Mitochondrial dysfunction in Parkinson’s disease

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Abstract

The cause of Parkinson’s disease (PD) is unknown, but reduced activity of complex I of the electron-transport chain has been implicated in the pathogenesis of both mitochondrial permeability transition pore-induced Parkinsonism and idiopathic PD. We developed a novel model of PD in which chronic, systemic infusion of rotenone, a complex-I inhibitor, selectively kills dopaminergic nerve terminals and causes retrograde degeneration of substantia nigra neurons over a period of months. The distribution of dopaminergic pathology replicates that seen in PD, and the slow time course of neurodegeneration mimics PD more accurately than current models. Our model should enhance our understanding of neurodegeneration in PD. Metabolic impairment depletes ATP, depresses Na⁺/K⁺-ATPase activity, and causes graded neuronal depolarization. This relieves the voltage-dependent Mg²⁺ block of the N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor, which is highly permeable to Ca²⁺. Consequently, innocuous levels of glutamate become lethal via secondary excitotoxicity. Mitochondrial impairment also disrupts cellular Ca²⁺ homeostasis. Moreover, the facilitation of NMDA-receptor function leads to further mitochondrial dysfunction. To a large part, this occurs because Ca²⁺ entering neurons through NMDA receptors has ‘privileged’ access to mitochondria, where it causes free-radical production and mitochondrial depolarization. Thus there may be a feed-forward cycle wherein mitochondrial dysfunction causes NMDA-receptor activation, which leads to further mitochondrial impairment. In this scenario, NMDA-receptor antagonists may be neuroprotective.

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Introduction

Parkinson’s disease (PD) was first described almost 200 years ago by the British physician, James Parkinson, who called the disease “paralysis agitans” (shaking palsy). The clinical entity he defined includes muscular rigidity, slowness of movement (bradykinesia), tremor at rest and postural instability (balance problems). Because PD is associated with a general paucity and slowness of movement, it is known as a hypokinetic movement disorder. The motor symptoms are the most prominent features of PD, but cognitive impairment and depression are common. PD typically begins insidiously in middle or old age and is slowly progressive over a period of 10–20 years. Juvenile onset, under age 30, is possible, but not common. Although it is commonly stated that PD affects about 1% of the population over age 50 and 2% of the population over age 70, it may be under-diagnosed. A recent community population study found that the prevalence of Parkinsonism was 14.9% for people 65–74 years of age, 29.5% for those aged 75–84 and 52.4% for those aged 85 or older [1].

The pathological hallmark of PD is the degeneration of pigmented, melanin-containing neurons in the substantia nigra pars compacta. The degenerative process is characterized by cytoskeletal changes that include the formation of Lewy bodies, which are ubiquitinated intraneuronal inclusions. The neurons of the substantia nigra pars compacta send their projections from the midbrain to two forebrain nuclei, the caudate and putamen, which together are called the striatum. Because these neurons use dopamine as a neurotransmitter, nigral degeneration leads to profound dopamine depletion in the striatum [2]. The degenerative process is not restricted to the substantia nigra, but can also involve other brain areas, such as the locus coeruleus, the ventral-tegmental area and the pedunculopontine tegmental nucleus [3].

Whether dopaminergic degeneration involves retrograde ‘dying back’ of nigrostriatal dopamine nerve terminals, or is primarily a disorder of the cell body, is uncertain. It is generally believed that Parkinsonian symptoms begin after about 80% of the substantia nigra dopaminergic neurons have degenerated; however, the degree of dopamine depletion in the putamen is much more severe than in the substantia nigra [4]. At a stage where 15% of control dopamine remains in the substantia nigra, the putamen has only about 2% of control values. This suggests that the pathogenesis of PD may involve dying back of dopaminergic nigrostriatal terminals followed by an eventual loss of the nigral neurons.

The cause of typical or ‘idiopathic’ PD is unknown, but a great deal of interest centres around the idea that mitochondrial impairment is responsible, either directly or indirectly, for the death of dopaminergic neurons. The rationale and evidence for this hypothesis are presented in this chapter.

