electron transport chain (ETC) genes and gene ontology families in a manner similar to that seen in the brains of patients with sporadic PD. Additionally, PD cybrids spontaneously form Lewy bodies and Lewy neurites, linking mtDNA expression to neuropathology, and demonstrate impaired organelle transport in processes [10]. In creating cybrids by hybridizing PD patient mtDNA with host cells, not only are defects in complex-I activity transferred, but also transferable are deficits in membrane potential, mitochondrial respiration, impaired mitochondrial biogenesis, and abnormal Ca^{2+} handling [10, 14, 15]. Mitochondrial biogenesis is a complex process that requires coordinated transcription of both nuclear and mitochondrial genes [16].

Previous chapters have discussed how mitochondrial toxins can lead to parkinsonism and how mutations in genes affecting mitochondria can contribute to PD and have proposed the therapeutic validity of separately targeting reactive oxygen species (ROS) production, mitochondria DNA damage, oxidative phosphorylation, and mitochondria dynamics. Targeting a very specific aspect of mitochondrial physiology could very well be the therapeutic answer. Another possibility is that activating a transcriptional program improving multiple aspects of mitochondrial function will have a larger positive impact on patient health. A number of molecular pathways including oxidative stress, mitochondrial dysfunction, protein dysfunction, apoptosis, and neuroinflammation are implicated in the pathophysiology of PD, and transcription factor modulation may be able to address these pathways (Fig. 12.1).

12.2 A Role for PGC-1α Dysfunction in PD Pathogenesis

A likely candidate for integrating a number of these pathways is transcription regulatory protein peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). A considerable body of work indicates that PGC-1α is the key regulatory factor in a complex network of transcription programs that culminate in mitochondrial biogenesis and cellular homeostasis (reviewed in [17]). PGC-1α is highly expressed in tissues with high-energy demand, such as brown adipose tissue, heart, skeletal muscle, and brain [18]. In addition to increasing mitochondrial biogenesis, PGC-1α enhances fatty acid oxidation and oxidative metabolism while protecting against oxidative stress [19–22]. PGC-1α also plays a central role in driving and coordinating gluconeogenesis and glucose transport, glycogenolysis, peroxisomal remodeling, and muscle fiber-type switching; PGC-1α is preferentially expressed in muscle

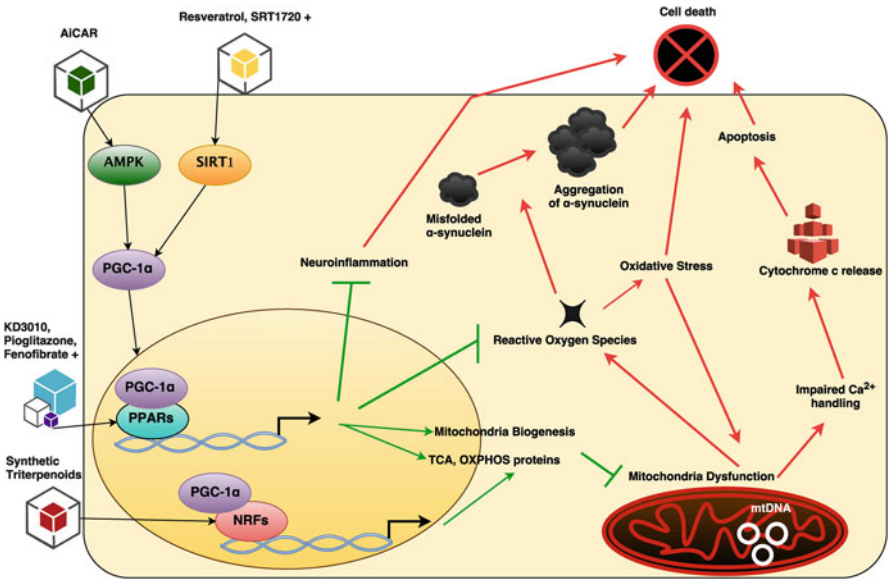

Fig. 12.1 Pathways of transcription modulation therapy in PD. Putative targets and their respective ligand activators for transcription factor modulation therapy are shown, as well as their expected actions and effects on pathological mitochondrial processes implicated in the pathogenesis of PD

enriched for type I myocytes and can convert type II myocytes to type I fibers [19]. In addition, PGC-1α protects against oxidative stress by regulating the expression of several ROS detoxifying enzymes, such as SOD1 and SOD2 (superoxide dismutases 1 and 2), catalase, and glutathione peroxidase-1 [20].

Recent studies have suggested that PGC-1α impairment is centrally involved in PD pathogenesis [23, 24]. From a meta-analysis of 17 independent microarray studies on multiple PD samples, including postmortem PD substantia nigra, strong evidence for PGC-1α transcription interference emerged, as set enrichment analysis revealed coordinate downregulation of 425 PGC-1α target genes [24]. These studies indicate that early in PD pathogenesis, defects in mitochondrial electron transport and glucose utilization appear. Furthermore, an independent study found evidence for a link between PGC-1α and PD due to recessive mutations in *parkin* and altered PGC-1α activation [23]. PARIS (parkin-interacting substrate) is a zinc finger protein that is highly expressed in the substantia nigra and is a substrate for parkin. PARIS represses the expression of PGC-1α and transcription factor nuclear respiratory factor 1 (NRF1). The site of interaction between PARIS and PGC-1α is a sequence involved in the regulation of insulin responsiveness and energy metabolism. Conditional knockout of parkin in adult mice led to progressive loss of dopamine neurons. Moreover, overexpression of PARIS led to the selective loss of dopamine neurons in the substantia nigra, and this was reversed by either parkin or PGC-1α co-expression [23]. Another study using A9 dopaminergic neurons derived from induced pluripotent stem cell (iPSC) lines carrying the pathogenic α-synuclein

A53T mutation, as well as isogenic mutation-corrected iPSC control lines, reported that PGC-1α transcriptional interference yielded mitochondrial dysfunction and cell death [3]. PD cybrids show reduced peroxisome PGC-1α levels, reduced cellular respiration, and molecular and mitochondrial respiratory properties similar to those observed in mitochondria in PD brain [7, 25]. All of these findings implicate impaired PGC-1α function in PD pathogenesis.

PGC-1α has recently emerged as a therapeutic target for several neurodegenerative disorders including PD and Huntington's disease (HD) [17, 26]. PGC-1α plays a protective role in models of different brain diseases [20, 27]. An increased vulnerability to MPTP-induced degeneration of nigral dopaminergic neurons was observed in PGC-1α knockout mice, suggesting a critical role of PGC-1α in neuroprotection [20]. The mechanisms involved in the neuroprotective effects of PGC-1α are, however, not fully understood. For example, this group found that by increasing PGC-1α levels and thus expression of several ROS detoxifying enzymes, such as SOD1 and SOD2, catalase, and glutathione peroxidase-1, neural cells were dramatically protected from oxidative stress and cell death [20]. Another group found that activation of PGC-1α increased the expression of nuclear-encoded subunits of the mitochondrial respiratory chain and prevented the dopaminergic neuron loss induced by mutant α-synuclein or the pesticide rotenone in cellular disease models [24]. Furthermore, it has been shown that PGC-1α knockdown increased α-synuclein accumulation and led to downregulation of the AKT/GSK-3β signaling pathway in human neuronal cells [28]. A recent study reported that PINK1 mutations impair parkin recruitment to mitochondria in neurons, increasing mitochondrial copy number and upregulating PGC-1α [29]. Other studies have shown that transgenic overexpression of PGC-1α or activation of PGC-1α by resveratrol protects dopaminergic neurons in the MPTP mouse model of PD [27]. These studies suggest compelling evidence for a role of PGC-1α in neurodegenerative diseases and as a good candidate for the treatment of PD.

Recently it was shown that adenoviral delivery of PGC-1α in the nigrostriatal system increased dopaminergic death [30]. This effect could be the result of excessive overexpression of PGC-1α, resulting in mitochondrial hyperactivity and increased production of ROS, increasing the vulnerability of dopaminergic neurons toward oxidative stress linked to enhanced mitochondria metabolism [30–32]. Additionally, PGC-1α overexpression was found to exacerbate β-amyloid and tau deposition in a transgenic mouse model of Alzheimer's disease [33]. The studies related to the role of PGC-1α in PD have provided inconsistent data regarding the effects of PGC-1α overexpression in PD [34].

