Oxidative Stress and Redox Signalling in Parkinson's Disease

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Issues in Toxicology No. 34

Print ISBN: 978-1-78262-188-1
PDF eISBN: 978-1-78262-288-8
EPUB eISBN: 978-1-78801-191-4
ISSN: 1757-7179

A catalogue record for this book is available from the British Library

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Printed in the United Kingdom by CPI Group (UK) Ltd, Croydon, CR0 4YY, UK

Preface

Parkinson's disease (PD) was first described in detail in 1817 by the London surgeon James Parkinson, who referred to the disorder as 'paralysis agitans' or 'shaking palsy'. PD is now defined as a chronic and progressive neurodegenerative disorder that currently affects ~1 million individuals in the US alone, and ~10 million around the world. Importantly, from 2006 to 2016 the population affected with PD doubled, while only a 10% increase in the US population was reported. The exact prevalence of PD is unclear as the disease is only diagnosed after the pathogenic process is far advanced. Thus, the exact number of individuals with PD is expected to be higher than the actual number of cases diagnosed. Economically, the estimated cost of PD in the US is ~$25 million per year in both treatment and lost income from the inability to work.

PD prevalence and incidence increase exponentially for individuals 65 to 85 years of age. While aging is considered the major risk factor for PD, the etiology of the disease is still largely unclear. Around 10% of diagnosed cases have been linked to inherited (familial) mutations in genes encoding proteins such as α-synuclein, Parkin, PINK1, DJ-1, and LRRK2, while other genetic modifications only increase the risk of developing the disease. The remaining ~90% of PD cases are sporadic, of which ~5% are linked to *de novo* mutations primarily in the α-synuclein and LRRK2 genes. For those sporadic cases without a clear delineated genetic background (~85% of total PD cases), it is hypothesized that environmental or occupational exposures are important contributors to neuronal cell loss. Thus, it is now considered that aging, genetics and environmental/occupational risk factors contribute to PD.

The primary clinical phenotype of PD is what is called parkinsonism, a movement disorder that is characterized by tremor at rest, bradykinesia, rigidity and postural instability, which is directly associated with the depletion of dopamine neurotransmission from degenerated dopaminergic A9

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Published by the Royal Society of Chemistry, www.rsc.org

neurons in the *substantia nigra pars compacta* (SNpc) innervating the striatum. Neuronal degeneration in PD affects other areas of the brain as the disease progresses, but SNpc dopaminergic neurons have been considered one of the primary and likely more sensitive targeted neuronal populations in PD. Importantly, pathological features in the olfactory bulb and brain stem that correlate with early nonmotor dysfunction in the sense of smell and sleep regulation, as well as the degeneration of dopaminergic neurons in the intestine leading to gastrointestinal dysfunction, are considered important components of the disease. To date, the bulk of research in the PD field has been focused on understanding the mechanisms behind the loss of SNpc dopaminergic neurons, but what makes these cells a primary pathological target in PD, and what makes them particularly sensitive to PD risk factors, remains unclear.

Since the late 1970s and early 1980s, oxidative stress has been recognized as a biomarker of the imbalance in redox homeostasis that occurs during the progressive loss of dopaminergic neurons in the brains of PD patients. Byproducts of protein, lipid and nucleic acid oxidation are found in post-mortem PD brain samples. It has been established that mitochondrial dysfunction, inflammatory processes, the pro-oxidant metabolism of dopamine, and metal-catalyzed free-radical formation combine to generate oxidative damage in dopaminergic neurons. Importantly, recent evidence has demonstrated that aside from non-specific oxidative damage, alterations in redox homeostasis associated with PD result in perturbations of enzymatic and signalling processes that are essential for neuronal physiology. Examples of these perturbations include (i) alterations in protein quality control mechanisms that lead to the accumulation of protein inclusions of α-synuclein (Lewy bodies); and (ii) energy failure that renders nigral dopaminergic neurons (which have high energy demands because of their extensive arborization and active pacemaking activity) very sensitive to ATP depletion.

This book aims to highlight recent advances regarding the role of alterations in redox homeostasis in PD pathogenesis. A complete overview of themes including mitochondrial dysfunction, iron and dopamine metabolism, antioxidants, protein aggregation/oxidation and others is reviewed. From this work emerges a strong sense of the rapid pace of discovery in the PD field, but also of substantial knowledge gaps that must be addressed if we are to meet the over-arching goals of identifying biomarkers for earlier diagnosis and developing disease-altering therapies.

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CHAPTER 1

Etiology and Pathogenesis of Parkinson's Disease

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1.1 Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder (after Alzheimer's disease), and it is estimated that PD affects approximately 10 million individuals worldwide, though many cases may go undiagnosed. With the growth of aging populations, it is estimated that PD will nearly double in incidence over the next 25 years, representing a major social and economic burden to provide long-term treatment and care for those affected.[1] This pressing concern has focused increased attention on the field of neurodegeneration, and while laboratory discoveries have begun translation into the clinic, one critical issue persists; the underlying causes of this progressive disorder remain, for the most part, unidentified. Inherited forms of PD strongly correspond to known genetic mutations in proteins involved with mitochondrial function, oxidative stress, and protein degradation pathways. However, inherited forms of PD only account for about 10% of PD cases, and sporadic PD has a much lower association with single gene mutations that are readily identified by specific protein dysfunction.[2,3] The pathogenesis of both familial and idiopathic PD involves several components;

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the gross manifestations of the disorder, the underlying neuronal death and cellular pathology, the molecular mechanisms behind progressive degeneration, and the genetic or environmental dysregulation of proteins responsible for cellular dysfunction.

Currently, no curative or 'disease-modifying' therapy is available to slow or stop the inevitable and inexorable progression of PD. While symptomatic treatment options are available, as the disease progresses and medication doses rise, the tolerability of PD drugs may decrease and side effects often become problematic. In order to develop therapeutic strategies that prevent the progressive loss of dopamine neurons, a clear understanding of the mechanisms behind cell death in PD must be elucidated. Many putative pathological mechanisms in PD can be linked to common pathways that converge on the mitochondrial production of oxidative stress. Here, the pathogenesis of oxidative stress in PD is examined in the context of the cellular pathology observed in the disease.