Mitochondrial impairment as a cause of PD: the case of MPTP

In the late 1970s, several users of illicit intravenous drugs were found to have developed acutely a severe Parkinsonian syndrome. Astute medical detec-
tive work by Langston and his colleagues [5] found the cause of the Parkinsonism. Apparently, sloppy synthesis of a new designer drug that was meant to be a meperidine analogue instead yielded a new neurotoxin. Analysis of the substance injected by these patients revealed 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) [5]. This was immediately believed to be the responsible toxin; however, it was subsequently demonstrated that MPTP is metabolized by monoamine oxidase type A to 1-methyl-4-phenylpyridinium ion (MPP\(^+\)). Blockade of this metabolic step prevented the toxicity of MPTP [6]. Thus it became clear that MPTP is a ‘pro-toxin’ and its metabolite, MPP\(^+\), is the active toxin. Conversion of MPTP to MPP\(^+\) is believed to occur in glial cells [7]. In experimental animals, mostly mice and monkeys, it was shown that systemic MPTP administration could reproduce accurately the clinical, neurochemical and neuropathological features of PD [8]. Nevertheless, the mechanism by which MPTP killed cells, and the reasons for selective vulnerability of dopaminergic neurons, remained to be explained.

In 1985, Nicklas and colleagues [8a] showed that MPP\(^+\) exerted its toxicity by inhibiting NADH-linked metabolism of mitochondria. That MPP\(^+\) was acting by selectively inhibiting complex I of the electron-transport chain was confirmed by Singer and colleagues [9]. Moreover, although MPP\(^+\) has a very low potency in complex-I preparations or in inverted mitochondrial particles (1–10 mM), it is concentrated many-fold in mitochondria [10], although the precise mechanism by which this occurs is uncertain [11]. Subsequent studies have demonstrated that MPP\(^+\) acts at the same site as the classical complex-I inhibitor, rotenone [12–14]. Toxicity of this compound is associated with ATP depletion [15], generation of oxygen free radicals [16,17], depletion of mtDNA [18] and, ultimately, cell death, which may be apoptotic or necrotic in nature [19].

It is obvious that mitochondrial respiration is essential for the health of all neurons in the brain. So, how does MPTP target with remarkable selectivity the dopaminergic neurons? In 1985, it was shown that MPP\(^+\), but not MPTP, was a substrate for the dopamine-uptake carrier, which is localized on dopaminergic neurons [20,21]. In addition, pharmacological blockade of the dopamine-uptake carrier in vivo prevented MPTP toxicity.

Thus, MPTP toxicity results from a defined sequence of events. Parenterally administered MPTP gains access to the brain, where it is converted in glial cells, by monoamine oxidase type A, to MPP\(^+\). Apparently, MPP\(^+\) is released by glia, whereupon it is selectively accumulated by dopaminergic neurons via the dopamine-uptake carrier. Once inside the dopaminergic neuron, MPP\(^+\) is concentrated within mitochondria where it acts to inhibit complex I. This results in ATP depletion, free radical generation and, finally, cell death. In one sense, the selective dopaminergic toxicity of MPTP seems quite contrived inasmuch as the toxin is a substrate for a carrier found exclusively in dopaminergic cells. However, the significance of the MPTP story rests not so much in the importance of MPTP as a cause of PD, but rather in the concept that mitochondrial impairment can produce a specific neurological disorder. In addition, the finding that a complex-I defect could cause experimental Parkinsonism led several laboratories to examine this enzyme complex in idiopathic PD.
In 1989, two independent groups reported that brain, muscle and platelets from patients with PD had depressed complex-I activity [22–24]. The finding of reduced complex-I activity in platelets has been replicated by several groups [25–28], but some laboratories have not found complex-I defects in PD platelets [29,30]. Results from muscle have been contradictory and controversial. The platelet abnormality of complex I does not appear to be related to anti-Parkinsonian medications [27,31], and does not result from loss of the catalytic ND1 subunit [32]. Variable defects in other electron-transport complexes are also reported [23,26,27]. Overall, there appears to be a consensus, but not unanimity, that there is a selective mild-to-moderate depression of complex-I activity in the platelets of PD patients. If so, this suggests that a systemic defect in this enzyme complex is somehow causing selective neurodegeneration in the substantia nigra.

