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Role of Reactive Oxygen Species in the Neurotoxicity of Environmental Agents Implicated in Parkinson's disease

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Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disorder with over one million cases in the United State alone [1]. The incidence of disease in the general population increases with age from 1–2% at 50 years to approximately 5% by the age of 85 [2,3]. The disease is clinically characterized by four major hallmarks which include rigidity, resting tremor, bradykinesia (slowness of movement), and postural instability. Non-motor symptoms such as cognitive impairment, sleep disturbances, olfactory dysfunction, and depression are also common and disabling manifestations of the disease resulting from damage to multiple areas in the brain [4]. Although PD is a multi-system disease, pathologically it is primarily characterized by a progressive degeneration of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) that project to the striatum and play an essential role in normal motor function. Clinical parkinsonian symptoms arise when a majority (60–70%) of SNpc DAergic neurons are lost, resulting in reduced DA levels in the nigrostriatal system [5]. Additionally, PD is distinguished by the presence of cytoplasmic inclusions called Lewy bodies in surviving DAergic neurons. Lewy bodies consist of numerous proteins including α -synuclein and ubiquitin [6]. Parkinsonian symptoms are not limited specifically to PD, as many other disorders affecting the nigrostriatal dopaminergic system have recently been characterized with similar clinical and pathological features [7]. These reports suggest that parkinsonism may arise from several different pathologies, although PD is the most common cause accounting for 80% of all cases [8].

Pesticide exposure as an environmental risk factor for PD

While the specific etiology of PD remains unknown, ageing is the strongest risk factor for developing the disease. Genetic susceptibility and environmental risk factors also have come under critical investigation. An estimated 5–10% of all parkinsonian cases are familial [9], with increased frequency of disease among relatives of patients. In recent years, several genes have been identified that contribute to both autosomal dominant and recessive inheritance in familial aggregates of PD including α -synuclein, parkin, DJ-1, LRRK2, and PINK1 [10]. However, studies revealed no difference in disease incidence in twins of patients with sporadic, late-onset PD [11,12], yet an increased concordance was observed in monozygotic twins with early-onset PD (<50 years) [11]. These data suggest that inherited factors are not likely to play a major role in sporadic PD, which represents a vast majority (~90%) of all cases. Therefore, extensive efforts have been made to identify other risk factors contributing to PD etiology.

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Epidemiological studies have regularly linked environmental factors with increased risk of PD incidence including rural living, farming, drinking well water, and exposure to agricultural chemicals [13,14]. Furthermore, a number of environmental toxicants have been associated with PD development including metals, solvents, and carbon monoxide [15–17]. However, pesticide exposure has received by far the most attention presumably due to the implications of widespread use of such agents on global public health. A comprehensive study of epidemiologic and toxicologic literature suggests a consistent correlation between pesticide exposure and PD with the strongest association resulting from long duration of exposure [18]. With respect to implications on human health risk, a number of pesticides have come under intense scrutiny regarding their potential neurotoxic actions resulting in the development of the “environmental hypothesis of PD”. This hypothesis speculates that chemical agents present in the environment are capable of selectively damaging DAergic neurons, thus contributing to the development in PD. Recently, the use of pesticides in toxin-based models of PD has become increasingly popular and provided valuable insight into the neurodegenerative process.

Oxidative stress and mitochondrial dysfunction in PD

The cause of nigral cell death in PD remains unclear, yet several hypotheses have emerged based on data from animal and cellular models and genetic studies. The “oxidative stress hypothesis” postulates that a disruption in the balance of reactive oxygen (ROS) and reactive nitrogen species (RNS) contributes to oxidative damage of cellular macromolecules ultimately leading to cell death. The premise of this hypothesis is based on landmark studies demonstrating the potential for the generation of hydrogen peroxide (H_2O_2) and other ROS during the oxidative metabolism of DA [19], which exposes DAergic neurons of the SNpc to chronic oxidative stress compared to other regions of the brain. Other factors contributing to the increased oxidative stress include the following: i) high basal levels of aerobic activity, ii) auto-oxidation of DA, its precursors and metabolites, to form quinones and semiquinones capable of adducting protein sulfhydryl groups, including glutathione (GSH), and iii) increased iron levels in the SNpc contributing to Fenton chemistry through the reduction of H_2O_2 to produce the highly reactive hydroxyl radical, $\cdot OH$ [20–22]. Analysis of postmortem brains from PD patients confirms the high level of oxidative stressing the SNpc marked by increased iron concentrations [23,24], decreased levels of GSH [25–27], increased lipid peroxidation [28–30], and DNA [31] and protein [32] oxidation.

Mitochondrial dysfunction is also an intrinsic aspect of this hypothesis due to their major role in the production of cellular ROS. Decreased complex I activity in the mitochondrial respiratory chain was observed not only in SNpc of PD patients [33], but also in platelets [34] and cybrid cell lines [35]. This inhibition can lead to the generation of ROS which when produced in the near vicinity, may target the respiratory chain leading to further inhibition with subsequent ROS production, and mitochondrial damage [36]. Mitochondrial related energy failure may also disrupt the vesicular storage of DA, leading to increased free cytosolic concentrations of the auto-oxidizable neurotransmitter [8]. Importantly, DAergic neurons of the SNpc have been shown to be uniquely sensitive to complex I inhibition [37].

Inflammation can also contribute to ROS production and has been implicated in the pathogenesis of PD. Microglial activation is associated with the degeneration of DAergic neurons in PD patients [38], although whether this protects or exacerbates neuronal loss is under debate. Activation of microglia in response to injury is associated with an upregulation of inducible nitric oxide synthase (iNOS) resulting in increased production of NO. Increased immunostaining for iNOS has been detected in the SNpc of PD brains [39], suggesting that RNS may also play a critical role in the disease. To support this notion, increased 3-nitrotyrosine staining, indicative of protein nitration, is also observed in Lewy bodies [40].

Therefore, nitrosative stress is a key component of the overall stress response associated with PD.

While these studies support a role for free radical damage in PD, intense debate exists regarding whether such processes are primary or secondary events in the pathogenic mechanism. Genetic- and toxin-based models have provided the strongest support towards a principle role for oxidative stress in PD. Popular toxin models of PD such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and paraquat (PQ) are based on their ability to generate ROS in neurons. Pesticide models such as rotenone, dieldrin, and maneb also support the role of environmental agents and oxidative stress in PD. Therefore, in order to gain insight on the pathogenesis of PD, understanding the mechanisms by which environmental agents and selective DAergic neurotoxins exert oxidative stress. Potential mechanisms leading to the production of free radicals, microglial activation and mitochondrial dysfunction are spotlighted and related to common themes in our understanding of the neurodegenerative process.

6-OHDA and MPTP: classic toxin-based models of PD

The first identification of specific DAergic neurotoxins over 30 years ago led to the development of toxin-based models to induce experimental PD. Since that time numerous models have been developed, although only a few have been well characterized in regard to their mode of inducing neurodegeneration. 6-OHDA and MPTP have been the most thoroughly studied models whereas agricultural chemicals such as paraquat, rotenone, dieldrin and maneb have received increased attention in recent years. While each of these toxins contribute to the death of SNpc DAergic neurons in part through the induction of oxidative stress their similarities and differences have provided intriguing details on possible mechanisms of neurodegeneration relating to PD.

