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ABSTRACT

This proposal represents our continuing our efforts to investigate the molecular mechanisms of Parkinson's disease (PD), a progressive neurodegenerative disorder that affects about 1 million people in the United States alone. Recent evidence suggests that inflammation plays a major role in the progressive nature of this disease. Thus, we are investigating the contributions of several pro-inflammatory enzymes that we found up-regulated in microglia following the use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP), the neurotoxin that is the cornerstone of PD research. We found that the number ofSNpc DA neurons in MPTP-treated IL-1 beta and IL-1R1 knockout mice did not differ from their non[knockout littermates. This suggests that IL-1 beta and IL-1R1 may not be involved in the inflammatory response related to the necrotic form of neuronal death characteristic of the acute MPTP dosing schedule. However, we did note that cyclooxygenase-2 (COX-2) and cPLA2are both up-regulated in SNpc DA neurons following acute MPTP. Because the acute MPTP dosing schedule is quite harsh, Core B characterized a subacute MPTP model of PD and found that this model exhibits at least 3 forms of neuronal death in theSNpc that are time dependent, necrosis, apoptosis and autophagy. This subacute model seems to be closer to human PD in that it does not present an immediate end-stage situation characteristic of many neurotoxin-based PD models. We have also found in this subacute model that the number of SNpc DA neurons in MPTP-treated IL-1 beta and IL-1R1 knockout mice did not differ from their non-knockout littermates during the inflammatory process which presents somewhat of a confusing situation. Thus, we are now using our neuronal/glial cultures and our neuronal and glial conditioned media to sort out the mechanisms involved in the initiation of the inflammatory response related to SNpc DA neuronal death.

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized primarily by resting tremor, rigidity, bradykinesia and postural instability (Fahn and Przedborski, 2000). Neurons associated with this component of PD are mainly, though not exclusively, the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). These neurons degenerate and die during the course of the disease (Fahn and Przedborski, 2000). The etiology of PD remains unknown, however our studies using the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of PD and tissues from PD brains indicate 1) that the superoxide radical and nitric oxide are implicated in in the initiation and progression of PD (Przedborski et al, 1992; Przedborski et al, 1996; Liberatore et al, 1999); 2) that there is a greater loss of dopaminergic terminals in the striatum than the loss of dopaminergic neurons in the SNpc (Jackson-Lewis et al, 1995); 3) that there is an inflammatory component to PD that may be the cause of its progressive nature (Liberatore et al, 1999; Wu et al, 2002); and 4) that there is an up-regulation of certain cytokines in the SNpc of both MPTP-treated mice and tissues from PD brains (Teismann and Przedborski, personal communication). Although there are a number of theories as to the etiology of PD, the oxidative stress hypothesis of PD seems to encompass all of the above-mentioned findings. Furthermore, while etiological factors may ignite PD's neurodegenerative process, additional factors must participate in its progressive nature as DA neurons continue to die, albeit at a lower rate for many years as shown in recent evidence from human brains of individuals who had injested MPTP (Langston et al, 1983). Our goal is to elucidate the pathogenic factors that contribute to the progression of PD, thus we have revisited its pathological picture in search of abnormalities that might give us clues as to these additional factors. Aside from a severe loss of DA neurons in the SNpc, there is also present a marked glial response in both the PD brain and the MPTP-treated brain (Langston et al, 1999). Interestingly, both astrocytes and microglia, the two main components of the microglial response, are up-regulated in both situations (McGeer et al, 1988; Forno et al, 1992; Czlonkowska et al, 1996; Oppenheimer et al, 1997; Banati et al, 1998; Mizra et al, 2000), however, the magnitude of their responses is quite different. Both astrocytes and microglia are thought to support DA neuron survival (Abbott, 1988). Astrocytes are thought to provide trophic support to the DA neuron (Abbott, 1988), while microglia are believed to represent a two-edged sword in that they can be supportive as well as deleterious (Banati et al, 1993). The astrocytic response is, at best, mild and in only a few instances, has this response been dramatic (Forno et al, 1992; Mirza et al, 2000). In contrast, the activation of microglia has been consistently strongest in the SNpc the area of the brain most affected by the neurodegenerative process (Vila et al, 2001) thus, possibly counter-productive to the survival of the DA neuron. As per Liberatore et al (1999) and Wu et al (2002), microglia produce the superoxide radical, nitric oxide and a host of deleterious cytokines (Banati, 1993) which are thought to contribute to the progressive nature of PD. Merely because the substantia nigra (SN) contains significantly more microglia than other brain areas (Smeyne et al, 2005), by virtue of this fact, DA neurons are in a microenvironment that can increase their vulnerability to damage, or worst, to death in the event of an oxidative stress situation. Activated microglia are also found in close proximity to any remaining dopaminergic neurons (McGeer et al, 1988), around which they sometimes

cluster to produce what resembles neurophagia. Microglial activation and neurophagia are indeed indicative of an active ongoing process of cell death (Langston et al, 1999) which is consistent with the progressive nature of PD. Few data exist on the temporal relationship between dopamine neuron death and microglial activation and cytokine production, pro-inflammatory compounds (Teismann and Przedborski, personal communication), which we think may be some of the factors which participate in the progressive nature of DA neuron death in PD. To this end, we have proposed to use the MPTP mouse model to sort out the process of gliosis as it relates to the time course and the events in SNpc dopaminergic neuron death.