As noted, there is also a selective complex-I defect in the brain. Schapira and colleagues reported a specific loss of complex I in the brains of patients dying with PD [22,33]. Janetzky and colleagues reported similar findings [34]. Paradoxically, whereas the platelet data would suggest a generalized defect, the brain complex-I reduction is restricted to the substantia nigra [35]. As in platelets, the reduced complex-I activity did not appear to result from medication use [35]. Moreover, in multiple system atrophy, another disorder that affects the substantia nigra, there was normal complex-I activity [35,36].

In summary, PD is associated with an apparent defect in complex-I activity of platelets and the substantia nigra pars compacta. It is not clear, however, whether this mitochondrial abnormality is important in PD pathogenesis. If it does play a pathogenic role, then the explanation of how an apparent systemic defect in mitochondrial function can produce selective degeneration of dopaminergic neurons will be of critical importance. One possibility is that...
nigrostriatal neurons have an inherent sensitivity to complex-I dysfunction. This is a testable hypothesis.

A novel model of PD: systemic inhibition of complex I

If dopaminergic neurons of the substantia nigra are selectively sensitive to defects in complex I in vivo, then with titration of systemic complex-I inhibition, it should be possible to demonstrate selective vulnerability of these cells. We have recently undertaken such a study. For this purpose, we have used rotenone, a classical, high-affinity inhibitor of complex I. Unlike MPP⁺, rotenone is highly lipid-soluble and easily crosses biological membranes such as the blood–brain barrier. It is not a substrate of the dopamine-uptake carrier and is not selectively accumulated by cells expressing the dopamine transporter (S.E. Stephans, A.I. Levey and J.T. Greenamyre, unpublished work).

Alzet™ osmotic minipumps were filled with a rotenone solution and were attached to Tygon microbore tubing. They were implanted under the skin of male Sprague–Dawley rats, and the right jugular vein was cannulated. Rotenone was delivered into the jugular vein in doses ranging from 1 to 12 mg/kg per day at rates of 2.24–9.87 μl per hour for 1–60 days. Animals were killed at various time points and the brains were sectioned mid-sagittally. One half of each brain was immersion-fixed and the other half was frozen for biochemical and histochemical studies.

Animals infused with rotenone did not gain weight as readily as controls and, in some cases, lost weight. At higher doses, animals appeared systemically ill. As infusions progressed, many animals required feeding by gavage and there was a relatively high mortality rate or need for early euthanasia. With prolonged infusions, animals tended to become bradykinetic or akinetic and rigid. One animal developed a spontaneous ‘rest’ tremor of a hind paw. Lower doses

Figure 2 TH staining of dopaminergic nerve terminals. Striatal sections from a control rat (a), a rat with a partial dopaminergic lesion of striatum (b) and a rat with near-total dopaminergic denervation of striatum (c) are shown. Note that even when striatal TH depletion is almost complete, there is relative sparing of dopaminergic terminals in the nucleus accumbens (arrow) and olfactory tubercle (arrowheads).
of rotenone were clearly better tolerated than higher doses. The behavioural effects of dopaminergic drugs have not yet been tested in a systematic fashion.

Rotenone binding is practically irreversible, so we can determine the proportion of complex-I rotenone-binding sites occupied using a [3H]dihydrorotenone-binding assay developed in this laboratory [37] (Figure 1). In animals with dopaminergic lesions, 55–74% of striatal complex-I-binding sites were occupied by the infused rotenone and in substantia nigra 61–82% of the sites were occupied. This is consistent with data indicating that complex I must be inhibited by about 70% before there is substantial inhibition of mitochondrial respiration or ATP synthesis [38]. In contrast, succinate dehydrogenase (complex II) and cytochrome oxidase (complex IV) activities were generally unchanged.