6-OHDA: the first catecholaminergic neurotoxin

6-OHDA was the first chemical agent discovered with specific neurotoxic effects on catecholaminergic pathways, including the nigrostriatal DA system [41–43]. 6-OHDA is a hydroxylated analogue of DA which uses the same transport system as DA and norepinephrine to produce specific degeneration of catecholaminergic neurons. Following stereotactic injection, 6-OHDA causes degeneration of DAergic neurons with dramatic loss of DA in the striatum [1]. The toxic mechanism of this compound is dependent on its oxidation with concomitant production of ROS and para- and semiquinone products. This process appears to occur via a one-electron reduction of oxygen resulting in superoxide ($O_2^{\cdot-}$) and semiquinone radical intermediates, as superoxide dismutase (SOD), a potent scavenger of superoxide radicals, significantly inhibits the oxidation of 6-OHDA [44]. Moreover, 6-OHDA toxicity is significantly inhibited in mice overexpressing cytosolic and mitochondrial forms of superoxide dismutase (SOD1 and SOD2, respectively) [45,46]. Like DA and other related compounds, 6-OHDA can be metabolized by monoamine oxidase to generate ROS. However, pretreatment with MAO inhibitors enhances the toxicity of 6-OHDA, offering evidence against the contributions of MAO-dependent ROS sources in the neurotoxic process. While the para- and semiquinone radicals produced during oxidation of 6-OHDA are capable of inducing cellular damage through reaction with nucleophiles such as protein and DNA, they do not appear to be primarily responsible for the toxic effects of 6-OHDA. Addition of ascorbic acid to recycle paraquinone enhances the neurotoxicity of 6-OHDA, suggesting a critical role for ROS and not electrophilic quinone species [47]. The complete oxidative reaction of 6-OHDA has been described in great detail elsewhere [44]. Although 6-OHDA does not reproduce all the characteristics of sporadic PD, namely Lewy body formation, this acute model has been used in the efficacy testing of many pharmacological anti-parkinsonian compounds.

MPTP: paving the way for the environmental hypothesis

In the early 1980s, MPTP was identified as the chemical agent responsible for producing a severe parkinsonian syndrome in a cohort of young adult drug users characterized by many clinical features of PD [48]. An impurity in the illicit synthesis of a narcotic merperidine analogue, MPTP first demonstrated that exposure to an environmental agent could produce an irreversible form of PD. Although risk of human exposure to this synthetic compound is limited, MPTP has been instrumental in the understanding of pathways contributing to degeneration of DAergic neurons. To date, MPTP remains the best-characterized PD model and has provided the strongest support for the role of oxidative stress in disease pathogenesis.

MPTP is highly lipophilic and readily crosses the blood-brain barrier (BBB). In the brain, MPTP is metabolized by monoamine oxidase B in glial cells to an unstable intermediate, followed by spontaneous oxidation yielding its toxic metabolite, 1-methyl-4-phenylpyridinium (MPP⁺) [49,50]. MPP⁺ is released to the extracellular space and gains entry into DAergic neurons via the dopamine transporter [51], where complex I of the mitochondrial respiratory chain is thought to be its primary target of inhibition. By disrupting the natural flow of electrons through the system, MPP⁺ is believed to cause an acute ATP deficiency and increased ROS production, particularly superoxide [50,52,53]. The fate of mitochondrial superoxide is governed by its reactivity with several targets that include manganese SOD (MnSOD, SOD2), nitric oxide (NO), susceptible iron-sulfur (Fe-S)-containing proteins, and other cellular macromolecules in close proximity. Many aspects of MPTP toxicity are attenuated in mice overexpressing SOD2 and exacerbated in mice with a partial deficiency in SOD2, confirming the role of mitochondrial superoxide [54,55]. The potential deleterious effects of mitochondrial superoxide and consequences on DAergic cell death are discussed later in this review. In addition to mitochondrial-induced ROS generation, MPP⁺ produces ROS via extrinsic mechanisms. ROS from the auto-oxidation of dopamine may result from the MPP⁺-induced release of the neurotransmitter from storage vesicles into the cytosol [56]. Furthermore, cyclooxygenase-2 induction following MPTP injection generates ROS via its peroxidase activity to form dopamine-quinones [57]. Although debate exists regarding the implications of each ROS source in MPTP, the toxic effects of superoxide are well demonstrated. Protective effects against MPTP have been observed following overexpression of SOD1 in cytosol and injection into the extracellular space of the SNpc [58,59]. Additionally, catalytic antioxidant synthetic compounds have proven effective in attenuating the toxic actions of MPTP in a variety of *in vitro* and *in vivo* models [60–62].

Although MPTP and 6-OHDA are commonly utilized to model of PD toxin, criticisms arise regarding the acute exposure of these synthetic compounds and the relevance to a progressive age-related neurodegenerative disease. Nevertheless, the exciting discovery of the toxic mechanisms of these compounds ground the foundation for the environmental hypothesis of PD and the search for potential environmental agents that mimics the chronic and progressive pathology associated with PD. As discussed below, PQ and rotenone were identified as potential DAergic toxins based on structural and mechanistic similarities to MPTP, respectively. The following sections focus on examining the toxic mechanisms of pesticides, the logic behind their study, and how they further classify the role of oxidative stress in PD.

Rotenone: strengthening the case for complex I inhibition

Deficiencies in the mitochondrial respiratory chain lead to decreased ATP synthesis, generation of ROS giving rise to oxidative stress, mitochondrial depolarization, and initiation of cell death processes. The central nervous system is highly dependent on aerobic respiration and maintaining a sufficient rate of oxidative phosphorylation is particularly critical to the survival of neurons. It is believed that subtle changes in respiratory chain complex activity may contribute to mild, late-onset neurological disorders. Support for this notion comes from

observations of a 15–30% reduction in complex I activity in non-familial sporadic PD patients [33]. Like MPTP, the toxic properties of rotenone stem from its ability to bind and inhibit complex I of the mitochondrial respiratory chain. Rotenone is a potent member of the rotenoids, a family of natural cytotoxic compounds extracted from tropical plants (Figure 1). It is most commonly used as an insecticide and to kill fish perceived as pests in lakes and reservoirs [17]. This pesticide is highly lipophilic, easily crosses the BBB and accumulates in subcellular organelles including the mitochondria [63]. Here, rotenone binds specifically to complex I, inhibiting the flow of electrons through the respiratory chain. The specific interactions of rotenone with complex I were demonstrated by replacing the endogenous subunit in human neuroblastoma cells with the single-subunit NADH dehydrogenase from *Saccharomyces cerevisiae*. The substitution of this rotenone-insensitive complex I attenuated rotenone's toxic effects [64]. In a manner similar to MPP⁺, rotenone-induced complex I inhibition results in acute ATP deficiency and generation of ROS. Following rotenone-induced inhibition, electron flow through complex I is slowed at upstream sites that are prone to electron leakage. Therefore, with electrons remaining in the site for a longer period of time than normal, molecular oxygen can react via an one-electron reduction to produce superoxide which is released in the mitochondria. Because rotenone and MPTP share the same principle mechanism of action, their toxic effects are assumed to be quite similar. In addition to the consequences of mitochondrial superoxide generation, the toxicity of both compounds is linked with glial cell activation. Microglial activation is pronounced in the rotenone rat model of parkinsonism, occurring prior to evidence of DAergic cell loss [65]. Knockout studies in mice have further identified microglial NADPH-oxidase mediated ROS generation as a key contributor to rotenone neurotoxicity [66]. Rotenone also inhibits the formation of microtubules leading to the accumulation of toxic tubulin monomers [67,68]. Rotenone exposure *in vitro* is characterized by a progressive depletion of GSH, oxidative damage to protein and DNA, and induction of apoptosis [64,69], all of which are blocked by the antioxidant α -tocopherol [64]. An inhibitor of DA synthesis also attenuated these effects, although its efficacy was limited to DAergic cells, further suggesting a selective vulnerability of these neurons to rotenone-induced oxidative stress [70].