Microglial cells are resident macrophage cells in the brain that have the ability to react promptly to brain injury and to subtle changes in the microenvironment surrounding their charges, which are, in the SNpc, the DA neurons (Kreutzberg, 1996). Normally, brain microglia are in a resting state in which they are barely visible and very few, if any, ramified processes can be detected. In contrast, in a pathological situation, microglia quickly become activated, proliferate, become hypertrophic, increase in size and produce a number of marker molecules that are either pro- or anti-inflammatory in nature (Banati et al, 1993; Kreutzberg, 1996). Pro-inflammatory compounds include the superoxide radical, nitric oxide (NO), prostaglandin E_2 (PGE_2), excitatory amino acids, and pro-inflammaroty cytokines such as interleukin-1-beta (IL-1-ß). Since activated microglia have been found in the SNpc of PD brains and in the MPTP mouse model of PD, and can produce such damaging molecules, it is important to discern their mechanism of activation in PD and in the MPTP mouse model of PD as well as to continue our efforts to identify compounds that exhibit neuroprotective qualities in the SNpc. Since this is a Program Project with several components, we will concern ourselves with the Project Core (Core A and Core B) and Project .1 (Przedborski).

Body of the Research

The general goal of this research project is the study of the pathogenesis of PD using the oxidative stress hypothesis as its basis and the MPTP mouse model as the vehicle for the study. Microglia are the object of this study as we have made several observations about these cells. These observations are as follows: (1) MPTP, a toxin that damages DA neurons in the SNpc as seen in PD, stimulates a robust activation of microglia which have been shown to produce cytotoxic factors; (2) NADPH oxidase, the main microglial producer of reactive oxygen species, is activated after MPTP administration; (3) we have data showing that microglia elevates other noxious mediators such as the pro-inflammatory cytokine interleukin-1β (IL-1β) following MPTP administration; (4) we have demonstrated that mediators such as the pro-inflammatory prostaglandin PGE_2 is also increased following MPTP administration. The above-listed findings suggest that inflammatory-related events are present in the SNpc following MPTP administration thus the possibility exists that these events may contribute to the progressive nature of PD. So, in our continuing efforts to investigate the pathogenesis of PD, we want to understand how microglia contribute to the degeneration and death of the DA neurons in the SNpc in PD and in the MPTP mouse model of PD. To do this, in **Specific Aim I**, our plan is to administer minocycline, a second generation tetracycline antibiotic that blocks microglial activation independent of its anti microbial effects, prior to administration of our acute

MPTP regimen, then assess the neuroprotective effects of minocycline in the SNpc using HPLC and Western blotting techniques. Also planned are cell culture experiments using neuronal/microglial cultures to study the individual mechanisms of microglial activation involved in the MPTP neurotoxic process. We also plan to assess the beneficial effects of pharmacological intervention in the MPTP neurotoxic process. In **Specific Aim II**, we plan to define the role of NADPH oxidase in the MPTP neurotoxic process at different time points in the MPTP mouse model of PD using both normal NADPH oxidase-deficient mice. We also plan to assess neuroprotection and the process of microglial activation. In **Specific Aim III**, we plan to assess the role of IL-1β in the MPTP neurotoxic process. To do this, we will determine the time course of IL-1β and IL-1 receptor 1 (IL-1R1) in those brain regions (striatum and ventral midbrain) affected by MPTP and in PD. We also plan to define the cellular origin of IL-1β production and IL-1R1 expression in this model. Furthermore, we will also assess the contribution of IL-1β in neurodegeneration by administering MPTP to IL-1 converting enzyme (ICE) and IL-1R1 knockout mice. Neuroprotection will be assessed as well. Lastly, in **Specific Aim IV,** we plan to examine the contribution of prostaglandin PGE_2 to the death of the SNpc DA neurons by assessing the roles of the PGE_2-synthesizing enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2 in the MPTP neurotoxic process in different brain regions and at different time points using different doses of the toxin. We also plan to use COX-1 and COX-2 deficient mice in these studies. These planned specific aims should provide valuable information about the mechanisms involved in the inflammatory response related to the MPTP neurotoxic process and to PD as well as identify targets for therapeutic intervention.