1.2 Clinical Manifestations of Parkinson's Disease

The motor signs and symptoms of PD – bradykinesia, resting tremor, rigidity, and postural instability – together with the patient's history, are the primary means for identifying the disorder, and current guidelines require two of the four main signs of the disease to be present, typically presenting with asymmetrical onset.[4] Most individuals are diagnosed over the age of 45, with only a small percentage (10%) of cases considered early-onset (under the age of 45).[4] Accompanying these movement deficits are non-motor symptoms of PD, such as decreased GI motility, loss of olfactory function, sleep disorders, and cognitive or behavioral changes.[5] Non-motor symptoms often occur prior to the onset of motor symptoms, though their presence alone has proven unreliable for detecting PD.[6] Motor symptoms of PD occur when there is approximately 80% loss of striatal dopamine levels, indicating that significant cell death and damage has occurred prior to emergence of visible symptoms of the disease.[7,8] This 'silent' period of pathogenesis is extremely problematic as it narrows the window for neuroprotective therapeutic intervention to the period after clinical diagnosis.

There is no definitive test for PD outside of the diagnostic criteria in the clinic and the response of a patient to levodopa (L-DOPA), which is the precursor of dopamine and the most efficacious symptomatic treatment for the disease.[9] Unfortunately, maintenance with L-DOPA and dopamine receptor agonists has limitations. On the one hand, dopamine mimetics are useful at abating many of the symptoms in PD such as bradykinesia, rigidity, and tremor, while on the other hand they contribute to a host of iatrogenic symptoms, including dyskinesias, 'wearing off' effects, and hallucinations.[10] In addition, treatment with dopamine agonists may exacerbate impulse control disorders, such as excessive gambling and reward-seeking behavior.[11,12] There are also indications that dopamine-replacement therapy itself contributes

to cellular toxicity, possibly enhancing the progressive loss of neurons associated with PD.[13] Several small molecule therapies aimed at inhibiting the progression of PD have entered the pipeline for novel drug development, many of which are anti-inflammatory therapies targeted at limiting oxidative stress.[14] It is critical to note, however, that no compound has been proven successful in Phase III clinical trials for this purpose, and the pursuit for new therapeutic strategies continues.

1.3 Neuropathology

Underlying many of the motor symptoms of PD is the selective loss of dopaminergic neurons of the *substantia nigra pars compacta* and their principal axon projections to the striatum. Degeneration of the nigrostriatal tract is considered the hallmark lesion of the disease; however, several other extranigral sites exhibit pathology: the locus coeruleus and subcoeruleus complex, reticular formation and raphe nuclei, dorsal nuclei of the Vagus, and nucleus basalis of Meynert may all display cell loss.[15] Aptly named for its dark appearance in the adult human brain, the *substantia nigra pars compacta* consists mainly of dopamine neurons that contain the pigment neuromelanin, a product of catecholamine metabolism, which is visibly reduced in intensity in the postmortem PD brain. Dopamine neuron loss does not occur uniformly throughout the *substantia nigra*, and the pattern of neuron death is distinct to the disease, differing from the typical loss of dopamine neurons associated with aging alone, which occurs predominantly in the dorsal tier of the *substantia nigra*.[16] Neurons of the nigrostriatal tract exhibit dieback, with the degenerative process beginning at the nerve terminal and extending retrogradely to the cell body with medioventral loss of the nigral regions showing more early lesions of PD and extending laterally.[17] The distinctive pattern of loss suggests that dopamine neuron death in PD is not merely due to mechanisms of accelerated aging.

The other key pathological feature of PD is the cellular accumulation of Lewy bodies (LBs) and Lewy neurites (LNs), which are protein accumulations within the somal cytoplasm of neurons (LBs) and their processes (LNs), in the *substantia nigra* and other regions.[18] Lewy bodies and neurites consist predominantly of aggregated α-synuclein protein with a variety of post-translational modifications, including phosphorylation, ubiquitination, nitration, and oxidation of several residues.[19]

The role of α-synuclein in the healthy brain remains somewhat unclear, though there is evidence that it participates in synaptic vesicle function.[15,20,21] α-Synuclein point mutations, (wildtype) gene duplications and triplications, and polymorphisms that increase expression of wildtype α-synuclein all cause PD.[22–25] This genetic information, together with the fact that α-synuclein accumulates in Lewy pathology even in idiopathic PD, suggests the central importance of this protein in almost all cases of the disease.[26] Neuropathological staging of PD progression is assessed by immunostaining

of accumulated α-synuclein, beginning in the caudal brainstem (or olfactory bulb) and spreading rostrally to the neocortex over the course of the disease.[18] According to this scheme, classical involvement of the nigrostriatal neurons occurs in the middle stages of the disease. It is currently unclear whether the spread of α-synuclein pathology represents prion-like cell-to-cell transfer of the protein (discussed below) or simply reflects the relative vulnerabilities of various neuronal populations over the long course of the disease.

α-Synuclein accumulation and aggregation and neuronal death are accompanied by a glial-driven neuroinflammatory response.[27] The involvement of glial cells in PD pathogenesis is proposed as both a beneficial response in an attempt to preserve damaged neurons, as well as a source of unregulated inflammation that can drive further neuronal damage.[14,28,29] What remains less obvious is whether neuroinflammation might sometimes be an inciting factor in PD, or merely a response to mitigate the damage that has already occurred in neurons.

The resident CNS macrophage cells, microglia, are the key enforcers of the immune response in the brain, regulating inflammatory protein expression and recruiting additional immunological participants, including t-lymphocytes, from the periphery.[30,31] It is also widely recognized that astrocytes, the most abundant cell type in the brain, are essential to the inflammatory response associated with progressive dopamine neuron loss in the *substantia nigra*.[32,33] Postmortem examination of microglia and astrocytes reveals an activated, hypertrophic cell phenotype within brains of individuals with PD, appearing most intensely in the ventral midbrain, but evident throughout the brain.[34,35] Reactive glial cells abundantly express pro-inflammatory proteins, which upregulate the production of many neurotoxic factors, including reactive oxygen species that further contribute to dopamine neuron damage. Several lines of experimental evidence have suggested that the glial-driven inflammatory response within the CNS may contribute to the progression of dopamine neuron death, indicating a possible role for anti-inflammatories as PD therapeutics.[30,31] Epidemiological data describing a link between long-term use of NSAIDs and a decreased risk for developing PD has supported this theory; however, no anti-inflammatory drug has been proven successful in clinical trials to halt the progression of the disease.[36] To this end, it is necessary to investigate the multifactorial nature of idiopathic PD, and the molecular factors that contribute to its etiology.