12.3 PGC-1α Activates the PPAR Family of Nuclear Receptors

The PPARs are ligand-inducible transcription factors that are part of the nuclear hormone receptor superfamily and transduce signals from environmental, inflammatory, and nutritional events into cell responses through gene transcription [35].

PPARs share significant conservation in their DNA-binding and co-activator domains, but diverge in their ligand-binding regions [36]. They are involved in the transcriptional control of genes regulating various physiological processes such as lipid homeostasis, glucose metabolism, inflammation, and cellular differentiation and proliferation [37, 38]. PPARs act as lipid sensors, being activated by small, lipophilic compounds, and thus regulate metabolism in response to dietary lipid intake and direct the subsequent metabolism and storage of lipids [36]. Three isoforms have been identified, PPARα, PPARδ (β), and PPARγ, which are encoded by different genes. These three isoforms differ in their tissue distribution, ligand specificity, and physiological role [39]. PPARα acts primarily to regulate energy homeostasis through its ability to stimulate the breakdown of fatty acids and cholesterol, driving gluconeogenesis and reduced triglyceride levels. PPARα acts as a lipid sensor, by binding fatty acids and initiating their subsequent metabolism. PPARδ also binds fatty acids and is involved in the regulation of fatty acid oxidation and lipid metabolism. PPARγ stimulates adipocyte differentiation in addition to regulating lipid and carbohydrate metabolism. The activation of PPARγ is linked to a reduction of glucose levels [39].

To modulate gene expression, a ligand-activated PPAR heterodimerizes with the retinoid X receptor (RXR) to bind PPAR-responsive elements (PPRE) in the promoter region of its gene targets [40]. After activation, the PPAR/retinoid X receptor heterodimer binds to a specific DNA sequence (peroxisome proliferator response element—PPRE) in the promoter region of PPAR target genes [38, 41] to modulate transcriptional activity. PPAR activity is also regulated by posttranslational modifications, such as phosphorylation and sumoylation [42, 43]. For example, there are several mechanisms involved in PPARγ inactivation: phosphorylation can negatively or positively affect PPARγ, depending on the specific protein residue modified [44–47]. PPARγ activity is also decreased via the ubiquitination degradation pathway [48]. Additionally, PPARγ sumoylation promotes the repression of inflammatory or adipocyte differentiation genes [42, 49].

PPARα is highly expressed in metabolically active tissues, such as the liver, kidney, intestine, heart, skeletal muscle, adrenal gland, and pancreas during fetal development of rodents [50, 51]. In adult rodent organs, the distribution of PPARα is similar to its fetal pattern of expression. PPARα is most highly expressed in tissues that catabolize fatty acids, such as the adult liver, heart, kidney, large intestine, and skeletal muscle; however, in the central nervous system (CNS), PPARα is expressed at very low levels, predominantly in astrocytes [52]. PPARδ is the most abundant isoform in the CNS and is expressed ubiquitously in virtually all adult tissues and also during fetal development [50–52]. In the CNS, PPARδ is found in the cerebellum, brain stem, and cortex, enriched in the dentate gyrus/CA1 region, and is also found in immature oligodendrocytes. Its activation promotes differentiation, myelin maturation, and turnover [53, 54]. In keeping with the role of PPARγ in regulating glucose and lipid metabolism, and promoting lipid storage and adipocyte differentiation, PPARγ is expressed in white and brown adipose tissue and in the CNS during fetal development of rodents [51, 55, 56], and it is present at lower levels in skeletal muscle, heart, and liver [50–52]. In the adult CNS, PPARγ is

expressed at low levels in several cell types including neurons, astrocytes, oligodendrocytes, and microglia [51, 57–59]. PPARγ is most highly expressed in the hypothalamus, where neuronal PPARγ immunoreactivity appears mainly as a nuclear labeling, although sometimes cytoplasmic staining is detectable [59].

All PPARs can be activated by polyunsaturated fatty acids with different affinities [60, 61]. PPARα ligands include fibrates that are commonly used for the treatment of hypertriglyceridemia and WY14,643 and GW7647. PPARδ binds and responds to VLDL-derived fatty acids, arachidonic acid, and eicosanoids, including prostaglandin A1. PPARδ agonists include synthetic compounds GW0742, GW501516, and KD3010 [62, 63]. Naturally occurring PPARγ ligands include long-chain fatty acids, other natural lipid ligands, eicosanoids, and the prostaglandin 15d-PGJ2. PPARγ ligands also include a few nonsteroidal anti-inflammatory drugs, such as ibuprofen, fenoprofen, and indomethacin [61, 64]. Synthetic thiazolidinediones (TZDs), including pioglitazone and rosiglitazone, were originally designed as PPARγ agonists and are currently in clinical use as insulin-sensitizing agents for the treatment of type 2 diabetes [61, 65].

12.4 PPARγ as a Therapeutic Target in PD

PPARγ agonists may have neuroprotective potential in treating various neurodegenerative diseases, as PPARγ regulates genes involved in glucose, amino acid, and lipid metabolism [66, 67]. Studies have suggested that PPARγ agonists can influence energy homeostasis [68] and ROS in the brain [69]. Recently, the neuroprotective effects of PPARγ agonists have been assessed in several in vitro and in vivo models of neurodegenerative conditions including PD [5, 70], Alzheimer's disease [71, 72], cerebral ischemia [73], and amyotrophic lateral sclerosis [73]. GW1929, a PPARγ agonist, is neuroprotective by increasing peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) levels and by enhancing cell respiration and mitochondrial biogenesis in cultured human dopaminergic neurons [74]. Treatment with GW1929 increased mtDNA copy number in dopaminergic neurons and the expression of NRF1 and mitochondrial transcription factor A (TFAM), two major factors in mitochondrial biogenesis [74].

12.4.1 PPARγ in the Rotenone Model of PD

PPARγ agonist pioglitazone was able to protect against rotenone-induced reduction of locomotor activity and decline in striatal dopamine levels [75]. In a recent study, rotenone irreversibly decreased mitochondrial mass, membrane potential, and oxygen consumption while increasing free radical generation and autophagy in human differentiated SH-SY5Y cells. Similar changes were seen in PINK1 knockdown cells, in which the membrane potential, oxygen consumption, and mitochondrial

mass were all decreased. In both models, all these changes were reversed by treatment with PPARγ agonist rosiglitazone, which increased mitochondrial biogenesis, increased oxygen consumption, and suppressed free radical generation and autophagy [76]. Rosiglitazone significantly increased the expression of proteins related to antioxidant defense and mitochondrial biogenesis (SOD1, nuclear factor (erythroid-derived 2)-like 2 (NRF2), NAD(P)H:quinone oxidoreductase 1 (NQO1), PGC-1, and TFAM). Thus, rosiglitazone was neuroprotective in models of mitochondrial dysfunction associated with PD through a direct effect on mitochondrial function (Fig. 12.1) [76].

12.4.2 PPARγ in the MPTP Model of PD

Results of preclinical studies showed that rosiglitazone protects dopaminergic neurons in MPTP models of PD, and pioglitazone can improve parkinsonian syndrome in rhesus monkeys treated with MPTP [70, 77, 78]. In the chronic MPTP mouse model of PD, treatment with rosiglitazone completely prevented motor and olfactory dysfunction and loss of dopaminergic neurons in the substantia nigra. Rosiglitazone partially protected against loss of striatal dopamine, whereas decreases in DOPAC and dynorphin mRNA in the striatum were completely abolished. Also astrogliosis and the number of activated microglia were reduced as assessed by GFAP and CD11b immunostaining, respectively, without affecting MPTP metabolism [78]. In the same model, treatment with rosiglitazone was also effective in protecting against partial degeneration of the substantia nigra and the decline of striatal dopamine [70]. Pioglitazone was shown to protect against chronic MPTP-induced neurotoxicity, with reduced activation of microglia, reduced induction of iNOS-positive cells, and fewer glial fibrillary acidic protein (GFAP)-positive cells in both the striatum and substantia nigra [79]. In the MPTP-treated rhesus monkey, pioglitazone was neuroprotective and anti-inflammatory, and it significantly improved clinical rating scores. Behavioral recovery was associated with preservation of nigrostriatal dopaminergic markers and reduced infiltration by CD68-positive macrophages in the nigrostriatal area [77]. More recently, the administration of a non-TZD partial PPARγ agonist was again shown to be neuroprotective in MPTP-induced neurodegeneration and was associated with downregulation of neuroinflammation, decreased oxidative stress, and modulation of PPARγ and PPARγ co-activator-1α (PGC-1α) expression [80].