Consistent with its potential as a PD toxicant, rotenone is selectively toxic to dopaminergic neurons as compared to GABAergic neurons in primary mesencephalic cultures [70,71], an effect that is greatly exacerbated in the presence of microglia [66]. In contrast with MPP⁺, rotenone does not appear to possess a specific transport system. Therefore, it is hypothesized that the DAergic neurons in the nigrostriatal pathway are selectively vulnerable to complex I inhibition, an assertion that has been supported *in vivo*. Chronic exposure to low doses of rotenone in rats resulted in uniform inhibition of complex I throughout the brain, while DAergic neuron numbers in the SN were decreased by 30% [37]. This selective degeneration of nigrostriatal neurons was accompanied by α -synuclein-positive LB-like inclusions. This study also demonstrated selective oxidative stress in the striatum of treated rats as measured by increased oxidative protein damage. Animals models utilizing rotenone have been complicated by three factors: 1) significant reductions in non-DAergic striatal neuronal populations [72], 2) wide variations in nigrostriatal effects within the same treatment paradigm, and 3) technical difficulty of rotenone use in animals [37]. Nevertheless, the presence of LB-associated DAergic neurodegeneration in this model proved a useful model for exploring the role of protein aggregates in neuronal death.

While the rotenone model has provided intriguing evidence for a role of complex I inhibition and subsequent mitochondrial dysfunction and oxidative stress in the etiology of PD, the risk of human exposure to this compound is limited. Rotenone breaks down readily in the environment, with a half-life ranging between 1 and 3 days in water and soil [1]. Therefore, despite its extensive worldwide usage the likelihood of PD being caused by environmental exposure to rotenone is uncertain. However, critical mechanistic insights have been obtained

from the rotenone model. Many environmental toxicants have shown the propensity to inhibit complex I in a similar manner, many of which possess a far greater exposure risk to humans [73]. The toxicity of several commercially available pesticides has been shown to be dependent on complex I inhibition with similar effects on neuroblastoma cells as compared to rotenone. Therefore, investigations of the potential neurotoxic mechanisms of such compounds is warranted in cellular and animal models, as they may account for the epidemiological data linking increased PD risk with pesticide exposure.

Paraquat: the role of redox cycling agents

PQ is one of the most widely applied herbicides in the world. While most European countries and the US have banned or restricted its use, PQ is still extensively utilized in other less developed nations. PQ is a quick-acting, non-selective herbicide that destroys green plant tissue on contact. Its primary uses include weed control in orchards and plantations and as a defoliant and desiccant to facilitate harvesting. PQ belongs to a class of bipyridyl herbicides characterized by a structural backbone of two covalently linked pyridine rings (Figure 1). Acute PQ poisoning is typically associated with lung toxicity due to selective, active uptake of the herbicide by diamine transporters in type II epithelial cells [74]. However, significant damage to the brain has been observed in individuals exposed to lethal doses of PQ [75,76]. Furthermore, epidemiological studies suggest an increased risk for developing PD following chronic exposure [77,78]. Increased risk for PD is also associated with diquat, another member of the family of bipyridyl herbicides often applied as a mixed preparation with PQ [79]. Exposure to DQ produces extensive damage to the central nervous system and was reported to cause parkinsonism in a farmer acutely exposed to a concentrated solution of the herbicide [80]. PQ was first recognized as a potential DAergic toxicant based on its structural similarity with MPP⁺ (Figure 1). Interestingly, MPP⁺ is a bipyridyl compound and was tested as an herbicide in the 1960s under the name of cyperquat [81]. Despite this striking resemblance, these two compounds exert their deleterious cellular effects via different mechanisms, although induction of oxidative stress is a shared property [82].

PQ's toxicity is related to its ability to redox cycle, accepting an electron from an appropriate donor with subsequent reduction of oxygen to produce superoxide while also regenerating the parent compound [83]. The standard reduction potential (E^0) of a compound is a measure of its affinity for an electron relative to a standard hydrogen electrode. PQ possesses an E^0 of -0.450 V. The E^0 values for several biologically important half reactions including NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ are -0.320 V and 0.324 V, respectively. Therefore, under physiological conditions PQ may accept an electron from such a redox couple. Many diaphorases, enzymes that transfer an electron from NAD(P)H , have shown the capacity to reduce PQ to its cationic radical ($\text{PQ}^{\cdot+}$). MPP⁺, on the other hand, has an E^0 value of approximately -1.180 V and is far less capable of accepting an electron from cellular systems. Following the gain of electron, $\text{PQ}^{\cdot+}$ undergoes the process of redox cycling through reduction of molecular oxygen. This redox reaction produces superoxide in addition to regeneration of the parent compound, PQ (Figure 2). SOD overexpression and SOD mimetics are capable of inhibiting the actions of PQ, supporting the notion that superoxide is a key mediator in the herbicide's toxicity [84–86]. Moreover, the redox cycling process is thought to deplete intracellular stores of NAD(P)H due to its increased oxidation, thus contributing to the herbicide's toxicity. $\text{PQ}^{\cdot+}$ can also reduce iron(III) and iron(III)-chelates, in turn catalyzing the formation of hydroxyl radicals via the Fenton reaction [87]. However, the high oxygen content in the brain presumably favors the reaction of $\text{PQ}^{\cdot+}$ with molecular oxygen over iron(III). The rate-limiting step in the redox cycling process is the reduction of PQ by an appropriate electron donor. Enzymes capable of initiating this reaction have been identified in microsomal, plasma membrane, and cytosolic cellular components in various organ systems (Table 2). Of particular

interest, NOS isoforms have been identified as PQ diaphorases, which is associated with a loss of the enzyme's normal NO-producing function [88].

In recent years, the involvement for mitochondria in the mechanism of ROS production by PQ has emerged, although studies examining a direct role of mitochondria in redox-cycling with PQ are extremely limited. Fukushima et al first showed that PQ may generate ROS by accepting electrons from purified complex I of the respiratory chain [89]. Subsequent inhibition of complex I occurs at millimolar concentrations of PQ or as a deleterious consequence of ROS production. Our laboratory recently demonstrated that PQ can be taken up into intact, respiring brain mitochondria which represent a major subcellular source in PQ-induced ROS production [90]. Furthermore, ROS production by PQ in brain mitochondria at micromolar concentrations was dependent on mitochondrial membrane potential and significantly attenuated by antimycin A, an inhibitor of complex III. Rotenone showed only limited inhibition, suggesting a role for complex III in PQ-induced mitochondrial ROS production. These results were further confirmed in intact rat primary midbrain cultures. Therefore, mitochondria disproportionately contribute PQ-induced ROS production in the brain via one or more sites of the respiratory chain.

An indirect excitotoxic mechanism in response to PQ has also been proposed. This is predicated on data showing that PQ stimulates glutamate efflux from neurons resulting in calcium influx through non-NMDA receptor channels. This influx of calcium can activate neuronal NOS, which may contribute to the formation of peroxynitrite and also participate in the redox cycling process [91]. Activation of the NADPH oxidase of microglia cells has also been shown as a key mediator of PQ toxicity in DAergic cells. Mixed neuron-glia primary cultures exposed to PQ exhibited a decrease in DA uptake and loss of tyrosine hydroxylase (TH)-positive cells. However, microglia-depleted cultures failed to show this response [92]. Furthermore, NADPH oxidase-deficiencies fail to reproduce the neurotoxic actions of PQ in both *in vitro* and *in vivo* models [92,93].