The earliest dopaminergic lesions were in the central portions of the striatum (Figure 2b). These could be seen as early as 1–2 days after beginning infusions of 7–12 mg/kg per day, but were highly variable. Figure 2(b) shows an early dopaminergic lesion in an animal killed 2 days after the start of a rotenone infusion at a rate of 7 mg/kg per day. A nissl stain revealed minimal damage to intrinsic striatal neurons in the region of depressed tyrosine hydroxylase (TH) staining (Figure 3b). At higher power, however, the density of neuronal profiles may have been slightly reduced (results not shown). In animals with small-to-moderate losses of striatal TH staining, there was generally no evidence of damage or loss of nigral dopamine neurons (Figure 4b). At a dose of 4 mg/kg per day, a similar striatal TH lesion was noted after 7 days of infusion; at a dose of 3 mg/kg per day, such lesions were seen at 8–18 days; and at a dose of 2 mg/kg per day, lesions were seen at 23 days.

When lesions progress to the point that the majority of striatal TH is lost (Figure 2c), there is still relative sparing of the dopaminergic innervation of the nucleus accumbens and near-complete sparing of the olfactory tubercle. Figures 2(c), 3(c) and 4(c) are from an animal infused at 4 mg/kg per day for 23 days. Even at this point, nissl staining fails to reveal much striatal damage.

Figure 3 Nissl-stained sections from the animals shown in Figure 2. Compared with the control striatum (a), the striatum with a partial dopaminergic lesion (b) shows little evidence of damage to intrinsic striatal neurons. Similarly, in the striatum with complete dopaminergic denervation (c), there is very little striatal damage. At high power, the sections shown in (b) and (c) may have some mild neuronal drop-out.
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Although at higher power there appears to be some mild thinning of neuronal profiles. With extensive striatal dopaminergic denervation, as in this animal, there is apparent retrograde damage or death of neurons in the substantia nigra pars compacta (Figure 4c). Consistent with the relative sparing of dopaminergic terminals in the nucleus accumbens, there is relative preservation of dopaminergic neurons in the ventral tegmental area (Figure 4c).

In summary, continuous, systemic administration of rotenone produces progressive dopaminergic denervation that starts in the central portion of the striatum, spreads to involve the entire striatum while sparing the nucleus accumbens and olfactory tubercle, and eventually proceeds in a retrograde fashion to the substantia nigra pars compacta (Figure 4c). Consistent with the relative sparing of dopaminergic terminals in the nucleus accumbens, there is relative preservation of dopaminergic neurons in the ventral tegmental area (Figure 4c).

From these preliminary studies, we conclude that systemic complex-I inhibition with rotenone causes selective damage to dopaminergic nerve terminals and that this damage proceeds in a retrograde manner to involve nigral cell bodies. The nigrostriatal neurons that die in PD appear to have an extraordinarily selective vulnerability to complex-I dysfunction. These results greatly strengthen the proposition that an acquired or inherited defect in complex I might be central to the pathogenesis of PD. This model should be extremely valuable for defining the mechanisms of neurodegeneration in PD and for testing neuroprotective therapeutic strategies. Some of the potential mechanisms involved in cell damage and death after complex-I inhibition are discussed below.

Complex-I defect: reactive oxygen species

Normally, about 98% of molecular oxygen is consumed by mitochondrial respiration. At complex IV, molecular oxygen is reduced by four electrons
to two molecules of water. The 1–2% of oxygen that is not reduced at complex IV is reduced to $\text{O}_2^{2-}$ and $\text{H}_2\text{O}_2$ [39]. Complex I is one of two sites in the electron-transport chain where free radicals, such as $\text{O}_2^{2-}$, are produced. The rate of superoxide production may be increased 6–7 fold under conditions in which partially reduced mitochondrial components accumulate. This would occur when complex I is defective or partially inhibited. For example, rotenone and MPP$^+$ increase free-radical production [40,41], and mitochondria isolated from patients with known mitochondrial defects produce excess $\text{H}_2\text{O}_2$ [42].