12.4.3 PPARγ in the Lipopolysaccharide Model of PD

Intrastriatal injection of lipopolysaccharide (LPS) in rats generates yet another model for degeneration of dopaminergic neurons in PD. In this model, pioglitazone prevented the loss of dopaminergic neurons and the decline in striatal dopamine

levels [81, 82]. Pioglitazone normalized cyclooxygenase-2 (COX-2) expression and increased the expression of uncoupling protein 2, which is located in the inner mitochondrial membrane, and affects the proton gradient necessary for mitochondrial function. Pioglitazone also increased the expression of mitoNEET, while iNOS induction and oxidative stress were reduced [81, 82]. In dopaminergic neuron–glial cultures, pioglitazone protected neurons from LPS by inhibiting abnormal microglial activation, interfering with phosphorylation of Jun N-terminal kinase and nuclear factor kappa-B, and by suppressing COX-2 expression and subsequent prostaglandin E(2) synthesis [83]. Pioglitazone also protected dopaminergic neurons against LPS damage by inhibiting iNOS expression and nitric oxide generation by differential regulation of p38 mitogen-activated protein kinase and the phosphoinositide 3-kinase/protein kinase B pathway [84]. Microglial activation has been implicated in the pathogenesis of PD and is believed to aggravate neuronal injury [70]. The anti-inflammatory actions of rosiglitazone against LPS were mediated by its ability to increase IL-4 expression [85].

12.4.4 PPARγ in the 6-OHDA Model of PD

The production of pro-inflammatory cytokines and microglial activation has been described in a model of PD in which 6-hydroxydopamine (6-OHDA) is used to selectively destroy dopaminergic and noradrenergic neurons [86]. A recent study demonstrated that pioglitazone did not exert any protection in the 6-OHDA model [87]. The lack of effect of pioglitazone in this model was attributed to the severity of the damage caused by 6-OHDA. However, in a parallel study by the same group, pioglitazone did protect against neuronal loss and motor behavior in the acute MPTP model [87]. In work by an independent group on 6-OHDA-lesioned rats, activation of PPARγ receptors by rosiglitazone significantly attenuated the production of both COX-2 and TNF-α (tumor necrosis factor-α) expression and increased GFAP expression in the striatum [78].

12.4.5 Mechanisms of PPARγ Agonist Neuroprotection

Potential mechanisms of neuroprotection by PPARγ agonists in PD include preventing mitochondrial dysfunction, oxidative stress, proteasomal dysfunction, autophagy, neuroinflammation, and apoptosis, all of which have been implicated in PD pathogenesis. Environmental factors and PD-inducing toxins directly induce both oxidative stress and mitochondrial dysfunction. PPARγ activation increased mitochondrial membrane potential and protected cells from apoptosis following growth factor withdrawal [88]. Pioglitazone also increased neuronal glucose uptake and restored brain ATP levels [89, 90]. Pioglitazone increased mitochondrial DNA content, oxygen consumption, PGC-1α, and TFAM in human adipose tissue and in the

neuronal-NT2 cell line [91–93]. Rosiglitazone induced both mitochondrial biogenesis and glucose utilization in mouse brain [94]. In addition, pioglitazone stabilizes MitoNEET, an iron–sulfur containing outer mitochondrial membrane protein, which regulates oxidative capacity [95–97]. In cooperation with PGC-1α, PPARγ agonists are able to promote mitochondrial biogenesis in nonneuronal cells, including fibroblasts and adipocytes [91, 93, 98]. Rosiglitazone was shown to protect human neuroblastoma cells against 1-methyl-4-phenylpyridinium iodide (MPP^+, the active metabolite of MPTP)-induced mitochondrial dysfunction through multiple mechanisms: induction of expression of antioxidants, SOD and catalase, and antiapoptotic activity by regulating the expression of Bcl-2 and Bax and by increasing mitochondrial membrane potential [99]. Recently it has also been shown that pioglitazone protected against MPTP-induced neurotoxicity by its inhibition of monoamine oxidase-B in the striatum (blocking the conversion of MPTP to MPP^+, via inhibition of monoamine oxidase-B) [100]. Additionally, the beneficial effects of PPARγ agonists are associated with reduced neuroinflammation and lower levels of pro-inflammatory cytokines produced by microglia cells in the brain [70, 77].

12.4.6 Limitations and Setbacks of Pioglitazone as a Potential PD Therapeutic

In the acute MPTP rodent model, the PPARγ agonist pioglitazone blocked dopaminergic neurodegeneration and reduced astrocytic and microglial activation. However, pioglitazone treatment did not alleviate MPTP-induced loss of tyrosine hydroxylase (TH) in the striatum and had only partially protective effects on the MPTP-induced decline in striatal tissue levels of dopamine [101]. In a MPP^+ mouse model, rosiglitazone treatment increased glutathione S-transferase activity, but did not alter SOD activity. In this study, the protective effects of rosiglitazone were not blocked by the PPARγ antagonist GW9662, suggesting that these effects may be independent of PPARγ activation [102]. Some of the metabolic effects of pioglitazone that occur independently of PPARγ may involve binding to the mitochondrial target of thiazolidinones (mTOT), a complex on the inner mitochondrial membrane that directly influences mitochondrial function [95, 103]. Novel compounds that bind to mTOT are being tested in animal models of PD and might offer similar benefits to pioglitazone, but with fewer adverse effects [104]. The potential of pioglitazone may be limited by adverse effects including an association with the development of bladder cancer [105–108].

A double-blind clinical trial (ClinicalTrials.gov: NCT01280123) in early PD patients failed to show any improvement in disease outcomes using pioglitazone [109]. These findings are in contrast to data in preclinical studies, raising concerns about how well toxin-induced animal models may recapitulate the course of human PD. However, while this compound was not effective, it is possible that other compounds that target PPARγ may be viable. Another consideration is that pioglitazone may not have had beneficial effects due to the low levels of expression of PPARγ in the CNS.

12.5 PPARδ Is a Promising Therapeutic Target in the CNS

While PPARγ can demonstrably have a neuroprotective role in the CNS [110], PPARδ may be a better therapeutic target. First, there are safety concerns over activation of PPARγ inducing adverse cardiovascular events like congestive heart failure [111, 112], while no cardiac toxicity has been seen with agonizing PPARδ. In addition, adipose tissue has the highest expression of PPARγ and the most notable gene expression changes in response to PPARγ agonists [113], while PPARδ is much more abundantly expressed in the CNS than PPARγ. Enhancing expression of genes involved in energy metabolism and mitochondrial biogenesis by promoting PPARδ activity may ameliorate PD neurotoxicity.

12.5.1 PPARδ Is a Regulator of Mitochondrial and Metabolic Function

While all three PPARs interact with PGC-1α to promote oxidative metabolism and metabolic activity, in skeletal muscle, mouse experiments have shown that pharmacologic [114] and genetic [115, 116] activation of PPARδ increases expression of genes involved in energy metabolism and mitochondrial biogenesis. Overexpression of constitutively active PPARδ in the skeletal muscle in transgenic mice dramatically favors a shift in muscle fibers to an oxidative metabolic status, thereby vastly improving exercise performance, even in untrained mice [116]. A greatly enhanced oxidative metabolic shift can also be achieved in the skeletal muscle of wild-type mice treated with the PPARδ agonist GW501516, when combined with exercise [114]. PPARδ expression is at least twofold higher in the brain than in the muscle, suggesting an important role for PPARδ in energy metabolism in the CNS [117]. PPARδ, the most abundantly expressed subtype of the PPARs in the CNS [118, 119], is widely expressed and also is a key regulator of metabolic pathways, promoting fatty acid oxidation, oxidative phosphorylation, and muscle fiber-type switching and mitochondrial biogenesis [115, 120]. PGC-1α expression can be controlled by PPARδ [120]. In addition to promoting mitochondrial biogenesis, PPARδ also regulates fiber-type switching in the muscle [114, 116]. PPARδ activation, like that of PPARγ, can inhibit the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [121]. PPARδ can also control the inflammatory status of monocytes and macrophages [121]. PPARδ agonists have neuroprotective effects in models of Alzheimer's disease and multiple sclerosis, which are concurrent with reduced glial cell activation [122, 123].