The ability of PQ to cross the BBB is severely limited following systemic exposure. However, the process occurs via a carrier-mediated mechanism involving a neutral amino acid transporter as pretreatment with valine or phenylalanine prevents PQ-mediated neurodegeneration in mice [94,95]. The passage of the herbicide into striatal neurons is less defined. While the process has been described as being sodium-dependent, the involvement of the dopamine transporter in PQ uptake has been proposed but remains controversial [82,93,96]. Despite these discrepancies, PQ reduces the number of DAergic neurons in rat organotypic midbrain cultures in a concentration-dependent manner, which is prevented by inhibitors of NMDA, NOS, and caspases [91]. These data emphasize the concept that several converging mechanisms are likely responsible for linking PQ-induced oxidative stress with ultimate neuronal demise.

Early studies in animal models failed to observe any changes in the nigrostriatal DA pathway following systemic exposure to PQ [97]. However, with the advent of stereological counting, sublethal dosing of PQ over a three-week time course has produced a decrease in TH-positive stained cells in the SNpc. In addition, DAergic neurons in the SN and striatum appear particularly sensitive to PQ, as other subpopulations of neurons were unaffected [98]. Reduced motor activity and dose-dependent losses of striatal DAergic nerve fibers were also reported in mice receiving multiple PQ injections [99]. While no significant depletion of striatal DA has been observed following PQ *in vivo*, evidence for enhanced DA turnover is suggested by increases in TH activity [98] and altered DA metabolite levels [100]. The selective toxicity of PQ to DAergic neurons in the nigrostriatal system is intriguing since these neuronal subpopulations are not expressing high activities of diaphorase activity [101]. Therefore, mechanisms of selective uptake, mitochondrial-dependent effects, or increased vulnerability of SN neurons may play critical roles in PQ-induced neurodegeneration. Additional evidence

to support PQ's status as a parkinsonian toxin comes from data demonstrating upregulation and aggregation of α -synuclein within SNpc neurons in treated mice [102]. Significant increases in 4-hydroxynonenol-positive neurons and nitrotyrosine immunoreactivity in nigral cells of PQ-treated mice provides further evidence for oxidative injury in the SN [103]. A direct role for oxidative stress in PQ-mediated neurodegeneration was observed in SOD1 or glutathione peroxidase (GPx) transgenic mice. In comparison to non-transgenic mice, SOD1 or GPx overexpression prevented reductions in DA metabolite levels and DA neurons following PQ treatment [104]. Moreover, synthetic SOD/catalase mimetics are capable of decreasing PQ-mediated DAergic neuronal cell death *in vivo* [105,106]. These studies have provided vital evidence for ROS and oxidative stress as critical events in the PQ model of PD. Landmark studies regarding PQ's mechanism of toxicity has well established the role of ROS and oxidative stress in a variety of organ systems. However, with the recent advent of PQ as a potential neurotoxicant, unique cellular targets have been identified in the brain. Further studies are therefore necessary to determine the contributions of these targets to PD neurodegeneration.

The advantages of using PQ as a PD model stem from its widespread usage as a pesticide and presence in the environment. Additionally, the chronic treatment paradigm provides a better representation of pathology associated with the disease, namely presence of α -synuclein and LB-like inclusions. However, PQ's usefulness as a PD model is limited by its failure to deplete DA levels.

Dieldrin and Maneb: the potential role of complex III in neurodegeneration

In close association with the implications of complex I inhibition by the environmental agents rotenone and MPP⁺, similar effects may be observed in deficiencies of other respiratory chain complexes. Two pesticides, dieldrin and maneb, have been shown to selectively inhibit complex III of the respiratory chain [107,108]. Although less is known regarding their potential for inducing oxidative stress compared to PQ, rotenone or MPTP, both of these compounds have helped shape the understanding of neurodegenerative processes.

Dieldrin

Dieldrin belongs to a broad class of organochloride pesticides which also includes dichloro-diphenyl-trichloroethane (DDT). The compound is classified as a chlorinated cyclodiene containing an unusually stable epoxide ring (Figure 1). Following its initial distribution as an insecticide in 1950, dieldrin was widely used around the world through the mid-1970s for the control of soil pests, seed treatment, and controlling the spread of tropical disease [109]. Due to potential carcinogenic actions and bioaccumulation, the United States restricted dieldrin's use in 1974 with an almost complete ban taking effect in 1987. While most developed countries have posed similar restrictions, dieldrin is still used as an insecticide in many developing nations. Despite the ban on its usage, significant levels of the pesticide are still detectable in the environment due to its lipophilicity and high stability. With a half-life of up to 25 years in the environment, dieldrin is listed among the most hazardous pesticides based on potential for human exposure [110]. As a result, post mortem studies found that brains of PD patients were more likely to have detectable levels of dieldrin than those who died of other illnesses [111]. In addition, elevated levels of the pesticide have been found in the caudate nucleus of PD brains compared to controls [112].

Due to its lipophilicity, dieldrin is capable of crossing the BBB, but it is also stored in adipose tissue with half-life in humans of approximately 300 days [113]. In the brain, neuronal ion channels are potential targets of the pesticide through inhibition of the GABA(A) receptor [114,115]. This action causes hyperexcitation and a massive influx of calcium via glutamate receptor channels. This calcium influx can induce neuronal NOS, further exacerbating the

production of ROS/RNS in the brain. Dieldrin also appears to target mitochondria by preventing electron flow at or near cytochrome *b* of complex III in the respiratory chain [107], suggesting the potential for a direct mechanism of ROS production and oxidative stress. Together with mitochondrial energy failure, this inhibition causes a build-up of electrons prior to the blockage at sites within complexes I and III where electron leakage can contribute to the generation of superoxide radicals. While direct evidence for respiratory chain-dependent ROS production by dieldrin is lacking, this process is well characterized with specific inhibitors of complex III [116,117]. Intracellular ROS levels are increased in several DAergic cell lines in response to dieldrin [16,118–120], which can be attenuated by pretreatment with SOD or the catalytic antioxidant, MnTBAP [118]. Extensive studies in PC12 cell lines have provided indirect evidence for the role of oxidative stress and mitochondrial dysfunction in dieldrin-induced toxicity. For example, dieldrin-induced DNA damage in PC12 DAergic cells is regulated by oxyguanosine glycosylase 1, an initiating enzyme in the excision repair of oxidized lesions [121]. Kitazawa et al showed that dieldrin alters mitochondrial activities and membrane potential, induces cytochrome *c* release, and activates caspase-3 and -9 of the apoptotic pathway. Importantly, induction of caspase-8, mediated through a death receptor pathway, was not activated [122]. Although these studies do not provide direct support for the involvement of oxidative stress, they indicate that apoptotic events in response to dieldrin are mitochondrially-mediated. Dieldrin also stimulates microglia to produce ROS, an effect that is significantly reduced with inhibition of NADPH oxidase [123].

In vivo studies have also shown evidence for oxidative stress in response to dieldrin. Slow infusion exposure over a two-week period produced global oxidative stress in mice as evidenced by increased lipid peroxidation and strong antioxidant and DNA repair responses in all mouse brain regions [124]. Thirty days of low dose treatment with the pesticide in mice led to decreases in total GSH, more oxidized GSH redox potential and increased protein carbonyls in the striatum [125].