1.3.1 Selective Vulnerability of the Nigrostriatal Dopamine Neuron

In PD, dopamine neurons within the *substantia nigra* are considered a selectively susceptible population of cells, whereas the adjacent dopamine neurons of the ventral tegmental area (VTA) are much more resistant to degeneration. The vulnerability of the nigrostriatal neurons is due to several factors, including their unique anatomy, physiology, bioenergetic profile,

and neurochemistry (reviewed in ref. 37). First, in rat brain, the length of the axon arbor of a single nigrostriatal neuron is up to 80 cm; in humans, this is estimated to be 4 m! In the rat, a single nigrostriatal neuron makes 100 000–240 000 synapses in the striatum. In humans, it is estimated that a nigrostriatal neuron makes 1 000 000–2 400 000 synapses. By contrast, the VTA neuron makes about 10-fold fewer synapses and therefore has a much lower bioenergetic demand. Further compounding the bioenergetic demand of the nigrostriatal neuron is the fact that their axons are unmyelinated. Thus, propagation of each action potential and subsequent repolarization requires much more energy than if the fibers were myelinated.

As discussed later, there is abundant evidence that mitochondria are dysfunctional in PD. If one conservatively estimates that there are 10 mitochondria per nigrostriatal synapse, then it is apparent that each neuron must maintain at least 10 000 000 mitochondria – and must do so over exceedingly long distances. As Paul Bolam has suggested, nigrostriatal neurons may have 'too many mouths to feed'. It seems reasonable to assume that those mitochondria most distant from the soma would be most vulnerable to 'wear and tear' and, as a consequence, they may produce less ATP and more ROS.[37] If so, this might explain why nigrostriatal degeneration begins at the terminals.

Dopamine itself may contribute to the vulnerability of the dopaminergic nigrostriatal neuron, particularly in the setting of mitochondrial impairment. Dopamine, a catecholamine, is produced from tyrosine by the sequential enzymatic actions of tyrosine hydroxylase and amino acid decarboxylase. A redox reactive molecule, dopamine is normally sequestered in synaptic vesicles at concentrations estimated to be in the high millimolar range.[38] Thus, even a small degree of dopamine leakage from vesicles could easily produce local cytosolic concentrations in the micromolar range. It is now clear that (i) mitochondrial impairment (complex I defects) and (ii) increased levels of alpha-synuclein, both of which have been implicated in PD, lead to redistribution of dopamine from vesicles to cytosol.[39] Cytoplasmic dopamine can undergo enzymatic oxidation or non-enzymatic auto-oxidation to produce dopamine quinone species (DAQ).[40] The electron-deficient DAQ readily reacts with cellular nucleophiles, predominantly reduced sulfhydryl groups on free cysteine residues, glutathione, and proteins, forming covalent cysteinyl-dopamine adducts.[41] Since the active sites of many proteins contain cysteine residues, DAQ modification can cause protein inactivation and loss of function.[42]

Dopamine oxidation has been associated mechanistically with (i) mitochondrial impairment, (ii) alpha-synuclein oligomerization, (iii) enhanced NMDA receptor function, and (iv) reduced proteasome function, each of which has been posited to play a central pathogenic role in PD.[39,43–45] In summary, the anatomy and physiology of nigrostriatal neurons predisposes them toward bioenergetic crisis, in contrast to VTA neurons. In the setting of bioenergetic impairment, dopamine may leak from vesicles to the cytosol where it can oxidize to DAQ and exacerbate the neurodegenerative process.

1.3.2 Mitochondrial Dysfunction in PD

It is well established that mitochondrial dysfunction is central to the etiology of dopamine neuron death and dysfunction in PD (reviewed in ref. 46). In addition to energy production *via* aerobic oxidative phosphorylation, mitochondria also regulate intracellular calcium levels, participate in lipid metabolism, as well as steroid, carbohydrate, and amino acid breakdown.[47] Mitochondria are responsible for signalling apoptosis pathways, and are the gatekeepers for apoptotic machinery to complete an organized cell death. The process of mitochondrial respiration within neurons requires aerobic oxidative phosphorylation, a process that naturally produces a high amount of oxidative byproducts, such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$).[47] In a healthy neuron, the mitochondria will detoxify these ROS using normal compensatory mechanisms such as antioxidant and reactive oxygen scavenger proteins (ex. glutathione peroxidase).[48] However, even under basal conditions, nigrostriatal dopamine neurons exist in a more oxidized state than other neurons.[49] Under pathological conditions, these compensatory mechanisms can be overwhelmed in the dopamine neuron, and ROS production from the mitochondria becomes a source of oxidative stress for the cell.[50] In addition to oxidative stress, mitochondrial dynamics are also an important factor in dopamine neuron pathology. Mitochondrial fusion and fission, the processes by which the outer membranes of two mitochondria join to form one membrane (fusion) or by which a single mitochondrial membrane becomes two (fission), are also potential sources of dysfunction in the neuron.[51] Mutations in genes regulating mitochondrial dynamics are a known cause of inherited forms of PD (ex. PINK1, Parkin, SNCA).[52] It is not surprising then, that genes controlling mitochondrial dynamics are central to dopamine neuron survival, given that synaptic maintenance, mitochondrial biogenesis, and neurotransmission are all regulated by this process.

1.3.2.1 Mitochondrial DNA Damage

The association of mutations in genes regulating mitochondrial function and PD is further supported by the regulation of mitochondrial DNA (mtDNA). Mitochondrial DNA encodes 37 genes, including 13 protein subunits for complex I-V of the electron transport chain (ETC). mtDNA is located in association with the inner mitochondrial membrane, neighboring where oxidative phosphorylation occurs. Therefore, it is constantly exposed to ROS.[53] The lack of histones and limited repair mechanisms causes mtDNA to be especially vulnerable to damage, such as strand breaks and base modifications; these in turn can lead to mutations.[54] Accordingly, mutations have been found at a higher rate in dopamine neurons of the *substantia nigra* in PD cases than age-matched controls, suggesting a role for mtDNA mutations in the pathogenesis of PD.[55–58]

Aside from frank mutations, mtDNA damage *per se* may be pathogenic, leading for example to blockage of mtDNA replication and cytotoxicity.