12.5.2 PPARδ in Preclinical Studies

The pesticide models of PD (paraquat, pyridaben, and maneb) elicit common signaling pathways in the ventral midbrain (VMB) and striatum of treated mice, which correspond with known signaling pathways identified in human PD, indicating that

these pathways contribute to the pathogenesis of sporadic PD. Not only was PPAR signaling identified as one of the concordant pathways, PPARδ was identified as a key gene in the global gene network analysis for both the VMB and the striatum of PD model mice. To evaluate the role of PPARδ repression in the CNS and the relevance of PPARδ dysfunction to neurodegenerative disease, a dominant-negative mutation at amino acid position 411 of PPARδ was introduced into transgenic mice with restricted expression to the CNS (Nestin-Cre) [63]. Transgenic mice overexpressing wild-type PPARδ have already been successfully produced with no negative neurological outcomes or motor abnormalities [115]. In contrast, the dominant-negative PPARδ mice displayed dramatic brain atrophy and motor abnormalities and significant neuron loss in the substantia nigra. Further histological examination of the PPARδ-E411P–Nestin-Cre mouse brain sections revealed post-developmental neurodegeneration and degeneration in the cortex and striatum, in addition to the loss of dopaminergic neurons in the substantia nigra [63]. These findings indicate that PPARδ dysfunction in the CNS can produce phenotypic abnormalities and histopathology findings relevant to PD. Because mitochondrial dysfunction and transcription interference with PGC-1α, the co-activator for PPARδ, are recognized features of PD pathogenesis [23, 24] and because there was a marked loss of TH+ dopaminergic neurons in the substantia nigra of dominant-negative PPARδ conditional transgenic mice, PPARδ agonist therapy should be considered as a potential treatment for PD.

Initial evaluation of the neuroprotective potential of PPARδ agonists in PD reported that PPARδ agonist treatment can ameliorate ischemic brain injury and reduce MPTP-induced striatal dopamine depletion in rodents [124]. PPARδ agonists provided a degree of neuroprotection against both cerebral infarcts and MPTP; however, the effects were not fully characterized [124]. In vitro administration of the PPARδ antagonist GSK0660 increased the detrimental effect of MPP^+ on cell viability, which was reversed by co-treatment with agonist PPARδ GW0742. Intrastriatal infusion of GW0742 reduced the MPTP-induced loss of dopaminergic neurons when compared to vehicle-infused mice [125]. GW0742 also can significantly improve cognitive impairments caused by MPTP in rats, and it can ameliorate MPTP-induced oxidative damage, DNA fragmentation, and TH levels [126].

12.5.3 The PPARδ-Specific Agonist KD3010 as a "Fast-Track" Therapy for PD

KD3010 ((*S*)-4-[*cis*-2,6-dimethyl-4-(4-trifluoromethoxy-phenyl)-piperazine-1-sulfonyl]-indan-2-carboxylic acid tosylate) is a highly selective and potent PPARδ agonist (US20060205736, US20070093504, and [127]). Thirteen-week toxicology studies differentiate KD3010 from known PPARγ and PPARα agonists through its lack of skeletal and cardiac muscle toxicity, weight gain, fluid retention, and hepatic toxicity, underscoring its PPARδ selectivity (Kalypsys, Inc.). Two phase I clinical studies indicate that KD3010 is well tolerated with a safety profile similar to placebo. Pharmacokinetic assays indicate dose proportionality without

accumulation, induction, or gender difference. Importantly, KD3010 traverses the blood–brain barrier, as a 10 % brain–plasma ratio has been demonstrated in mice [63]. KD3010 has already demonstrated neuroprotective effects in a mouse model of Huntington's disease, preventing neuron loss and rescuing motor function [63]. Further evaluation of the neuroprotective potential of PPARδ agonists is necessary in PD models.

12.6 Other Nuclear Receptors Co-activated by PGC-1α as Therapeutic Targets

Nuclear respiratory factors 1 and 2 (NRF1 and NRF2), a target of both PGC-1α and the PPARs, are pivotal transcription factors involved in the regulation of redox balance and mitochondrial biogenesis (reviewed in [17]). NRF2 is normally sequestered by its inhibitor Keap1 in the cytoplasm. In response to oxidative stress, NRF2 translocates to the nucleus and dimerizes with another member of its family of transcription factors [18], activating transcription by binding to an antioxidant response element (ARE) located in the promoter of multiple antioxidant genes, including NQO1, heme oxygenase-1 (HO-1), and glutathione S-transferase [19, 20]. Several studies have suggested that NRF2 and NQO1 protect against cellular dysfunction in different models of PD [21, 22, 25]. Recently, it was demonstrated that rosiglitazone, the PPARγ agonist, increased expression of NRF2 and the antioxidant enzyme HO-1, enhancing elimination of ROS in hepatocytes [26].

Synthetic triterpenoids inhibit oxidative stress and cellular inflammatory processes by potently activating the ARE-NRF2-Keap1 signaling pathway. Triterpenoid activation of NRF2 leads to dissociation of NRF2 from Keap1, translocation to the nucleus, and binding to the ARE promoter sequences. This results in the coordinated induction of multiple cytoprotective genes, including antioxidant and anti-inflammatory genes. Neurons from NRF2 knockout mice are more susceptible to mitochondrial electron transport chain complex inhibitors such as MPP^+ and rotenone-mediated oxidative stress [25]. Injection of the mitochondrial complex-II inhibitor 3-NP causes increased motor deficits and striatal lesions in the NRF2 knockout ($NRF2^{-/-}$) mice, which were protected by adenoviral-mediated overexpression of NRF2 [27]. Significant neuroprotective effects of the synthetic triterpenoid CDDO-MA in the 3-NP rat model and MPTP PD mouse model were observed [28]. CDDO-MA reduced ROS generation, attenuated MPTP-induced nigrostriatal dopaminergic neurodegeneration, prevented dopamine depletion, and reduced 3-NP-induced striatal lesions (Fig. 12.1) [28]. Another group found that triterpenoids improve behavioral phenotypes and survival in transgenic mouse models of HD [29]. In addition to acting as a transcriptional co-activator for PPARs and NRFs, PGC-1α can regulate other nuclear receptors, such as the thyroid hormone receptor, the estrogen receptor, and the estrogen-related receptor α [22]. Additionally, PGC-1α acts as a co-activator for other transcription factors such as TFAM, myocyte enhancer factor 2, FOXO receptors, and hepatic nuclear factor 4 [22].

12.7 Upstream Regulators of PGC-1α as Therapeutic Targets

12.7.1 Adenosine Monophosphate-Activated Protein Kinase (AMPK)

Adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine kinase, is an upstream regulator of PGC-1α. AMPK is a critical metabolic switch activated by an increase in the cellular AMP/ATP ratio, which indicates low energy status. Activation of AMPK triggers increases in glucose uptake, oxidative metabolism, and mitochondrial biogenesis (Fig. 12.1) [128]. AMPK has been shown to control PGC-1α and mitochondrial enzyme gene expression [129]. AMPK activation leads to increased PGC-1α expression [130, 131], and AMPK requires PGC-1α activity to modulate the expression of several key players in mitochondrial and glucose metabolism [129]. A closer link has been provided by recent findings showing that AMPK can directly interact with and phosphorylate PGC-1α, increasing the transcriptional activity of PGC-1α [129]. AICAR (5-aminoimidazole-4-carboxamide-1-*b*-D-ribofuranoside) acts as an AMP analog and has been used extensively as an activator of AMPK and stimulator of mitochondrial biogenesis [132–136]. AICAR has been described as an exercise mimetic because of its ability to stimulate oxidative metabolism gene programs and to increase endurance similar to exercise [114]. AICAR has also shown positive effects in both acute and chronic disease models characterized by mitochondrial dysfunction. Preconditioning of rats with AICAR was protective against cardiac ischemia/reperfusion injury through enhancement of myocardial glucose uptake [137]. AICAR was also shown to limit renal ischemia/reperfusion injury in the rat kidney [138]. Chronic administration of AICAR prevented the development of hyperglycemia and insulin resistance in Zucker diabetic rats [139].

12.7.2 Sirtuins

Sirtuins are a family of protein deacetylases linked to mitochondrial biogenesis and mitochondrial function. SIRT1 is the most highly studied due to its positive regulation by the oxidized coenzyme NAD^+ and its ability to act as a positive transcriptional regulator of PGC-1α and other mitochondrial-associated genes through promoter deacetylation [132, 140]. Recent studies have shown that PGC-1α plays a key role in cell metabolism with SIRT1, a NAD^+-dependent deacetylase that can regulate and activate PGC-1α by deacetylating it at specific lysine residues, thereby resulting in increased expression of PGC-1α target genes [141, 142]. In corresponding results, PD cybrids show reduced SIRT1 phosphorylation, in addition to reduced PGC-1α activity levels [7, 25].