In accordance with its potential as a parkinsonian toxicant, dieldrin is selectively toxic to DAergic neurons compared to GABAergic neurons in mesencephalic cultures [126] and also can stimulate α -synuclein fibril formation and aggregation [127,128]. As a result, many studies have focused on changes in DA handling in response to dieldrin. Pretreatment with a monoamine oxidase inhibitor, deprenyl and inhibiting DA synthesis through inhibition of TH both attenuated dieldrin-induced ROS production in PC12 cells [118]. This indicates a critical role for DA and its metabolites in the generation of ROS with increased susceptibility of DAergic neurons to dieldrin. In addition, other organochloride pesticides related to dieldrin are capable of inhibiting vesicular monoamine transporter (VMAT2) function indicating that a failure to sequester DA may increase the risk of oxidative stress [129]. It remains to be determined if this potentially toxic event is a direct result of dieldrin interaction with the transporter or related to mitochondrial energy failure. While single high dose administration of dieldrin in mice fails to induce any significant changes in striatal DA and metabolite concentrations [130], chronic dosing paradigms show depletion of brain DA levels in a variety of animal models [125,131–133]. Furthermore, changes in dopamine transporter and VMAT2 levels [134,135], decreased DA metabolite levels, and increased cysteinyl-catechol adducts in the striatum [125] suggest alterations in the uptake, storage, and metabolism of DA in the nigrostriatal pathway. However, it is important to note that despite these changes, there appears to be no evidence of DA neuron loss. Interestingly, developmental (perinatal exposure during gestation and lactation) exposure to low dieldrin levels alters DAergic neurochemistry in offspring and exacerbates MPTP toxicity later in life [134].

Studies of dieldrin's associations with PD are less extensive as with other pesticides and environmental agents. While dieldrin shows many of the characteristics of a parkinsonian toxicant including ability to induce DA depletion, mitochondrial dysfunction, oxidative stress,

α -synuclein aggregation and apoptosis, its use as a research tool has remained limited. In order to adequately link dieldrin with increased risk of PD, detailed mechanistic studies to determine the manner in which dieldrin produces these effects are necessary. Two critical issues that remain to be resolved are: 1) the selective nature of dieldrin toxicity in DAergic neurons, and 2) whether induction of oxidative stress is a primary or secondary event in dieldrin-mediated neurotoxicity.

Maneb

Dithiocarbamates (DTC) have been in use for over fifty years with steadily increasing worldwide application on a broad range of crops. DTCs have been linked to a range of neurobehavioral abnormalities with adverse effects on both glutamate and DA systems [136]. Part of the toxic mechanism of these compounds has been associated with the catalyzation of DA oxidation and chelation of metals leading to alterations in cellular redox status, contributing to oxidative stress [137,138] Maneb is an organomanganese fungicide that belongs to the DTC family mainly used in the control of field crop pathologies [17] (Figure 1). To support maneb's potential role in the etiology of PD, permanent parkinsonism has been reported following chronic occupational exposure to the fungicide [139].

Maneb's ability to cross the BBB is assumed based on studies showing neurochemical and behavioral changes following exposure, although no specific transport mechanism has been identified [140]. The major active component of this fungicide is manganese ethylene-bis-dithiocarbamate (Mn-EBDC), while minor reagents are also present that are lacking clearly defined functions. It has been demonstrated that the parent compound maneb and not its major metabolites are responsible for neurotoxicity [141], but these other constituents may be contributing factors. While Mn-EBDC is relative stable, it may breakdown to manganese and EBDC, both of which are potentially neurotoxic. Manganese is known to cause brain damage, and chronic exposure has been linked to PD-like symptoms [142,143], although recent evidence suggests that the globus pallidus is affected rather than the SN [144]. Substituting zinc (Zn-EBDC) for manganese failed to reduce toxicity in primary neuronal cultures, suggesting that EBDC is a principle component contributing to toxicity [145]. EBDC also enhances the neurotoxicity of MPTP [146]. Direct injection of Mn-EBDC to lateral ventricles in the rat brain produces selective DAergic cell neurodegeneration and extensive striatal DA efflux, implicating a direct role for this compound. In isolated rat brain mitochondria, Mn-EBDC preferentially inhibits mitochondrial complex III, contributing to ROS production and mitochondrial dysfunction [108]. Furthermore, non-toxic doses of maneb in primary mesencephalic cultures decrease ATP levels, inhibit NADH-linked respiration, and cause mild to moderate uncoupling suggesting impairment of normal mitochondrial function [141]. Maneb produces a decrease in TH-positive neurons [136] along with the generation of ROS, increased levels of GSH and GSSG, and altered heme-oxygenase-1 protein levels, providing evidence of an oxidative stress response in DAergic cultures [136,147]. Additionally, pretreatment with buthionine sulfoximine to deplete GSH levels, exacerbates insult upon treatment with maneb.

Animal studies examining the role of maneb in PD have not produced significant proof for selective toxicity in the nigrostriatal system, although depressant effects on the central nervous system with partial involvement of DAergic systems has been observed [148]. However, striking evidence has come from animal models utilizing co-administration of DTCs such as maneb and other parkinsonian environmental toxicants.

Multi-hit hypothesis of environmental PD risk

The majority of work identifying potential DAergic toxicants associated with PD comes from studies examining mechanisms and risk arising from a single chemical. However, human environmental exposures are much more dynamic and likely to involve numerous risk

modifiers including multiple or mixtures of chemicals. The multi-hit hypothesis, as it relates to neurodegeneration and PD, suggests that the brain may be capable of withstanding the effects of an individual chemical that targets the DAergic system. However, when multiple chemicals target numerous sites within the DAergic system, defense mechanisms may be compromised resulting in cumulative damage and neuronal death [149]. Therefore, the toxic effects resulting from a mixture of environmental chemicals may vary in terms of potency, time of onset, and effects in comparison to single chemical exposures. Studies supporting this hypothesis have largely been focused on the ability of DTCs and other environmental toxicants, including those applied in geographically overlapping areas, to act in an additive or synergistic manner to produce a PD-like pathology.

The first model of synergistic toxicants interactions in regard to PD was demonstrated with co-treatment of MPTP and the pesticide, diethyldithiocarbamate (DDC) [150]. This study showed that MPTP neurotoxicity was enhanced in mice pretreated with DDC, as assessed by striatal dopamine levels. Further studies using co-treatment of DDC and MPTP showed the following: 1) concomitant behavioral changes in co-treated mice [151,152], 2) additive inhibition of mitochondrial respiration chain activity [153], and 3) nigral cell loss and dopamine depletion with DDC at non-toxic doses of MPTP [154]. The enhancing effects with DDC appear to result from increased brain concentrations MPP⁺ caused by changes in the biodisposition of MPTP [155]. Similar effects have also been observed with PQ in combination with DDC [96], suggesting that DTCs may enhance the neurotoxicity of compounds by altering their kinetics and bioavailability in the brain.