Whereas a defect in complex I can enhance free-radical production, local free-radical production can selectively damage complex I. Numerous studies in vitro have demonstrated that mitochondrial free-radical generation irreversibly inhibits complex I [40,41]. Cleeter et al. [43] showed that MPP$^+$ can cause irreversible inhibition that is prevented by free-radical scavengers. Thus it is possible to envisage a feed-forward cycle of complex-I impairment causing free-radical formation and further complex-I damage.

The superoxide radical produced by mitochondria, which increases in the setting of complex-I impairment, can react with nitric oxide to form peroxynitrite. Peroxynitrite is a biologically generated, reactive and highly toxic substance that can oxidize proteins, membrane lipids, sugars and DNA. Strong evidence links nitric oxide to the degeneration of dopaminergic neurons induced by MPTP in mice [44]. The cellular source of the nitric oxide is unknown and whether this mechanism is important in PD is currently speculative. Nevertheless, it is well established that peroxynitrite can also inhibit complex I.

What is known of the biochemical pathology of PD supports the idea that a complex-I defect and oxidative mechanisms play important roles in PD pathogenesis. As noted, there is evidence for impaired complex-I activity in the substantia nigra of PD brains. This would be expected to enhance production of reactive oxygen species. Consistent with this hypothesis, decreased levels of GSH have been found in the substantia nigra of PD brains [45]. GSH plays an important role in removing peroxides; its depletion in PD suggests oxidative stress. In addition, lipid hydroperoxides and malondialdehyde, which are products of free-radical damage to lipids, are increased in PD substantia nigra [46,47]. Based on these data, it is reasonable to hypothesize that anti-oxidants may provide protection against oxidative damage.

**Complex-I defect: excitotoxicity**

Glutamate, in addition to being the predominant excitatory neurotransmitter in the brain, has neurotoxic properties under some circumstances. In particular, neuronal bioenergetic status has a major impact on whether glutamate functions as an excitatory transmitter or an ‘excitotoxin’. With impairment of electron transport, there is a concomitant decrease in ATP levels. Neurons require mitochondrially derived ATP to fuel the $\text{Na}^+$/H$^+$-ATPase that maintains membrane polarity. With depletion of ATP, $\text{Na}^+$/H$^+$-ATPase activity is depressed, and we have demonstrated that mitochondrial toxins cause neuronal depolarization [48]. Neuronal depolarization
relieves the voltage-dependent Mg\(^{2+}\) blockade of the N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor [49]. Consequently, normal levels of extracellular glutamate may cause excitotoxic activation of NMDA receptors and excessive elevation of calcium. Mitochondria also take up large amounts of cytosolic calcium [50]. When electron transport is impaired, there may be decreased mitochondrial calcium uptake. In this situation, for a given amount of calcium influx to the cytosol, levels of calcium rise excessively, because it can no longer be sequestered in the mitochondria [48]. Thus, mitochondrial impairment leads to aberrant activation of NMDA receptors while simultaneously rendering neurons less able to withstand this insult (for reviews, see [51,52]). Whether or not this sequence of events leads to excitotoxic cell death, it certainly creates an additional metabolic stress, as neurons must struggle to handle the abnormal calcium load. It should also be noted that activation of NMDA receptors leads to mitochondrial production of reactive oxygen species [53,54]. The potential involvement of excitotoxicity in the cell

Figure 5 NMDA-receptor-induced mitochondrial calcium accumulation is qualitatively different from that induced by other stimuli. (a) After stimulation of non-NMDA receptors with kainate, cytosolic calcium uptake is substantially faster than mitochondrial calcium uptake. Similar results were obtained if cells were stimulated with KCl or ionomycin. (b) In contrast, after NMDA-receptor stimulation, the cytosolic and mitochondrial transients cannot be resolved temporally. (c) The rise time or \(t_{1/2}\) of mitochondrial calcium uptake is twice as fast after NMDA stimulation as compared with other stimuli. F, fluorescence.
death induced by metabolic impairment raises the possibility that glutamate antagonists may be neuroprotective.