Drugs such as resveratrol can act through SIRT1 to decrease PGC-1α acetylation, producing a subsequent increase in activity of PGC-1α and its downstream target genes involved in oxidative phosphorylation and mitochondrial biogenesis (Fig. 12.1) [143–145]. In addition to enhancing mitochondrial biogenesis and oxidative metabolism, resveratrol has been shown to be protective in animal models of cardiovascular disease, neurodegeneration, and metabolic syndrome [146–152]. Evaluation of resveratrol in humans has shown positive effects on insulin resistance and glycemic control in diabetic patients [153]. The therapeutic application of resveratrol is limited by its low potency and poor bioavailability, which has led to the development of a new class of synthetic small-molecule SIRT1 activators, including SRT1720, SRT1460, SRT2104, and SRT237. These synthetic compounds have been shown to improve insulin resistance and to lower plasma glucose in type II diabetic animals in addition to improving mitochondrial oxidative capacity [145]. SRT1720 significantly increased life span in obese mice fed a high-fat diet [154]. Additionally, SRT1720 can promote recovery from renal ischemia/reperfusion injury through activation of SIRT1/PGC-1α and stimulation of mitochondrial biogenesis [155]. Other naturally available compounds in addition to resveratrol can induce SIRT1 and positively regulate mitochondrial biogenesis, including quercetin, a flavonoid found in multiple different foods, and daidzein and genistein isoflavones found in soybeans [156, 157].

In addition to SIRT1, SIRT3, which acts within the mitochondrial matrix, is critical for mitochondrial function. SIRT3 regulates a wide range of mitochondrial processes, including efficiency of the electron transport chain [158], maintenance of mtDNA integrity [159], and regulation of mitochondrial ROS production [158, 160]. SIRT3 has been shown to be a critical downstream mediator of the protective effects of AICAR treatment in renal injury models [138]. AICAR was also shown to protect against cisplatin-induced renal injury in mice through SIRT3-mediated enhancement of mitochondrial dynamics and function [161]. These data suggest that sirtuins are critical mediators of mitochondrial homeostasis and that direct or indirect pharmacological activation of sirtuins can positively affect disease outcomes through improved mitochondrial function.

12.8 Conclusion

By activating transcriptional programs addressing multiple aspects of mitochondria dysfunction, certain targets and compounds may ameliorate a number of mitochondrial-related disease pathways, including oxidative stress, autophagy, and neuroinflammation (Table 12.1). Improvements on multiple fronts, however, are needed, and combinatorial approaches may be necessary to elicit a greater impact on patient health in PD. Indeed, candidate compounds will need to be vigorously evaluated, as effectiveness in preclinical mouse models of PD has not translated into improvements in patients in human clinical trials thus far. Whether compound testing in PD

Table 12.1 List of mitochondrial effects and selected pharmacological activators

Target	Compounds	Mitochondrial effects
Transcriptional co-activator		
PGC-1α		Increases mitochondrial biogenesis, mtDNA content, expression of TCA and OXPHOS genes[a], gluconeogenesis, ROS detoxification
PGC-1α activators		
AMPK	AICAR	Increases PGC-1α activity and its effects
SIRT1	Resveratrol, SRT1720, SRT1460, SRT2104, SRT2379	Increases PGC-1α activity and its effects
Nuclear receptor agonists		
PPARα	Fibrates (fenofibrate)	Activates AMPK and PGC-1α and their effects
PPARγ	Thiazolidinediones (rosiglitazone, pioglitazone)	Increases the effects seen with PGC-1α; also prevents release of pro-inflammatory cytokines
PPARδ	GW0742, GW501516, KD3010	Increases the effects seen with PGC-1α; also prevents release of pro-inflammatory cytokines
Targeted by PGC-1α and PPARs		
NRFs	Triterpenoids (CDDO-MA)	Increases the expression of TCA and OXPHOS genes[a]

[a]TCA = tricarboxylic acid cycle; OXPHOS = oxidative phosphorylation

models of iPSC-derived A9 dopaminergic neurons should supplement studies in rodents will be an important issue to address, as PD therapy development proceeds.

12.9 References

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Chapter 13
Delivery of Biologically Active Molecules to Mitochondria

Diana Guzman-Villanueva and Volkmar Weissig

13.1 Introduction

Mitochondria are subcellular organelles responsible for regulating essential cellular functions including energy production and metabolic processes such as oxidative phosphorylation, gluconeogenesis, and fatty oxidation. In addition to these vital roles, mitochondria also play pivotal roles for cell survival and cell death, which makes them attractive as drug targets in particular for potential anticancer chemotherapies [1].

During oxidative phosphorylation, reactive oxygen species (ROS) are continuously being produced due to the incomplete reduction of oxygen [2]. While ROS at low levels are essential signaling molecules needed for the regulation of the entire metabolome, any uncontrolled overproduction of ROS (oxidative stress) has been linked to many disorders including neuronal (Alzheimer's, Parkinson's), cardiovascular (ischemia–reperfusion injury), and metabolic (diabetes) diseases. Removing any excess of ROS, therefore, appears to be key for the treatment of those pathologies.

Although the cell is equipped with its own antioxidant defense system comprised of superoxide dismutase (SOD), catalase, glutathione, peroxidase, and others, any excess of ROS can potentially overwhelm this system resulting in oxidative damage to lipids, proteins, and nucleic acids. Damage to the respiratory chain, in turn, may trigger a further increase in ROS production eventually leading to a vicious cycle. To protect cells from oxidative damage, strategies for the delivery of antioxidants have been explored for several decades, unfortunately only with very limited success with respect to developing clinical protocols. Among several other obstacles, any antioxidant treatment has to overcome the membrane impermeability of enzymes with antioxidant activities, such as SOD or catalase, as well as the limited

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intracellular bioavailability of antioxidants like vitamin E or coenzyme Q_{10} [3]. Moreover, although scavenging agents like SOD mimetics have shown to be cell permeable and very effective in reducing oxidative stress in in vitro models, they do not selectively accumulate within mitochondria. To solve the general problem of low bioavailability of administered antioxidants and subsequently to replenish the endogenous antioxidant defense system with crucial components, mitochondria-targeted pharmaceutical nanocarrier systems such as nanoparticles and nanovesicles are being increasingly explored.

13.2 Lipophilic Cations

Since revealing the existence of the mitochondrial membrane potential (MMP), which is generated by the electron flow through the respiratory chain [4], a number of synthetic ions have been demonstrated to selectively accumulate inside the mitochondrial matrix in response to the MMP. Skulachev showed that anions such as phenyl dicarbaundecaborane (PCB^-) accumulate in submitochondrial particles in an energy-dependent manner, and cations like dimethyl ammonium, tetrabutyl ammonium, and triphenylphosphonium (TPP) penetrate mitochondria [5–7]. In addition, Johnson screened multiple rhodamine compounds and described that only those positively charged, like rhodamine 123 and other lipophilic fluorescent probes, were able to stain mitochondria in living cells, while those uncharged or negatively charged did not, suggesting that the selectivity of rhodamine 123 to stain mitochondria was a result of the relatively high electric potential in the mitochondrial membrane [8]. Later, based on physicochemical characterization, quantitative structure–activity relationship (QSAR), and the Fick–Nernst–Planck model, Horobin developed a model to predict the cellular uptake and selective accumulation of low molecular weight compounds within mitochondria [9].

During the last 15 years, the utilization of lipophilic cations has become a well-known approach for targeting drugs and biologically active molecules to and into mitochondria. Driven by the large MMP of the inner mitochondrial membrane (negative inside, positive outside, 140–180 mV), lipophilic cations readily accumulate within the mitochondrial matrix without requiring a particular transport mechanism (see Fig. 13.1a).