In recent years, the use of PQ and maneb has emerged as a multi-hit model in which the two pesticides act synergistically in producing a PD-like pathology. Animals treated with PQ + maneb show reduced motor activity and increased damage to both striatal nerve terminals and nigral cell bodies in comparison to those treated with either agent alone [156]. The use of this dual pesticide model is based on the marked geographic overlap in agricultural usage of PQ and maneb in areas throughout the US. This suggests a risk of simultaneous exposure to both compounds in human populations, and supports the environmental multi-hit hypothesis in the etiology of PD. Mice exposed to the two pesticides alone or in combination once per week for 4 weeks, showed increased levels of DA, metabolites, and turnover immediately following intraperitoneal injections and returning to control levels within 48 hours only with PQ + maneb. Reductions in TH immunoreactivity also were detected in the dorsal striatum but not the nucleus accumbens only with combined pesticide exposure following the last injection [156]. A similar treatment paradigm over 6 weeks demonstrated additional changes in the nigrostriatal system including decreased DAT immunoreactivity in the striatum and reduced cell counts and TH immunoreactivity in the SN [100]. A critical role for oxidative damage in the toxicity of PQ + maneb was confirmed using transgenic mice overexpressing SOD1 or GPx. In contrast with non-transgenic controls, these mice showed no significant changes in behavioral and pathological markers, including lipid peroxidation and dopaminergic cell number in the SNpc [104]. In accordance with the ability of both pesticides to independently generate ROS, these data support the notion that protective mechanisms against neurodegeneration induced by PQ + maneb exposure involves modulation of ROS levels and oxidative stress.

Common mechanisms contributing to pesticide-induced neurodegeneration

The chemicals discussed in this review all possess unique characteristics in terms of exposure risk, brain region localization, and molecular targets. However, all of these chemicals appear to possess similar intrinsic mechanisms of toxicity relating to the generation of oxidative stress. Among age-related neurodegenerative disorders, PD represents the best example of a disease for which mitochondrial oxidative stress has been strongly implicated [157]. Therefore, the realization that environmental agents linked to the development of PD target the mitochondria

is not surprising. All of the agents presented here possess the common ability to alter normal function of the respiratory chain. While the precise inhibitory mechanisms of maneb, dieldrin, and PQ are not fully elucidated, information learned from complex I inhibition by rotenone and MPP⁺ is useful towards understanding the potential deleterious consequences resulting from an initial mitochondrial insult.

Mitochondrial oxidative stress

Through either inhibition of the mitochondrial respiratory chain or in the case of PQ which may utilize these complexes to redox cycle, these agents are able to generate ROS, specifically superoxide, in the mitochondria. The critical involvement of mitochondrial superoxide in the neurodegenerative process is demonstrated in mutant mice overexpressing or deficient in SOD2, which show attenuation or increased susceptibility, respectively to PD toxicantss. The fate of mitochondrial superoxide is determined by its reactivity, proximity, and availability of biological targets including SOD2, NO, and susceptible Fe-S centers (Figure 3). SOD2, present in the mitochondrial matrix, acts as the first line of antioxidant defense against superoxide, converting the majority of these radicals to H₂O₂. Unlike superoxide, H₂O₂ may gain access to the cytosol and other cellular components by passing through biological membranes. Furthermore, both superoxide and H₂O₂ may participate in the production of stronger oxidants with deleterious cellular effects. Therefore, mitochondrial-generated superoxide resulting from respiratory chain inhibition is likely to contribute to oxidative damage within the mitochondria, while H₂O₂ may exert oxidative damage both inside and outside of mitochondria.

An important target of mitochondrial superoxide is NO. NO is a highly diffusible signaling molecule generated by the action of NOS, forms of which are found in neurons and glial cells. The spontaneous reaction of superoxide with NO occurs at a rate comparable to that with SOD2 and generates peroxynitrite, a powerful oxidizing species. Cellular damage induced by peroxynitrite includes depletion of thiol-dependent antioxidants, DNA strand breakage, lipid oxidation, and protein nitration [158]. The detection of 3-nitrotyrosine residues with many of these PD toxicantss provides evidence for the role of RNS. Interestingly, both SOD2 and the complexes of the respiratory chain have been identified as potential targets of nitration by peroxynitrite and RNS [159]. Subsequent inactivation of these enzymes sets up a vicious cycle of escalating ROS/RNS production as the build up of electrons in the inactivated respiratory chain leads to an increase in superoxide production in the mitochondria that cannot be efficiently detoxified. This deleterious sequence can also be initiated by superoxide in a similar manner involving oxidative damage to respiratory chain components [36].

Another major cellular target of mitochondrial superoxide is the direct oxidation and inactivation of susceptible Fe-S proteins. The unique and preferential sensitivity of these Fe-S proteins towards reaction with superoxide stems from the presence of an unligated, electrophilic iron atom in the active site of the enzyme. Following oxidation, this labile iron atom is lost from the cluster with concomitant inactivation of the enzyme. Labile Fe-S containing enzymes, such as aconitase are particularly abundant in the brain and act as a major target for superoxide [160]. The inactivation of mitochondrial aconitase may have two major consequences on cellular toxicity. First, Fe²⁺ and H₂O₂, simultaneously released during the enzyme's inactivation, may participate in the Fenton reaction resulting in hydroxyl radical ([•]OH) production [161,162]. This highly reactive radical is capable of oxidizing proteins, DNA, and lipids while contributing to mitochondrial dysfunction. Secondly, mitochondrial aconitase is an enzyme of the TCA cycle, catalyzing the dehydration of citrate to isocitrate. Based on the brain's dependence on oxidative energy metabolism, any interruption in TCA cycle activity resulting from even partial inhibition of mitochondrial aconitase may greatly affect neuronal viability. MPTP treatment inactivates mitochondrial aconitase in the SN prior to overt cell loss [163]. Furthermore, MPTP mobilizes of a novel pool of chelatable iron in the mitochondria

that parallels aconitase inactivation with MPTP. Aconitase inactivation and increased mitochondrial iron were attenuated in mice overexpressing SOD2 and exacerbated in mice with a partial deficiency of SOD2, thus suggesting the role of mitochondrial superoxide in this process [163].

The consequences of mitochondrial oxidative stress on overall cell viability are well documented in the MPTP mouse model. Resultant mitochondrial dysfunction can lead to a cellular energy deficiency and initiate the apoptotic process. In addition, the release of DA from intracellular storage vesicles is associated with mitochondrial energy failure and may further exacerbate the disruption in cellular redox status and ROS levels. Each of the environmental agents discussed in this review are capable of altering normal mitochondrial function in a manner that leaves this critical organelle susceptible to the generation of ROS. Therefore, it is believed that deleterious consequences of mitochondrial oxidative stress occur in a similar fashion as those induced by MPTP. However, other sources of superoxide and ROS including both cytosolic and extracellular, may be equally important in toxicant-induced neurodegeneration.

Genetic studies have additionally revealed that mitochondrial oxidative stress implicated in the toxicity of environmental agents discussed in this review are similarly involved in the deleterious effects caused by genetic abnormalities. Notably, mitochondrial dysfunction and oxidative stress have been linked with several genes associated with PD, including α -synuclein, parkin, DJ-1, PINK1, and LRRK2 [164]. While a detailed discussion is beyond the scope of this review, supporting evidence from DJ-1 and PINK1 is particularly intriguing. PINK1 is a mitochondrial localized kinase that may play a critical role in protecting mitochondria from oxidative stress and apoptosis. This protection is lost by specific mutations affecting kinase function [165,166] or suppression of PINK1 [167], while overexpression attenuated markers of apoptosis induction [168]. DJ-1 is a ubiquitously expressed protein and localizes to the mitochondrial matrix [169]. Interestingly, DJ-1 is a proposed antioxidant protein, capable of reducing ROS formation directly [170] and stabilizing the major antioxidant gene regulating transcription factor, Nrf2 [171]. Overexpression of DJ-1 protects against a wide variety of oxidative stress, including that of MPTP and 6-OHDA [172,173]. Moreover, nigrostriatal DAergic neurons in mice lacking DJ-1 expression show increased vulnerability to MPTP [174]. This critical new data arising from genetic studies provides further support for the role of mitochondrial dysfunction and oxidative stress in the development of PD.