An excess of abasic sites (lacking a purine or pyrimidine base) has been detected in dopamine neurons of the *substantia nigra* in postmortem PD tissue; however, such mtDNA damage was not found in cortical neurons, suggesting that mtDNA damage may be specific to the dopaminergic cell pathology.[59] Furthermore, mtDNA abasic sites within dopamine neurons have also been reported in rats following rotenone treatment, a phenomenon that occurs prior to dopamine cell loss.[59] Given these data, it has been proposed that the detection of mtDNA damage, such as abasic sites, may provide a novel biomarker for early pathological changes in PD.[60]

1.3.2.2 Complex I Inhibition

There is a strong connection between mitochondrial complex I dysfunction and death of dopamine neurons in PD. In addition to the observation of diminished complex I activity in postmortem PD tissue, complex I inhibition induced by exogenous chemical agents is the basis for causing selective dopamine neuron death in neurotoxic models of PD.[61,62] First demonstrated with 1-methyl-2-phenyl-1,2,3,6-tetrahydropyradine (MPTP) toxicity, complex I inhibition results in a potent and selective loss of neurons from the *substantia nigra* and a parkinsonian phenotype.[63] MPTP is bioactivated in the astrocyte *via* monamine oxidase B (MAO-B) to its reactive metabolite MPP^+, which has structural specificity for the dopamine transporter (DAT) expressed on the dopamine neuron. MPP^+ accumulates in the mitochondria where it impedes the flow of electrons in the ETC at complex I, resulting in ATP depletion, reactive oxygen species accumulation, and mitochondrial dysfunction.[64]

Another model of PD is based on the classical complex I inhibitor, rotenone. Aside from being a biochemical tool, rotenone is an organic pesticide still employed in the United States for killing invasive aquatic species.[65] It is distinct from MPTP toxicity in that it does not require bioactivation in the astrocyte, nor does it have a specific affinity or requirement for the DAT. The use of rotenone in animal models of PD results in a lesion of the *substantia nigra* and striatum, with accumulation of α-synuclein protein in the cytoplasm of dopamine neurons, and neurobehavioral deficits.[66] This model is additionally useful in demonstrating the selective toxicity of complex I inhibition to dopamine neurons, given that systemic injection of rotenone affects all cells, but results in a nigrostriatal lesion. The rotenone model of PD correctly predicted the transferrin receptor 2-dependent accumulation of iron in the *substantia nigra*, which was later confirmed in human PD cases.[67] It also predicted the accumulation of mtDNA abasic lesions in nigrostriatal neurons in PD.[59] Translational discoveries such as this exemplify the importance of neurotoxin models in the discovery of pathological mechanisms behind PD. It is of course impossible to fully recapitulate the complex disease state of PD using any animal model, and therefore understanding our limitations using these models is equally important in their employment.

1.3.3 Oxidative Stress

Superoxide ($O_2^{\bullet -}$) is the principal reactive species released from the mitochondria, emanating from complex I, when ATP is not being produced and therefore protonmotive force (Δp) is high, or when NADH/NAD^+ ratio is high within the mitochondrial matrix.[47] Superoxide can be converted by superoxide dismutase (SOD) to the less toxic hydrogen peroxide (H_2O_2). Mitochondria express their own form of SOD (MnSOD), suggesting that there is a biologically relevant purpose for superoxide production from the mitochondria.[47,53] It is proposed that the production of H_2O_2 from mitochondria serves as a redox signal in cells, by transiently altering protein-thiol redox status.[68] Under normal conditions, H_2O_2 is detoxified by catalase or glutathione peroxidase (GPX), maintaining homeostasis between normal mitochondrial signalling and scavenging enzymes.

Pathological increases in reactive oxygen species may result from aberrant enhanced production or impaired detoxification, or some combination thereof. Although H_2O_2 has low relative toxicity, it is possible to form highly reactive radicals from H_2O_2.[50] Within the brain, nitric oxide (NO) is abundant, and when combined with H_2O_2, leads to production of peroxynitrite ($ONOO^-$) and the hydroxyl radical ($OH^{\bullet}$). The highly toxic hydroxyl radical is capable of binding to cellular macromolecules causing widespread protein dysfunction, lipid oxidation, and creating strand breaks in DNA. Peroxynitrite attacks tyrosine residues and causes protein dysfunction, which is readily observed in both animal models of PD as well as in the human disease.[69,70] The function of tyrosine hydroxylase within dopamine neurons can be compromised by peroxynitrite, a process that may contribute to the dysfunction of dopamine production even in preserved neurons in the *substantia nigra*.[71]

It is clear that oxidative stress plays a key role in the pathogenesis of this disease. Tissue from PD *substantia nigra* contains evidence of lipid peroxidation, decreased glutathione levels, and increased iron content.[27,72] Iron and other redox sensitive transition metals (copper, manganese) present within the cell contribute to oxidative damage through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$) resulting in the production of the hydroxyl radical. Several lines of evidence support the theory that iron metabolism is disrupted in PD, leading to both increased accumulation of iron and oxygen radicals in the *substantia nigra*.[73,74] Iron deposits are also observed to interact with α-synuclein, contributing to Lewy body formation.[75] Proteins that store iron in an unreactive state (ferritin, transferrin receptor) are an important component of iron regulation within the brain, and disruption of these proteins may contribute to neurodegenerative processes.[76]

The relative paucity of antioxidants in the CNS is also likely to play a role in the oxidative damage observed in PD. The major detoxifying peptide antioxidant of the CNS, glutathione (GSH), can act to remove ROS either through nonenzymatic reduction or catalysis with glutathione peroxidase, leading to oxidized glutathione (GSSG). The depletion of GSH in the brains of PD patients is one of the earliest biochemical changes that occurs, which

suggests that it may contribute to progression of the disease.[48] This may conceivably result from diminished GSH synthesis, due to decreased levels of the GSH precursor, cysteine, circulating levels of which have been reported to decline with age.[77] Additionally, the amino acid carrier 1 (EAAC1), which transports cysteine into neurons has been shown in mouse models to be modified following MPTP treatment.[48,78] EAAC1 may be inactivated through protein nitration (caused by oxidative stress), thereby reducing the amount of GSH production by up to 30% in neurons exposed to MPTP[78] and nearly 50% reduction after rotenone treatment.[79] Because ROS produced by complex I are predominantly detoxified in the neuron by GSH, neuronal damage is widespread after GSH depletion. More recently, Swanson and colleagues reported that EEAC1(-/-) mice show a progressive loss of nigrostriatal dopamine neurons associated with excessive protein nitration. In this model, the cell-permeable GSH precursor, *N*-acetylcysteine, which bypasses EEAC1, was protective.[80]

GSH depletion may also affect the proteasome system, as described in one *in vitro* study that observed inhibition of 26S proteasome activity in cultured SH-SY5Y cells when GSH dropped below 50% of control.[81] Evidence for GSH depletion and inflammatory activation also exists in the c-Jun-N-terminal kinase (JNK) pathway, which becomes activated when GSH levels are decreased.[82] Abnormal activation of cellular inflammatory pathways through this mechanism may contribute to pathologic neuroinflammation that enhances dopamine neuron toxicity. It was shown that gene deletion of JNK2 and JNK3 in mice significantly protected dopamine neurons following MPTP treatment, indicating that JNK pathways stimulated by GSH depletion likely contribute to exacerbated inflammatory responses.[83]

1.3.4 Dopamine Metabolism

Dopamine, an essential neurotransmitter, also has the capacity to produce reactive intermediates, which may cause collateral oxidative damage within the cell.[84] Cellular toxicity resulting from dopamine metabolism has been shown to occur *via* two main pathways; the production of reactive intermediates from enzymatic metabolism of the catecholamine, and the autoxidation of the molecule to a highly electrophilic dopamine quinone.[41] The metabolism of dopamine by the monoamine oxidase-B enzyme (MAO-B) into its primary metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) produces H_2O_2 as a byproduct.[85,86] If cellular compensatory mechanisms (catalase, GSH) cannot readily detoxify this intermediate, Fenton cycling of electrons may occur upon interaction with transition metals present in the brain (Fe^{2+}, Mn^{2+}, Cu^{2+}) resulting in the production of hydroxyl radicals.[87] In addition, autoxidation of the neurotransmitter produces a highly reactive dopamine quinone species, which has been shown to be capable of binding and modifying cellular proteins.[28] Nucleophilic protein residues, such as sulfhydryl groups, are especially vulnerable to covalent modification by the dopamine quinone, a process that likely results in protein dysfunction.