Mike Murphy pioneered the use of triphenylphosphonium (TPP) cations for selectively targeting biologically active molecules to and into mitochondria in living mammalian cells in vitro and in vivo. He and his group successfully linked TPP to a large variety of low molecular weight compounds, in particular to molecules with antioxidant activities in order to prevent and protect cells and tissues against oxidative damage. Lipoic acid, ebselen, and tocopherol are some examples of antioxidants conjugated to TPP cation [10]. MitoE, MitoPBN, MitoPeroxidase, and MitoCP are other TPP-conjugated antioxidants which have been extensively studied [3, 11, 12]. However, by far the most studied mitochondria-targeted antioxidant seems to be MitoQ (see Fig. 13.2).

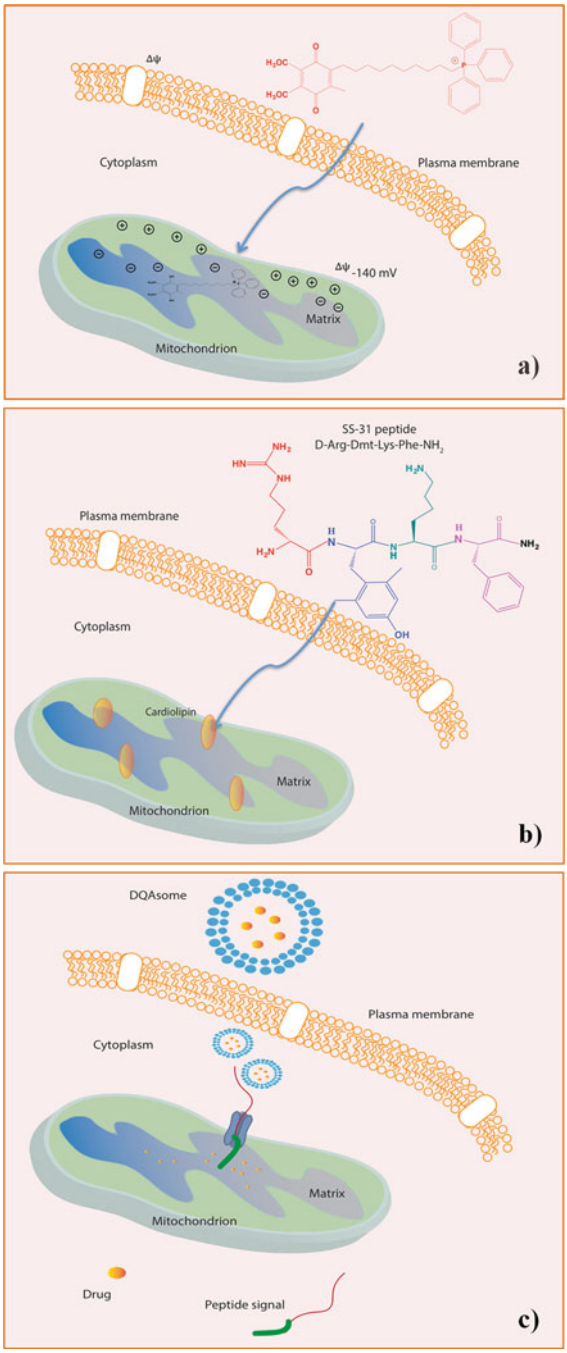


Fig. 13.1 Schematic illustration of mitochondriotropic drug delivery systems: (**a**) triphenylphosphonium (TPP) conjugates, (**b**) peptide antioxidants, and (**c**) DQAsomes

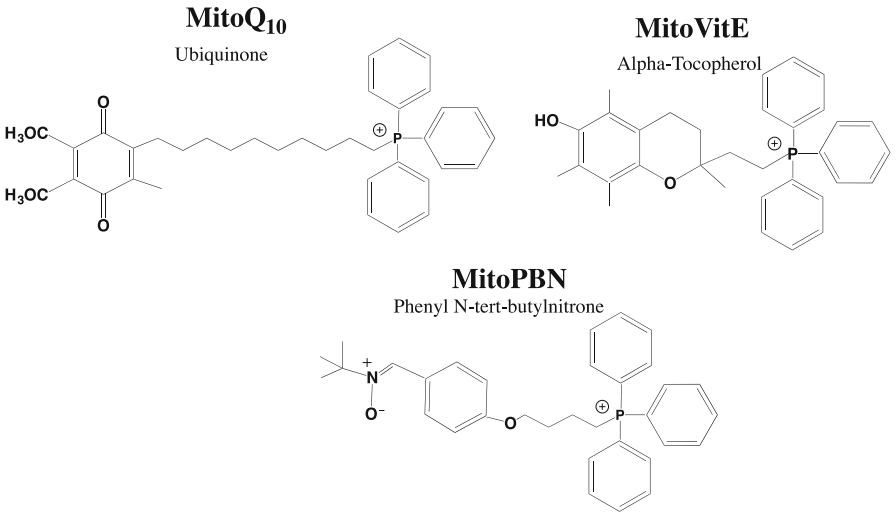


Fig. 13.2 Structures of mitochondriotropic antioxidants

MitoQ is a ubiquinone covalently linked to the lipophilic TPP cation via an alkyl chain. Once inside mitochondria, the ubiquinone residue is incorporated into the lipid bilayer and reduced to ubiquinol, which ultimately acts as an efficient antioxidant preventing lipid peroxidation and oxidative damage induced by hydrogen peroxide radicals [13].

MitoQ has been demonstrated to exert protective effects against oxidative stress in a number of in vitro and in vivo models. MitoQ was well tolerated in a Phase 1 trial, but the following Phase 2 study failed to show efficiency with respect to slowing the progression of Parkinson's disease of 128 enrolled patients. The reasons for this failure can be multifold and are beyond the scope of this chapter. However, toxicity issues have raised a concern. In particular, MitoQ has been linked to adverse effects such as nausea and vomiting in a dose-dependent manner [14] at concentrations above 500 nmol.

One possible reason for this toxic effect appears to be the extensive accumulation of the lipophilic cation within mitochondria, which eventually may lead to membrane depolarization [10]. This naturally raises the question of drug metabolism. Any design of antioxidant conjugates able to selectively accumulate inside the mitochondrial matrix has to include features to make them biodegradable during normal drug metabolism, an aspect which currently appears to be underappreciated. Alternatively, naturally and not chemically modified antioxidants could be also encapsulated or incorporated into pharmaceutical biodegradable nanocarriers such as liposomes. Boddapati et al. hydrophobized the TPP cation with a stearyl residue (STPP) in order to prepare so-called mitochondriotropic liposomes, loaded them with ceramide, and demonstrated their efficiency by inducing apoptosis in cancer cells presumably via directly acting on mitochondria [15]. Malhi et al. functionalized

doxorubicin-loaded STPP liposomes with surface-bound folic acid in order to make them cancer cell specific and called such liposomes "mitocancerotropic" [16].

To reduce the toxicity of alkyl TPP cations, Biswas et al. attached TPP to the distal end of polyethylene glycol-phosphatidylethanolamine (PEG-PE) (TPP-PEG-PE) and incorporated this conjugate into liposomes loaded with paclitaxel. The resulting formulation proved to be less toxic in comparison with STPP or PEG-STPP liposomes, improved the anticancer effect of paclitaxel, and efficiently accumulated in mitochondria [17]. Benien et al. conjugated the TPP cation via a linker group to commercially available functionalized phospholipids, while Guzman-Villanueva et al. attached TPP directly to the head group of phospholipids (Fig. 13.3). The resulting mitochondriotropic liposomes maintained their selectivity for mitochondria and showed significantly reduced toxicity in comparison with STPP liposomes [18, 19]. Table 13.1 shows an overview of other pharmaceutical nanocarriers, which have been rendered mitochondriotropic via conjugation to TPP cations.

13.3 Mitochondriotropic Peptides

Szeto–Schiller (SS) peptide antioxidants represent an alternative to lipophilic cations to deliver antioxidants to mitochondria. These small, water-soluble tetrapeptides have been demonstrated to accumulate in the inner mitochondrial membrane (IMM) (Fig. 13.1b) and to efficiently scavenge ROS [3].

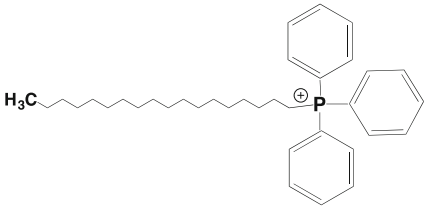
With respect to their amino acid sequence, SS peptides usually alternate aromatic amino acids like phenylalanine (Phe), dimethyltyrosine (Dmt), or tyrosine (Tyr) with the basic amino acids arginine (Arg) and lysine (Lys) [28], as seen in Fig. 13.4. As described by Berezowska, the synthesis of SS peptides was carried out by solid-phase methods [29]. SS-01 (Tyr-D-Arg-Phe-Lys-NH_2), SS-02 (Dmt-D-Arg-Phe-Lys-NH_2), SS-20 (Phe-D-Arg-Phe-Lys-NH_2), and SS-31 (D-Arg-Dmt-Lys-Phe-NH_2) are some examples of these novel SS peptide antioxidants [30].