Inflammation and microglial activation

The loss of DAergic neurons in PD is characterized by an increased number of activated microglia cells in the surrounding brain tissue [38], while nonsteroidal anti-inflammatory drugs have been associated with a reduced risk of developing PD [175]. In accordance, the neurotoxic actions of several environmental agents including MPTP, rotenone, and PQ are mediated by an inflammatory response, as evidenced by increased staining for activated microglia in the SNpc [65,93,176]. These studies also showed that gliosis occurs prior to the disappearance of DAergic cells from the SNpc, suggesting that inflammation is a significant contributor to the neurodegenerative process. Microglia activation is associated with increased expression of iNOS and NADPH oxidase, two enzymes capable of generating free radicals in the forms of NO and superoxide, respectively. Therefore, levels of NO and superoxide are increased both within cells and the extracellular space surrounding neurons upon toxicants exposure. The increased levels of these free radicals leads to their reaction generating peroxynitrite, whose detrimental cellular effects include lipid peroxidation, protein nitration, and DNA strand breaks. The involvement of intracellular and extracellular superoxide in MPTP toxicity has been demonstrated using SOD1 overexpression in mice. Transgenic mice expressing increased levels of the cytosolic enzyme showed minimal damage to DAergic neurons of the SNpc

following MPTP administration [59]. Furthermore, stereotaxic injection of SOD1 which remains in the extracellular space, showed protection of striatal TH-positive fibers confirming the role of extracellular superoxide in the toxicity of MPTP [58]. In accordance with these results, NADPH oxidase-deficient mice exhibited less DAergic neuronal loss from the SNpc than littermate controls after MPTP injections [58]. Similar results supporting a critical role for NADPH oxidase have also been observed with rotenone and PQ models [65,93]. In addition, the usage of iNOS-knockout mice and NOS antagonists to diminish NOS activity, thus reducing the production of NO, have also proven effective in significantly attenuating MPTP-induced neurotoxicity [177–179]. Collectively, these data provide strong evidence that both microglial-mediated production of both NO and superoxide are key components in the degeneration of the nigrostriatal pathway brought about by environmental toxicants.

Studies utilizing MPTP both *in vitro* and in animal models have provided strong support for the role of oxidative stress in PD. While MPTP studies have been the most comprehensive, other environmental agents have confirmed the notion that free radical production from a variety of cellular sources are critical components in the disease pathogenesis. While similarities exist between MPTP and the environmental agents discussed in this review relating to toxic mechanisms and downstream consequential effects, it is equally important to recognize those differences that are present. Most importantly, primary cellular targets must be verified to determine if oxidative stress is a direct consequence of a compound's toxic action, especially in the case of those environmental agents where debate remains or little is known regarding their foremost toxic mechanism.

Concluding remarks

The environmental hypothesis of PD began with the discovery of MPTP in the early 1980s and has since developed to include a variety of environmental agents. Pesticides have received the most attention due their global application and widespread implications on human health. In recent years, both epidemiological and toxicological studies have provided evidence that pesticides have the potential to act as DAergic toxicants contributing to the development of PD. While these studies have been useful in promoting our understanding of both the etiology and pathogenesis of this PD, one must be cautious when interpreting these two data sets together.

This review examined molecular mechanisms of neurodegeneration underlying environmental agents used to model PD *in vitro* and *in vivo*. Each of the toxicants examined in this review presents critical information regarding shared and unique mechanisms of neurodegeneration, although the limitations of studying these environmental agents must also be recognized. For example, no single chemical reproduces all the characteristics of idiopathic PD. Each model possesses its own strengths and weaknesses and information obtained from studies of these chemicals, along with genetics and epidemiology must be assessed to achieve a comprehensive view of the neurodegenerative process. Furthermore, models of PD have prominently focused on aspects of motor system effects resulting from nigrostriatal damage. A non-motor symptoms resulting from damage to olfactory bulb, cortex, and brain stem among other regions are also quite disabling to the patient. A drawback of current models of PD is that the non-motor symptoms are overlooked. Therefore, future studies warrant examination of potential damage in brain regions other than the nigrostriatal DA system induced by environmental agents. Lastly, caution must be exercised when extrapolating data from these *in vitro* and *in vivo* studies. These models are based on relatively high dose and acute exposure to a single toxicants, in contrast to the majority of human cases where disease risk is associated with low level, chronic exposure over many years to decades to a host of environmental agents. Therefore, correlations between toxicant concentrations in these cases must be scrutinized in terms of contributions to the death of DAergic neurons. Another major question that needs to be

addressed is if non-voluntary exposure to environmental agents can lead to significant concentrations of these compounds in the brain necessary to produce an oxidative insult similar to that seen in experimental models?

Despite some drawbacks and limitations, PD models utilizing chronic administration of environmental chemicals may better reproduce the pathogenesis of a neurodegenerative disease than acute treatment with MPTP or 6-OHDA. As a result, studies with PQ, rotenone, maneb, and dieldrin have been critical in identifying target systems and mechanisms involved in the loss of DAergic neurons in PD. Regardless of their direct role in PD, the use of these agents as experimental models will likely further our understanding of disease pathogenic mechanisms. Consequently, a more complete understanding of which specific environmental agents pose the greatest threat to human health and contributions to PD pathogenesis is vital for establishing improved usage regulations and reduction of exposure risks.

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List of Abbreviations

6-OHDA	6-hydroxydopamine
BBB	blood-brain barrier
DA	dopamine
DAergic	dopaminergic
DDC	diethyldithiocarbamate
Fe-S	iron-sulfur
GSH	glutathione

MPP⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NOS	nitric oxide synthase
PD	Parkinson's disease
PQ	paraquat
RNS	reactive nitrogen species
ROS	reactive oxygen species
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
TH	tyrosine hydroxylase
VMAT	vesicular monoamine transporter

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Figure 1. Chemical structures of rotenone, MPP⁺, PQ, dieldrin [16], and maneb. Note the structural similarity between MPP⁺ and PQ.

Figure 2.

Redox cycling mechanism of PQ. *E* represents enzymes capable of donating electrons to PQ. A list of identified enzymes is presented in table 1.

Figure 3.

Proposed mechanisms of mitochondrial superoxide production by environmental agents. Rotenone and MPP $^+$ inhibit electron flow through complex I, whereas dieldrin and mane b target complex III to produce mitochondrial superoxide. PQ can utilize both complexes I and III in the redox cycling process to generate superoxide. The fate of mitochondrial superoxide is controlled by its reactivity with several targets that include SOD2, NO, and Fe-S centers of proteins such as aconitase. The consequences of reaction with each target is discussed in the text.

Table 1
Referenced studies in relation to rotenone toxicity.