Indeed, intrastriatal dopamine injections in a rat model produced a significant lesion within the striatum, as well as a dose-dependent increase of cysteinyl protein binding by dopamine and DOPAC.[88] These lesions were attenuated by the coadministration of GSH, suggesting that quenching of such reactive species by antioxidants is a key component in maintaining dopamine homeostasis and limiting cellular toxicity.[88,89]

In addition to the production of reactive intermediates, there is evidence that oxidized dopamine may directly contribute to mitochondrial dysfunction, resulting in the swelling of brain mitochondria and opening of the mitochondrial permeability transition pore.[27] Isolated rat mitochondria exposed to dopamine quinone showed characteristics of ETC uncoupling, as measured by an increase in resting state respiration.[27] These data also indicated that the addition of GSH could attenuate dopamine quinone-mediated damage to mitochondria, while reactive oxygen scavengers such as catalase and superoxide dismutase did not, suggesting that formation of the dopamine quinone may directly alter thiol-containing mitochondrial coupling proteins.[41] Another line of evidence suggests that the aldehyde metabolite of dopamine, dihydroxyphenylacetaldehyde (DOPAL), elicits mitochondrial dysfunction and inhibits tyrosine hydroxylase in dopaminergic PC6-3 cells.[90] Together, these data indicate a strong association between the dopamine neurotransmitter and oxidative damage within the cells responsible for producing the molecule.

Given these findings, it is unsurprising that packaging and storage of dopamine is a highly regulated cellular process; sequestration of dopamine into synaptic vesicles provides a storage condition that limits autooxidation of dopamine, by maintaining a low pH and limiting exposure to MAO-B.[15] The vesicular monoamine transporter (VMAT2) has been shown to be critical for the proper handling of dopamine molecules, and knockdown of VMAT2 in mice results in a PD-like progressive loss of dopamine neurons from the *substantia nigra*.[91] There is also evidence that α-synuclein may play a role in forming these synaptic vesicles from early endosomes by interacting with phospholipase D2 (PLD2), and mutations in the α-synuclein gene would likely disrupt the formation of these vesicles.[92] Increases in free dopamine within the presynaptic cytoplasm is a plausible contributor to the amplified amount of oxidative damage observed in PD, as well as the observed reduction of functional dopamine neurotransmitter levels. In addition, it was shown that spontaneously forming dopamine-quinone molecules can modify α-synuclein, creating adducts that inhibit the transformation of synuclein protofibrils to fibrils,[43] which implies that dopamine oxidation may play a role regulating Lewy body formation and accumulation of soluble α-synuclein oligomers.[20] It is clear that the oxidative sequelae related to dopamine dyshomeostasis are critical in the pathogenesis of PD. This also presents a unique challenge in the treatment of the disorder, where on the one hand dopamine replacement is extremely valuable to remediate motor symptoms, but on the other hand may contribute to cellular stress.

1.3.5 Neuroinflammation

Inflammatory processes are associated with a broad spectrum of neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis, multiple system atrophy, multiple sclerosis, Huntington's disease, and PD. There is mounting evidence that inflammation-associated oxidative stress plays a central role in the pathogenesis of PD. An increase of pro-inflammatory cytokines in conjunction with decreased glutathione-related genes have been reported in a microarray study of the SN in PD brain tissue.[93] In addition, positron emission tomography (PET) scans assessing *in vivo* microglial activation in patients with PD revealed a significant elevation in inflammatory response within brain regions most affected by the disease.[94] Activated microglia were seen in the basal ganglia, striatum, and neocortical regions of the brain regardless of how advanced the disease, suggesting that microglial activation in PD is an early and continuous process.[94] It is postulated that activated microglia within the midbrain release pathological levels of pro-inflammatory cytokines, leading to an increase in oxidative stress in the already sensitive dopamine neuron population. Microglia contribute to oxidative damage through their antimicrobial defenses, including respiratory bursts, which release superoxide generated from NADPH oxidase enzymatic reactions.[31,50,95] Additionally, pro-inflammatory gene expression within the microglia, such as nuclear factor (NF)-κB, mitogen-activated protein kinases (MAPK), and activator protein (AP)-1, results in secondary production of ROS and nitric oxide (NO).[96]

Inflammatory gene expression within the microglia is dynamically regulated; pro-inflammatory gene transcription must be suppressed under basal conditions, but capable of rapid induction upon occupation of surface receptors for cell damage or pathogens.[97] Redox-sensitive transcription factors, such as NF-κB, are capable of upregulating hundreds of downstream pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), which converts L-arginine and NADPH to citrulline and NO.[98] In addition, NF-κB activation increases the expression of proteins that promote neurotoxicity including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ.[99] These proteins also activate surrounding astrocytes, which enhance the expression of many pro-inflammatory proteins, including iNOS, resulting in high levels of NO.[100] Several lines of evidence have substantiated that astrocyte-mediated iNOS production is involved in the loss of dopamine neurons from the SN, including *iNOS* gene deletion studies in mouse that result in protection from MPTP-induced toxicity.[101]

Impairment of negative regulation of inflammatory pathways within glia may also be important in PD. Normal inflammatory resolution in the brain involves the expression of anti-inflammatory molecules including TGFβ, IL-10, and glial-derived neurotrophic factors. Suppression of inflammatory transcription is also a target for negative feedback mechanisms involving nuclear corepressors such as histone deacetylaces (HDAC1-3) and nuclear corepressor proteins (NCoR1/2). Saijo *et al.* (2009) suggested a novel role

for the TH-regulating protein, Nurr1, in nuclear suppression of inflammatory gene transcription within astrocytes. Their findings indicated that the orphan nuclear receptor Nurr1 is recruited to the p65-NF-κB transcription factors bound to inflammatory gene promoters, where it recruits the CoREST corepressor complex, which removes chromatin bound-NF-κB transcription factors.[102] Conversely, the knockdown of astrocytic Nurr1 in adult mice exacerbated dopamine neuron loss following the peripheral injection of bacterial lipopolysaccharide (LPS).[102] This represents a novel role for Nurr1, which had previously only been characterized as a protein required for the development of tyrosine hydroxylase in dopamine neurons, the genetic mutation of which is also linked to a rare form of late-onset inherited PD.