The mechanism by which these peptides exert their antioxidant effect is attributed to the presence of Dmt and Tyr, which scavenge ROS leading to the formation of unreactive tyrosyl or dityrosine radicals, which also react with superoxide radicals to form tyrosine hydroperoxide [30]. Although tyrosine possesses already good antioxidant properties, the methylation of the phenolic ring seems to further increase the antioxidant potency of the SS peptides. Moreover, using a FluoroProbe it was demonstrated that SS-3 selectively binds to cardiolipin in the IMM by electrostatic and hydrophobic interactions, thus inhibiting cardiolipin peroxidation and the onset of mitochondrial permeability transition (MPT) [31] (Fig. 13.1b).

Despite SS peptides bearing a 3^+ net charge at physiologic pH, these peptides are taken up into cells and accumulate within the mitochondria inner membrane up to 1000-fold in a concentration-dependent, energy-independent, and non-saturable manner [11, 28]. Since the mitochondrial uptake of SS peptides is not driven by the mitochondrial membrane potential, their delivery to the mitochondrial matrix is not hampered by pathologically reduced IMM potentials. SS peptides have shown

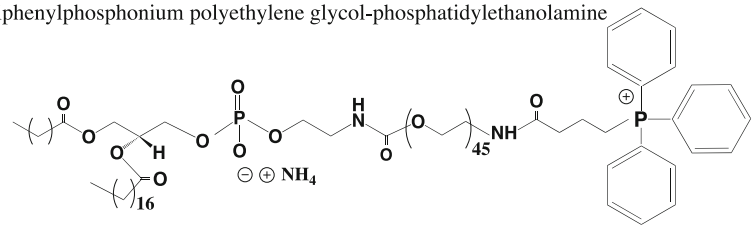
STPP

Stearyl triphenylphosphonium



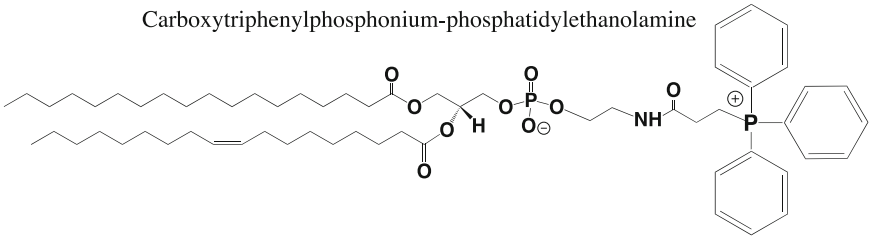
TPP-PEG-2000-PE

Triphenylphosphonium polyethylene glycol-phosphatidylethanolamine



CTPP-PE

Carboxytriphenylphosphonium-phosphatidylethanolamine



DPTPP

Diplamitoyl triphenylphosphonium

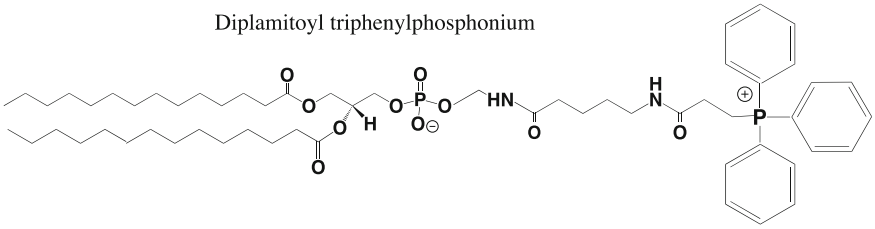


Fig. 13.3 Schematic representation of mitochondriotropic anchors

Table 13.1 Pharmaceutical nanocarriers rendered mitochondriotropic via conjugation to TPP cations

TPP conjugate	Cargo	Carrier
Stearyl triphenylphosphonium (STPP) [15]	Ceramide	Liposomes
Triphenylphosphonium-PEG-PE (TPP-PEG-PE) [17]	Paclitaxel	Liposomes
PEI-triphenylphosphonium (PEI-TPP) [20]	Doxorubicin	Nanoparticles
Triphenylphosphonium-PE (CTPP-PE) [19]	–	Liposomes
Folic acid stearyl triphenylphosphonium (FA-STPP) [16]	Doxorubicin	Liposomes
Dioleoyl, dimyristoyl, dipalmitoyl triphenylphosphonium [18]	–	Liposomes
D-α-tocopheryl poly(ethylene glycol) 1000-triphenylphosphine (TPGS1000-TPP) [21]	Paclitaxel	Liposomes
Poly(amidoamine)-triphenylphosphonium (G(5)-D-Ac-TPP) [22]	–	Dendrimers
Triphenylphosphonium-*N*-(2-hydroxypropyl) methacrylamide (TPP-HPMA) [23]	–	
Poly(ethylene glycol)-polycaprolactone-triphenylphosphonium (PEG-PCL-TPP) [24]	Coenzyme Q10	Micelles
Poly(D-L-lactic-*co*-glycolic acid)-*block*-poly(ethylene glycol)-triphenylphosphonium (PLGA-*b*-PEG-TPP) [25]	Ionidamine, α-tocopheryl succinate, curcumin, 2,4-dinitrophenol	Nanoparticles
Gold-chitosan-triphenylphosphonium (AuNC-TPP) [26]	–	Nanoclusters
Triphenylphosphonium-HDL-apoA-I [27]	Apolipoprotein	Quantum dots

to be safe at concentration of up to 100 μM without causing any depolarization of mitochondria [30]. The presence of D-amino acids and amidation of the C-terminus confer peptidase resistance to all SS peptides [28]. SS peptides were shown to protect linoleic acid as well as low-density lipoproteins (LDL) against oxidation by H_2O_2 [28, 30].

After intravenous injection in sheep, SS-02 was rapidly distributed to the brain and also showed a high affinity for opioid receptors [32]. In mice SS-02 proved to be 36 times more potent than morphine by producing an analgesic response for up to 12 h. Due to its inherent affinity for opioid receptors, SS-02 can potentially cause constipation and respiratory depression just like morphine. In contrast to SS-20, SS-31 displays reduced affinity for opioid receptors, and its daily administration (5 mg/kg) for 5 months did not show significant side effects [33].

SS peptides are readily taken up by Caco-2 cells (15 min) as well as by isolated mouse liver mitochondria (2 min) [30]. When neuronal cells (N2A) were stressed with *tert*-Butyl hydroperoxide (*t*BHP), the SS-02 and SS-31 peptides significantly reduced the intracellular ROS production and prevented oxidative cell death while completely preserving mitochondrial functionality [33]. Thus, it appears that low concentrations of SS peptides can potentially protect cells from oxidative stress [30, 34, 35].