**Selectivity of NMDA-induced mitochondrial calcium uptake**

The NMDA receptor is a glutamate-gated, high-conductance calcium (and sodium) channel. Depending on subunit composition, other types of glutamate receptor (non-NMDA receptors) can also flux calcium. Entry of calcium through the glutamate receptors is an important component of excitotoxic damage. Although NMDA receptors tend to allow more calcium entry than non-NMDA receptors, even when calcium fluxes are normalized, NMDA-receptor activation appears to be more toxic [48,55]. It has been suggested that there is a preferential relationship between NMDA-receptor activation and mitochondrial generation of free radicals [54]. In addition, there may be a functional compartmentalization of NMDA receptors with mitochondria, in terms of calcium buffering [54]. However, the precise interrelationships between NMDA-receptor activation, cytosolic calcium increase and mitochondrial calcium uptake remain obscure. To reliably, independently and simultaneously detect cytosolic and mitochondrial calcium-concentration changes in the same cell, we loaded primary striatal neurons with two calcium indicators, calcium-green 1N and rhodamine-2, and visualized the fluorescence signals from single neurons with laser scanning confocal fluorescence microscopy [56].

When neurons were stimulated by continuous exposure to the non-NMDA agonist, kainate, cytosolic calcium increased with a $t_{1/2}$ of 2.93 ± 0.11 s, whereas mitochondrial calcium lagged behind, having a $t_{1/2}$ of 6.15 ± 0.25 s.
(P < 0.0001; Figure 5A). Similar results were obtained after stimulating neurons with KCl or ionomycin. In marked contrast, the cytosolic and mitochondrial calcium fluxes could not be resolved temporally after NMDA receptor stimulation (2.71 ± 0.09 versus 2.89 ± 0.17 s, respectively; Figure 5B). The difference between NMDA-receptor stimulation and other stimuli was a result of faster mitochondrial calcium accumulation after NMDA-receptor activation (Figure 5C). From these experiments, it is clear that calcium loads that enter the cytoplasm during NMDA-receptor stimulation have more rapid, or ‘privileged’, access to mitochondria. This probably accounts for the selectivity of NMDA-receptor activation in producing mitochondrial damage via calcium-dependent generation of free radicals, collapse of mitochondrial membrane potential and opening of the permeability transition pore.

A unified scheme of pathogenesis

Complex I may play a central role in the pathogenesis of PD, but oxidative stress, depletion of GSH and NMDA-receptor-mediated excitotoxicity have also been implicated. It is possible to put all of these mechanisms into a unified hypothesis of PD pathogenesis (Figure 6). If one assumes that complex-I defects play a primary role, then all other mechanisms can come into play. Defective complex I can lead to increased production of reactive oxygen species and depletion of GSH, as noted previously. The reactive oxygen species can further damage complex I and cause a worsening impairment of mitochondrial function. In turn, mitochondrial dysfunction can cause activation of NMDA receptors and excessive uptake of calcium into mitochondria. This can cause or exacerbate mitochondrial production of free radicals. As seen in Figure 6, it is possible to envisage interacting feed-forward loops by which neuronal damage and, ultimately, cell death occur. The process of neurodegeneration could be initiated at any of the steps shown in Figure 6, and would eventually lead to each of the other mechanisms.

At present, although it is not possible to define unambiguously the inciting insult in PD, the scheme shown here does suggest potential therapeutic strategies. For example, anti-oxidants or pharmacological manipulation of the glutathione system may be beneficial. Because mitochondrial dysfunction can initiate the apoptotic cascade, strategies targeting steps in apoptosis may prevent nigral cell death. The mitochondrial permeability transition pore is also an attractive target since it only appears to open catastrophically under pathological conditions. In addition, if secondary excitotoxicity plays a role in pathogenesis, glutamate antagonists may be neuroprotective in PD. Glutamate-receptor activation also imposes large energetic demands on neurons. Therefore, even in the absence of frank excitotoxicity, glutamate antagonists may ease the bioenergetic burden of dopaminergic neurons. In this regard, it is worth noting that amantadine, a weak NMDA-receptor antagonist, has been shown to be an independent predictor of improved survival in patients with PD [57].

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References

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