1.4 Genetics of Parkinson's Disease

A detailed review of the genetics of PD is beyond the scope of this chapter, and readers are referred to recent reviews.[103,104] In brief, a refined knowledge of the genetic factors involved in familial PD has led to better understanding of pathological cellular processes that may be common to all forms of the disease, including mitochondrial dysfunction, oxidative stress, and abnormal protein processing.

The most commonly inherited form of PD is caused by an autosomal dominant mutation in the leucine-rich repeat kinase 2 (LRRK2) protein, a kinase whose substrates have been elusive.[105–107] Mutant LRRK2 has been associated with dysregulation of macroautophagy as well as mitochondrial abnormalities.[108,109] LRRK2 mutations display variable, age-dependent penetrance and have also been found in idiopathic PD, suggesting that other risk factors for the disease affect the risk associated with LRRK2 mutations.[110] The degree to which LRRK2 contributes to idiopathic disease is uncertain, but there is hope that LRRK2 kinase inhibitors may be beneficial for at least some forms of PD.

The first genetic association with PD was a point mutation in α-synuclein; several other point mutations have also been described, all of which are rare.[23] It was subsequently found that PD could be caused by gene duplications or triplications of the *wildtype* gene, indicating that simply producing too much *normal* α-synuclein protein causes neurodegeneration.[24] The presence of α-synuclein in the Lewy pathology of typical idiopathic PD cases further emphasizes its central importance in the disease.[111,112] How α-synuclein causes toxicity is uncertain, but there is evidence that it impairs autophagy[113] or specifically disrupts mitochondrial protein import.[114] Therapeutic strategies aimed at reducing levels of α-synuclein, preventing its aggregation, or inhibiting certain post-translational modifications are under investigation.

Parkin is an E3 ubiquitin ligase, mutations of which cause an autosomal recessive form of PD. The protein is important in proteasome-mediated proteostasis and for lysosomal degradation of proteins. Under certain conditions, Parkin is translocated to mitochondria where it helps to recruit

autophagy machinery for mitochondrial quality control.[115] In the same cellular pathway, mutations in PINK1, the protein responsible for recruiting Parkin to the mitochondrial membrane, also result in an early-onset phenotype of PD.[116,117] There is growing interest in Parkin as a therapeutic target, and gene therapy and small molecule Parkin 'activator' approaches are being studied.

Aside from Parkin, several genes associated with inherited PD are related to protein degradation pathways within the cell. As one example, mutations in the glucocerebrosidase (GBA) gene impair autophagic function and are a major risk factor for development of PD.[118,119] Interestingly, Rocha and colleagues have demonstrated that glucocerebrosidase activity is also reduced in idiopathic PD.[120] Small-molecule glucocerebrosidase chaperones to enhance activity are currently under development.

Given the role of oxidative stress in PD, it is not surprising that mutations that affect proteins involved in antioxidant defenses might be associated with PD.[121] 15 is an oncogene that acts as a redox-sensitive protein chaperone and regulates several antioxidant pathways within the cell.[122] Mutations in DJ-1 cause an autosomal recessive, early-onset form of PD.[123] Interestingly, mice with gene deletion of DJ-1 show neuronal impaired mitochondrial complex I function.[124,125] It is noteworthy that the expression of DJ-1 in human brain is higher in astrocytes than neurons, and is elevated in this cell type in postmortem tissue of PD patients.[72,126,127] This suggests that a primary defect in a glial protein – DJ-1 – may result in a neuronal phenotype. Efforts to selectively enhance DJ-1 expression in astrocytes are underway.[153]

In summary, a large number of genetic mutations have been associated with PD. In general, these genes fall into categories affecting mitochondria, proteostasis/autophagy, and oxidative stress. The role of these proteins in idiopathic PD is an area of active investigation and appears to be leading to new therapeutic strategies.

1.5 Environmental Exposures and the Risk of Parkinson's Disease

1.5.1 Pesticides

Until the discovery of α-synuclein mutations in 1998, PD was thought to result primarily from environmental exposures, and genetics was thought to play little, if any, role in pathogenesis. In contrast, it had been reported that early-age exposure to rural environment and well water were associated with enhanced risk of PD.[128] It was hypothesized that well water was a vehicle for a 'causal agent' that might be toxic. In this context, there has been intense interest in the potential role of pesticides in PD. Many correlative studies have been limited in their ability to control for outside variables, leading to only weak associations with pesticides and PD. Recent data from rigorously controlled studies, however, have linked paraquat and rotenone exposure to

a measured increase in PD risk.[129,130] Consistent with mechanisms believed to be important in PD, rotenone is a complex I inhibitor[131] and paraquat is a redox cycling pro-oxidant compound;[131] both compounds have been used in rodents to model PD.[132,133] Thus, experimental studies have provided 'biological plausibility' for epidemiological studies.

1.5.2 Metals

A strong correlation exists between occupational metal exposure and neurotoxicity, best exemplified by manganism, a parkinsonian disorder that results from inhalational overexposure to manganese.[134] It has traditionally been believed that manganism differs clinically from idiopathic PD; however, some recent studies suggest that the clinical phenotype of manganism overlaps substantially with that of PD.[135] Many studies investigating the association of transition-metal exposure and PD have suggested a role for mitochondrial dysfunction as a result of metal accumulation in the basal ganglia.[136] In a case-control study of occupational exposure to iron, copper, manganese, zinc, mercury, and lead, there were significant correlations to PD incidence in those exposed to manganese and copper for more than 20 years.[137] Additionally, correlational studies investigating lifelong exposure to airborne manganese in the Valcamonica region of Italy, where ferroalloy plants operated from 1902–2001, indicated that individuals in this population were more likely to develop motor, cognitive, and sensory dysfunction than in surrounding areas.[138] At this point, however, it remains controversial whether manganese exposure causes typical idiopathic PD.