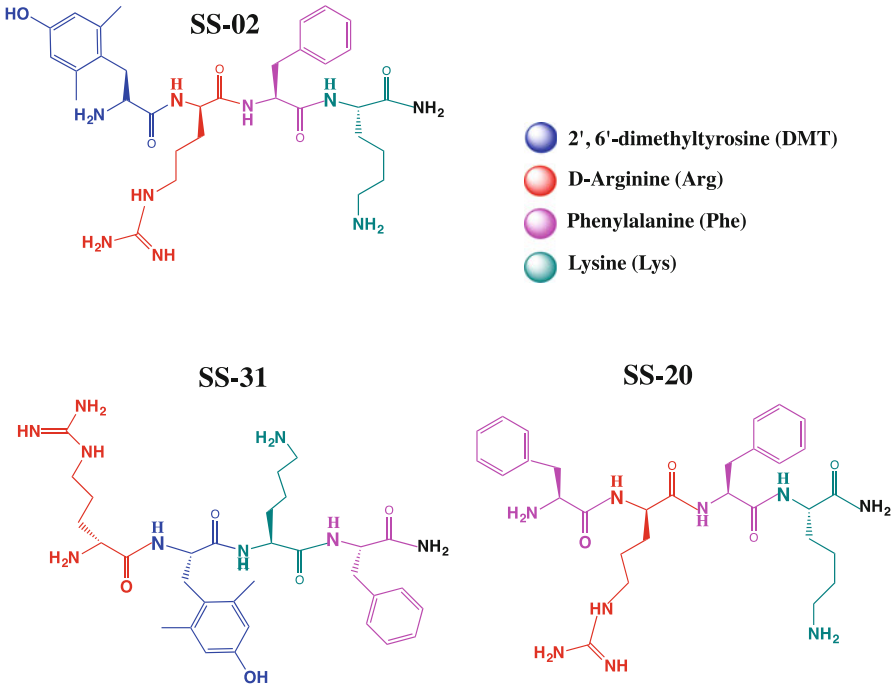


Fig. 13.4 Structure of SS (Szeto–Schiller) peptide antioxidants

When evaluating the scavenging activity of SS peptides during ischemia–reperfusion in an ex vivo animal study, SS-02 and SS-31 protected the myocardium from "stunning" when administered during reperfusion [30, 36]. The progressive loss of contractile force observed during 30 min ischemia and 90 min reperfusion in guinea pig hearts was significantly reduced after hearts were perfused with SS peptides [36]. Additional evidence showed that the pre-ischemic treatment with SS-20 and SS-31 peptides reduced contractile dysfunction during reperfusion [3]. These studies demonstrate the potential of SS peptides to significantly reduce ischemia–reperfusion injury.

In an infarct size rat model where the left anterior descending (LAD) coronary artery was occluded for 60 min, rats that received SS-20 or SS-31 prior to occlusion and 5 min before reperfusion showed higher ATP content and significantly reduced infarct size and lipid peroxidation [37].

In the same context, SS-31 reduced infarct size and hemispheric swelling and avoided glutathione depletion when given immediately after reperfusion in a model of cerebral ischemia–reperfusion in mouse [38].

Based on the extensive data showing the benefits of SS peptides as a potent antioxidant in in vitro and in vivo models, as well as their low toxicity and excellent pharmacokinetic properties, these peptides can be considered as powerful therapeutic agents for the treatment of disorders associated with ROS production. In addition, due to physicochemical properties like solubility and membrane permeability, these

Table 13.2 Ongoing clinical trials for Bendavia peptide listed on www.clinicaltrial.gov

Identifier number	End point	Subjects	Status
NCT01754818	Pharmacokinetics/tolerability	Healthy	Completed
NCT01518985	Endothelial dysfunction	Healthy	Completed
NCT01786915	Safety/pharmacokinetics	Healthy	Completed
NCT01513200	Pharmacodynamics/pharmacokinetics	Healthy	Completed
NCT01755858	Pharmacodynamics (renal flow and renal function)	Patients	Recruiting
NCT02245620	Safety/efficacy (skeletal function)	Patients	Recruiting
NCT01572909	Effectiveness/safety/tolerability	Patients	Completed
NCT01115920	Safety/tolerability/pharmacokinetics	Healthy	Completed
NCT02388529	Safety/tolerability/pharmacokinetics/efficacy	Patients	Not yet recruiting
NCT02388464	Safety/tolerability/pharmacokinetics	Patients	Recruiting
NCT02367014	Safety/tolerability/efficacy	Patients	Recruiting
NCT02436447	Safety/pharmacokinetics	Patients	Recruiting
NCT02314299	Safety/efficacy	Patients	Recruiting

peptides can easily be administered by intravenous, intraperitoneal, subcutaneous, or even intracerebroventricular injections. SS-31 under the name Bendavia or MTP-131 is currently undergoing multiple clinical trials to assess its therapeutic potential for the treatment of cardiovascular, kidney, neurodegenerative, and genetic mitochondrial diseases (see Table 13.2).

In healthy volunteers in Phase 1, Bendavia (NCT01115920) proved to be well tolerated at doses of 10, 50, and 100 mg. At the highest peptide concentration, only minimal changes in the serum sodium levels and other electrolytes were observed [39]. Currently in a Phase 2 study, the safety, tolerability, and efficacy of intravenously administered Bendavia are being evaluated in patients undergoing coronary intervention and stenting (NCT01572909). In addition to these studies, Bendavia is being evaluated in skeletal muscle disorders, mitochondrial myopathy, acute coronary syndrome, heart failure, and acute kidney injury, along with other disorders (Table 13.2).

Despite the large number of clinical trials and nonclinical data validating its therapeutic activity, Bendavia failed to reduce heart tissue damage in a Phase 2 study in patients with acute coronary events, as presented in the American College of Cardiology's 64th Annual Scientific Session.

13.4 Dequalinium Vesicles

During the last 15 years, a large variety of pharmaceutical mitochondria-targeted nanocarriers have been described, most recently reviewed in [40]. This development began at the end of the 1990s with the accidental discovery of the vesicle-forming capacity of dequalinium chloride (DQA) [41]. Dequalinium (1,1′-decamethylene bis-4-aminoquinaldinium chloride) is a cationic bolaamphiphile composed of two

quinaldinium rings linked by ten methylene groups (Fig. 13.5). DQA was found to be able to self-assemble into liposome-like vesicles named DQAsomes (DeQAlinium-based lipoSOMES) at that time. The strong affinity of DQA for mitochondria, combined with its ability to form cationic liposome-like vesicles (DQAsomes), led to the idea of using DQAsomes as a potential mitochondria-targeted drug and DNA delivery system [42, 43]. Follow-up studies by the same and by other authors confirmed the suitability of DQAsomes for the delivery of biologically active compounds to mitochondria.

Lyrawati et al. demonstrated the ability of DQAsomes to deliver an artificial mini-mitochondrial genome construct encoding green fluorescence protein (GFP) to the mitochondrial compartment of a mouse macrophage cell line, which resulted in the expression of GFP mRNA and protein [44]. Lyrawati's paper is the very first publication describing functional transgene expression within mitochondria in living mammalian cells.

Formulations of paclitaxel with DQA showed to increase the solubility of the drug in comparison with free paclitaxel by a factor of roughly 3000, thereby representing an alternative to Cremophor-based formulations of the highly insoluble paclitaxel. A series of other in vitro and in vivo studies demonstrated an increase of paclitaxel's efficiency in triggering apoptosis by directly acting on mitochondria [45].

A follow-up study by Vaidya et al. has confirmed the increased apoptotic activity of DQAsomal encapsulated paclitaxel [46]. These authors also successfully conjugated folic acid to the surface of DQAsomes in order to make them specific for tumor cells overexpressing the folate receptor, which further increased the apoptotic activity of DQAsomal paclitaxel.

Most recently, the preparation of curcumin-loaded DQAsomes for pulmonary delivery has also been described [47]. Curcumin is a potent antioxidant with anti-inflammatory properties; however, it has a very low bioavailability following oral administration due to its water insolubility. Curcumin encapsulated into DQAsomes increased its antioxidant activity in comparison with free curcumin. Curcumin-loaded DQAsomes have been proposed by these authors as a potential inhalation formulation with mitochondrial-targeting ability which would eventually open up a

Dequalinium Chloride

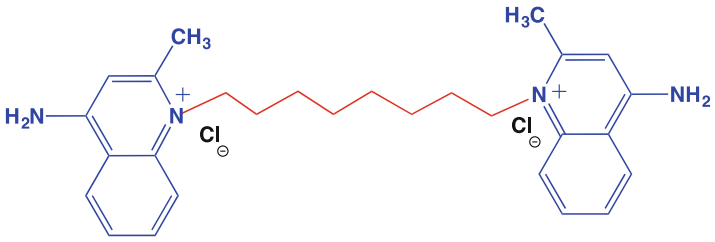


Fig. 13.5 Chemical structure of dequalinium chloride

new strategy for curcumin delivery aimed at the treatment of acute lung injury [47]. DQAsomes can nowadays be considered the prototype for all vesicular mitochondria-specific nanocarriers.

13.5 Concluding Remarks

The last two decades have seen tremendous progress in recognizing and appreciating the central role mitochondria play for human health. It has become well established that mitochondrial dysfunctions are associated with a large number and variety of clinical disorders including Alzheimer's and Parkinson's disease. Consequently, since the end of the 1990s, an increasing number of strategies have emerged to selectively deliver biologically active molecules to mitochondria in order to either probe or manipulate mitochondrial functions. Promising in vitro data as well as successful animal studies will eventually lead to new clinical approaches for the therapy of human diseases caused by mitochondrial malfunctions.

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