Model	Concentration/Dose	Major Findings	References
<i>In vitro</i>			
Chinese hamster ovary cells	12 μ M	Inhibition of microtubule assembly	Brinkley et al. 1974 [68]
Mouse primary mesencephalic cultures	5 nM	Selective DAergic neuron death	Marey-Semper et al. 1995 [71]
Rat primary mesencephalic cultures	1–30 nM	NADPH oxidase dependent microglial ROS production	Gao et al. 2002 [66]
	100 – 100 nM	Role of endogenous DA and superoxide in selective DAergic neuron death	Sakka et al. 2003 [70]
SK-N-MC human neuroblastoma cells	10 nM – 1 μ M	ATP depletion, GSH depletion, and cell death dependent on complex I inhibition	Sherer et al. 2003 [64]
Rat midbrain slice culture	50 nM	DAergic neuronal loss, increased protein carbonyls	Sherer et al. 2003 [64]
<i>In vivo</i>			
Rat	3 mg/kg per day, 5 weeks, s.c.	Decreased TH immunoreactivity in nigrostriatal system, increased protein carbonyls in midbrain and olfactory bulb	Betarbet et al. 2000 [37], Sherer et al. 2003 [64]
	2–3 mg/kg per day, 4–5 weeks, s.c.	Uniform complex I inhibition, DAergic neuron degeneration in SN, α -synuclein-positive cytoplasmic inclusionsmicroglial activation in SNpc and striatum	Betarbet et al. 2000 [37], Sherer et al. 2003 [65]
	2.5 g/kg per day, 28 days, i.v.	Reductions in non-DAergic striatal neurons	Hoglinger et al. 2003 [72]

Table 2

Enzymes proposed to participate in PQ redox cycling with production of superoxide.

Enzyme	Reference
NADPH cytochrome c reductase	Clejan et al 1989 [87]
Microsomal NAD(P)H-dependent reductase	Dicker and Cederbaum 1991 [180]
NADH:ubiquitin oxidoreductase (Complex I)	Fukushima et al 1993 [89]
NADPH:ferredoxin oxidoreductase	Liochev et al 1994 [181]
Nitric oxide synthase	Day et al 1999 [85]
NADPH oxidase	Bonneh-Barkey et al 2005 [182]
Ubiquitin:cytochrome c oxidoreductase (Complex III)	Castello et al 2007 [90]
Thioredoxin reductase	Gray et al 2007 [183]

Table 3
Referenced studies in relation to PQ toxicity

Model	Concentration/Dose	Major Findings	References
<i>In vitro</i>			
Rat lung slices	~200 μ M	Active uptake by diamine transporters	Smith. 1981 [74]
Complex I preparations	100 μ M – 10 mM	Complex I-dependent ROS production	Fukushima et al. 1993 [89], Cocheme et al. 2008 [184]
Rat organotypic midbrain slice cultures	10 – 50 μ M	Selective DAergic neuron death, excitotoxic mechanism of ROS production	Shimizu et al. 2003 [91]
Rat primary mesencephalic cultures	0.5 – 1 μ M	Selective reduction in TH- positive cells, decreased DA uptake, NADPH oxidase- dependent microglial ROS production	Wu et al. 2003 [92]
	250 μ M	Role of complex III and mitochondrial membrane potential in ROS production	Castello et al. 2007 [90]
Rat brain mitochondria	250 μ M	Mitochondrial uptake of PQ, role of complex III and mitochondrial membrane potential in ROS production	Castello et al. 2007 [90]
<i>In vivo</i>			
Rat	5 – 20 mg/kg, s.c.	Role of neutral amino acid transporter in PQ brain uptake	Shimizu et al. 2001 [94]
Mice	10 mg/kg, once per week, 1–3 weeks, i.p.	Role of neutral amino acid transporter in PQ brain uptake, selective decrease of TH-positive neurons in SNpc, striatal DAergic nerve fiber loss, enhanced DA turnover, α -synuclein upregulation and aggregation, increased 4-HNE and nitrotyrosine immunostaining	McCormack et al. 2003 [95], McCormack et al. 2002 [98], Brooks et al. 1999 [99], Thiruchelvam et al. 2000 [100], Manning-Bog et al. 2002 [102], McCormack et al. 2005 [103]
SOD1- and GPX-transgenic mice	5 mg/kg, twice per week, 9 weeks, i.p.	Protection against striatal DA depletion, DAergic neuron loss, and lipid peroxidation	Thiruchelvam et al. 2005 [104]
gp91 ^{phox} -knockout mice	10 mg/kg, once per week, 1–2 weeks, i.p.	Microglial activation and NADPH oxidase-dependent DAergic neuron loss	Purisai et al. 2007 [93]

Table 4
Referenced studies in relation to dieldrin toxicity.

Model	Concentration/Dose	Major Findings	References
<i>In vitro</i>			
Rat liver mitochondria	~500 nM	Inhibition of complex III	Bergen. 1971 [107]
Rat primary cortical neurons	10 μ M – 10 mM	Inhibition of GABA _A -receptor channels	Pomes et al. 1994 [114]
Rat primary dorsal root ganglion neurons		Inhibition of GABA _A -receptor channels	Ikeda et al. 1998 [115]
Rat and mouse primary mesencephalic cultures	10 nM – 100 μ M	Selective DAergic cell death, decreased DA uptake	Sanchez-Ramos et al. 1998 [126]
PC12, rat pheochromocytoma cells	30 – 500 μ M	DA depletion, decreased mitochondrial membrane potential, increased ROS, DNA fragmentation	Kitazawa et al. 2001 [118], Stedeford et al. 2001 [121], Kitazawa et al. 2003 [122]
SN4741 immortalized mouse nigral clonal cells	40 μ M	Increased ROS, apoptotic cell death	Chun et al. 2001 [119]
N27 immortalized rat mesencephalic clonal cells	~300 μ M	Increased ROS, apoptotic cell death, α -synuclein aggregation	Kanthasamy et al. 2003 [120], Sun et al. 2004 [128]
Primary mouse microglial cultures	0.1 nM – 1 μ M	Microglial NADPH-oxidase dependent ROS production	Mao et al. 2007 [123]
<i>In vivo</i>			
Mallard ducks	Unknown	DA depletion	Sharma et al. 1976 [132]
Rats	Unknown	DA depletion	Wagner et al. 1978 [133]
	3 mg/kg per day,	Increased DAT binding	Purkerson-Parker et al. 2001 [135]
Ring doves	1–16 ppm, 8 week diet	DA depletion	Heinz et al. 1980 [131]
Mice	3 – 48 mg/kg over two weeks, s.c.	Increased lipid peroxidation and DNA repair response	Sava et al. 2007 [124]
	0.3 – 3 mg/kg, every 3 days for 30 days, i.p.	DA depletion, decreased GSH, decreased DAT expression, increased α -synuclein, increased cysteinyl-catechol adducts	Hatcher et al. 2006 [125]

Table 5
Referenced studies in relation to maneb toxicity.

Model	Concentration/Dose	Major Findings	References
<i>In vitro</i>			
PC12, rat pheochromocytoma cells	50 – 1000 ng/ml	Increased GSH and GSSG, altered HO-1 levels	Barlow et al. 2005 [136],
	30 μ M	ROS generation, mitochondrial uncoupling,	Domico et al. 2007 [141]
Rat primary mesencephalic cultures	10 –120 μ M	Decreased ATP levels, mitochondrial dysfunction	Domico et al. 2006 [141]
Isolated rat brain mitochondria	~4 μ M	Inhibition of complex III	Zhang et al. 2003 [108]
<i>In vivo</i>			
Rats	20 nM, 2 weeks, intraventricular	Striatal DA efflux	Zhang et al. 2003 [120]
Mice	200 – 1000 mg/kg	Depressed locomotor activity	Morato et al. 1989 [148]