Iron accumulates abnormally in the brains of individuals with PD, although there is little evidence that exposure to iron increases the risk of PD. On the other hand, there is evidence that genetically determined dysregulation of iron may contribute to PD risk.[139] There is also experimental evidence that altered iron homeostasis may contribute to nigrostriatal neurodegeneration.[67,140]

1.5.3 Pathogens

The historical significance of a pathogenic infection and neurodegeneration is most clearly defined by the 1918 Spanish influenza postencephalitic cases of parkinsonism. A large spike in parkinsonism prevalence occurred in the years following the widespread H1N1 influenza infection,[141] although the clinical symptoms were clearly distinct from typical PD. Interestingly, persons *in utero* during the 1918 influenza pandemic had an increased risk for developing PD (2–3 fold), suggesting a role for immune-based neuroinflammation, possibly without direct exposure to viral particles.[142–144]

Intriguingly, it has been reported recently that contemporary strains of influenza virus, such as H5N1, can travel from the peripheral nervous system to the central nervous system and cause neuroinflammation and degeneration of nigrostriatal dopamine neurons.[143,144] Moreover, there is evidence

that systemic inflammation may be important. For example, intraperitoneal injection of bacterial lipopolysaccharide (LPS) in pregnant mice can also lead to a decreased expression of dopamine neurons in offspring.[145] It has been suggested that the medical community should be prepared to monitor for the emergence of parkinsonism related to current and future influenza pandemics.[143]

1.6 Gene–Environment Interaction

With only ~10% of PD cases strongly linked to inherited mutations, a widely held hypothesis suggests that low-penetrance susceptibility genes interact with environmental exposures and contribute to the majority of idiopathic PD incidence. One such example may be in populations exposed to large amounts of pesticides, with relative risk determined in part by polymorphisms in enzymes required to detoxify these chemicals (SOD, NADPH, quinone reductase, NQO1, and MAO).[146] The decreased ability to eliminate pesticides linked to PD, such as rotenone or paraquat, could significantly impact dopamine neuron survival, given the selectivity for complex I inhibition that many pesticides exhibit. Indeed in a study examining the odds ratio of polymorphisms in detoxifying enzymes and pesticide exposure, individuals with anomalies in SOD or NQO1 were nearly 2.5 times as likely to develop PD.[146]

Genes involved in the metabolism of xenobiotics may also contribute to polymorphic expression of PD susceptibility. Numerous polymorphisms of the P450 heme-oxygenase enzyme system are well characterized, leading to the altered distribution of chemical compounds normally metabolized by these proteins. Among P450 proteins, certain CYP2D6 polymorphisms have been correlated with a 2–3 fold increased risk of PD.[147,148] As a primary xenobiotic metabolizing enzyme, CYP2D6 is involved in the elimination and detoxification of many chemicals. Therefore, compromised CYP2D6 function may be linked to poor metabolism of these chemicals, leading to increased neurotoxicity from compounds, such as pesticides, known to be toxic to the dopamine system.[149]

Another potential source for heightened susceptibility to neurodegeneration is the differential accumulation of toxic compounds in the brain due to functional polymorphisms in multi-drug resistance transporters (MDR1) in the blood–brain barrier. It has been reported that a polymorphism associated with decreased MDR1 protein expression and function is increased in relation to the severity of PD, with early-onset cases showing a higher frequency of the polymorphism than late onset cases, which in turn, had a higher frequency than controls.[150] Such inter-individual differences in transporter-mediated xenobiotic access to the brain might have significant impact on the long-term consequences of environmental exposures. In this context, polymorphisms affecting blood–brain barrier permeability may influence susceptibility to PD.[151]

Gene–environment interactions involving downstream mechanisms after xenobiotic exposures may also be important. For example, as noted

previously, selective mitochondrial DNA (mtDNA) damage in the form of apurinic/apyrimidinic (abasic) sites has been found in the vulnerable nigral neurons in Parkinson's disease (PD). The persistence of abasic sites suggests an ineffective base excision repair (BER) response in PD. In addition, Sanders *et al.* showed that pesticide exposure, which has been linked to PD risk, can cause mtDNA damage.[60] A recent study of 619 PD patients early in disease and 854 population controls found that polymorphisms in BER enzymes, APEX1 and OGG1, were not by themselves associated with PD; however, when combined with pesticide exposures, the polymorphisms markedly increased the risk of PD – and the highest risk was associated with polymorphisms in *both* genes together with pesticide exposure.[152]

In summary, it appears that gene–environment interactions influence both the extent of xenobiotic exposure, as well as the relative efficiency of potential compensatory mechanisms.

1.7 Conclusions

While it is still common practice to discuss 'Parkinson's disease', it is now apparent that PD is actually multiple diseases, with a common phenotype, which may be caused by a variety of distinct genetic mutations, or the cumulative effects of low-penetrance mutations or polymorphisms, or environmental exposures, or some combination of these. We are beginning to understand the key players and cellular pathways leading to degeneration and, as such, are beginning to devise therapeutic strategies to slow or halt the otherwise inevitable progression of the disease. The crucial task ahead is to translate these findings into clinically useful treatments that will impact the lives of those affected by PD.

Acknowledgements

Some of the work described here was supported by NIH grants NS095387, ES020327, ES020718 and training grant T32 NS086749 and the American Parkinson Disease Association, the Blechman Family Foundation, the DSF Charitable Foundation and the Consolidated Anti-Aging Foundation.

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CHAPTER 2

Oxidative Stress and Redox Signalling in the Parkinson's Disease Brain

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2.1 Introduction

Dopaminergic neuronal cell loss in the *substantia nigra* (SN) *pars compacta* (SNpc) is considered the pathological hallmark of Parkinson's disease (PD). Since the early 1990s, oxidative stress has been suggested to exert a causative role in the loss of dopaminergic cells.[1] Accordingly, the three major risk factors linked to PD (aging, environmental exposures and genetic alterations) have been linked to an increased generation of reactive oxygen species (ROS) and the accumulation of byproducts of oxidative damage. Indeed, postmortem brain-sample analyses have reported an increased accumulation of oxidized proteins, nucleic acids and lipids in PD brains. In this chapter, we will provide an introductory overview of the pathogenic mechanisms involved in the alteration of redox homeostasis that occurs in PD. We will also discuss the intrinsic properties of SNpc dopaminergic neurons that make them vulnerable to neurodegeneration. The mechanisms by which these alterations

Issues in Toxicology No. 34
Oxidative Stress and Redox Signalling in Parkinson's Disease
Edited by Rodrigo Franco, Jonathan A. Doorn and Jean-Christophe Rochet

Published by the Royal Society of Chemistry, www.rsc.org

in the cellular redox homeostasis contribute to dopaminergic neurodegeneration will be discussed in greater detail in the following chapters.

2.2 Oxidative Stress and Antioxidant Systems

Molecular oxygen (O_2) is vital for the survival of aerobic organisms. In normal cells, 30–32 molecules of ATP are generated per O_2 during aerobic metabolism. During cellular respiration O_2 is consumed by mitochondria, and at the terminal step of the oxidative phosphorylation (OXPHOS), it is reduced to H_2O. Electrons travel along the respiratory electron transport chain (ETC) composed of four complexes: complex I (nicotinamide adenine dinucleotide [NADH] ubiquinone oxidoreductase), complex II (succinate ubiquinone oxidoreductase), complex III (ubiquinone-cytochrome c oxidase), complex IV (cytochrome c oxidase), plus ubiquinone and cytochrome c. The ETC is leaky, and leaked electrons reduce O_2 resulting in the generation of superoxide anion radicals, $O_2^{\bullet-}$ (Figure 2.1).[2]

Oxidative stress is defined then as an increase in the steady-state levels of ROS and its reactive byproducts (including reactive nitrogen species [RNS]) that surpasses cellular antioxidant defenses.[3] Oxidative stress leads to the oxidation of biomolecules (proteins, nucleic acids and lipids). Cells have evolved not only antioxidant defenses, but also a wide range of mechanisms for the removal or repair of oxidative damage that involve reduction (when oxidation is reversible) and degradation of oxidized biomolecules or organelles. When oxidative damage persists it not only affects a single cell, but also the tissue in which it resides, thus having important implications for overall organism physiology.[3,4]

2.2.1 Reactive Oxygen and Nitrogen Species: Sources

ROS include radical (an atom or molecule with a single unpaired electron, •) and non-radical species such as $O_2^{\bullet-}$, singlet $^1O_2$, hydrogen peroxide H_2O_2 and the hydroxyl radical ($^{\bullet}OH$). Mitochondria metabolism is the primary source for ROS. In the mitochondria, $O_2^{\bullet-}$ is generally considered to be generated in the matrix by electron leakage from complex I,[5] and in both the matrix and the inner membrane space (IMS) by electron leakage from complex III.[6] $O_2^{\bullet-}$ generated in the matrix and the IMS has been proposed to be released to the cytosol *via* the mitochondrial permeability transition pore (mPTP)[7] or the voltage-dependent anion channels (VDAC),[8] respectively (Figure 2.1). However, at least 10 different sites for $O_2^{\bullet-}/H_2O_2$ production in the mitochondria have been described including 2-oxoglutarate dehydrogenase (OGDH), pyruvate dehydrogenase (PDH), complex II (flavin site), glycerol 3-phosphate dehydrogenase (mGPDH), as well as the less well-characterized electron transferring flavoprotein/ETF:Q oxidoreductase (ETF/ETF:QOR) system of fatty acid β-oxidation, proline dehydrogenase, aconitase, dihydroorotate dehydrogenase and monoamine oxidase (MAO). MAO is an enzyme located

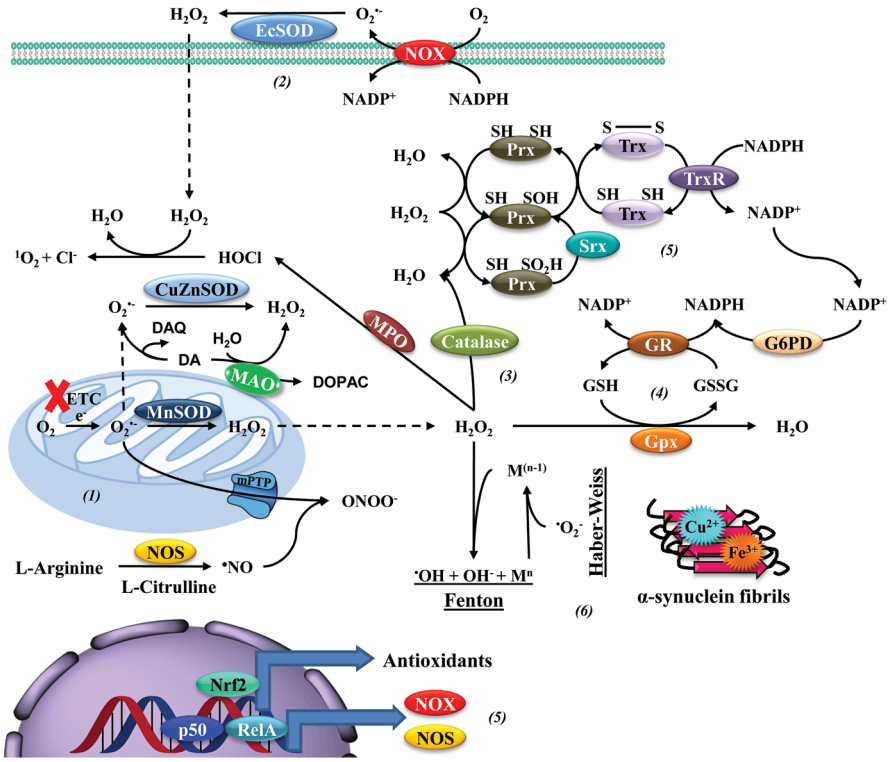

Figure 2.1 Oxidative stress and catalytic antioxidant systems. Cells have intrinsic antioxidant mechanisms to maintain a tight homeostatic control of ROS/RNS generated under physiological conditions, and to detoxify their excessive accumulation in pathological situations. (1) Mitochondria are the primary source of ROS. In PD, a dysfunction in mitochondrial ETC leads to increased formation of $O_2^{\bullet-}$. SODs dismutate $O_2^{\bullet-}$ to O_2 and H_2O_2. MnSOD is localized in the mitochondrial matrix, CuZnSOD in the IMS, peroxysomes, nucleous or cytosol, and EcSOD in the extracellular space (2). (3) Catalase catalyzes the decomposition of H_2O_2 to water and O_2 and is primarily localized in the peroxisomes. (4) Gpx reduce peroxides using GSH as substrate. Gpx isozymes encoded by different genes vary in their cellular location and substrate specificity. GR, which reduces GSSG back to GSH, requires NADPH as electron-donor reductant, and G6PD is indispensable for the regeneration of NADPH from $NADP^+$ in the cytosol. (5) Prxs are ubiquitous thiol peroxidases that scavenge peroxides. Catalysis of H_2O_2 is mediated by the reaction of their active Cys residue with H_2O_2. The catalytic activity of Prxs requires reducing equivalents provided by the Trx/TrxR system. (6) Misfolded aggregates of α-synuclein and metals also contribute to oxidative damage by Fenton reaction and $^{\bullet}OH$ formation. (7) In glial cells transcriptional regulation of NOX and NOS is mediated by inflammatory signalling *via* the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Both NF-κB (in neurons) and Nrf2 (glial cells primarily) activation regulates the expression of antioxidant defenses.