Key Research Accomplishments

Core A

Core A continues its work as the administrative arm of this project. It provides the necessary centralized scientific leadership of this project by reviewing all of our findings, by discussing the data with the researchers and by guiding the researchers in the proper reporting of results. Because of Core A's close supervision of this project, no part of this project is not completed or is not in progress.

Core B

Core B is the centralized MPTP facility for this project. Dr. V. Jackson-Lewis is the PI of this facility. It is located on the 19th floor of the Black Building within the Institute of Comparative Medicine and is a self-contained unit. The primary role of Core B is to support the activities of the project by planning the MPTP experiments, by performing the MPTP injections, by sacrificing the MPTP-treated mice, by dissecting the needed MPTP tissues and by training our researchers in the safety and handling of MPTP. All injections are performed by Dr. Jackson-Lewis. Samples are either shipped to other researchers or used here at Columbia. Quality controls are run frequently using HPLC to insure that samples are prepared properly. Drs. Jackson-Lewis and Przedborski are now deemed as experts in the use of MPTP receiving at least a dozen calls or e-mails per week concerning the use of MPTP or how to establish a MPTP facility.

Core B continues its efforts to define and refine the MPTP mouse model of PD. In this respect, we have examined, in depth, the subacute MPTP mouse model of PD. Also, Core B has begun to examine the neuroprotective effects of another SOD mimetic in the acute MPTP mouse model.

Specific Aim I

In Vivo Experiments

The in vivo experiment in this specific aim is completed. The results have been presented at the Society for Neuroscience Meeting in 2002 and published in the Journal of Neuroscience under the title **Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydripyridine mouse model of Parkinson disease in 2002 (J Neurosci 22: 763-771).** The major findings in this study were as follows: (1) that minocycline protects SNpc DA neurons against the damaging effects of MPTP; (2) that minocycline inhibits microglial activation; (3) that minocycline prevents nitroyrosine formation; (4) that minocycline prevents pro-inflammatory cytokine formation; (5) that minocycline confers resistance beyond iNOS (inducible nitric oxide synthase) ablation.

Specific Aim II

The proposed studies for Specific AIM II have been completed and results from this study were presented at the Society for Neuroscience Meeting (2003) and have been published in the Proceedings of the National Academy of Science under the title: **NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydripyridine mouse model of Parkinson disease (PNAS 100:6145-6150, 2003).** The findings of this study are as follows: (1) NADPH oxidase is induced in the SNpc of MPTP-treated mice; (2) NADPH is expressed in microglia in the SNpc of MPTP-treated mice and this expression is time-dependant; (3) ablation of the gp91 subunit of NADPH oxidase attenuates the production of reactive oxygen species as demonstrated by hydroethidium fluorescence in tissues; (4) mice deficient in the gp91 subunit show less damage in SNpc TH-positive neurons than their non-deficient littermates; (5) less protein carbonyl content was noted in gp91 deficient mice than in their wild-type counterparts; (6) MPTP-induced SNpc DA neuron damage is lessened by infusion of extracellular SOD into the striatum of MPTP-treated mice.

Specific Aim III

In Specific Aim III, we plan to assess the role of IL-1β in the MPTP neurotoxic process. In our preliminary data, following MPTP administration to normal C57bl/6 mice from Charles River Laboratories, we found increased levels of IL-1β and IL-1R1 in the ventral midbrain of the treated mice compared to their saline-treated counterparts. Thus, as per this specific aim, we investigated whether SNpc DA neurons would be protected in IL-1β and IL-1R1 knockout mice.

Specific Aim IV

MPTP stimulates the formation of PGE2 which apparently enhances SNpc DA neuron death through the exacerbation of the inflammatory response. We presented data from this part of the study at the Society for Neuroscience Meeting (2003). Results from this work were published in PNAS under the title **Cyclooxygenase-2 is instrumental in**

Parkinson's disease (PNAS 100:5473-5478, 2003. In this publication, we reported that (1) the COX-2 (cyclooxygenase-2) enzyme rather than the COX-1 enzyme is up-regulated in SNpc DA neurons in PD brains and in the MPTP mouse model of PD; (2) the up-regulation of COX-2 in PD and in the MPTP mouse model of PD occurs through Jun kinase/c-Jun-dependent mechanism; (3) COX-2 inhibition as well as COX-2 ablation in the striatum of the MPTP mouse model attenuates the production of 5-cysteinyl dopamine which is known to be neurotoxic; (4) COX-2 inhibition and ablation attenuates TH-positive neuron in the SNpc of MPTP-treated mice.

Reportable Outcomes for 2005-2006

Core B

This year brought more telephone calls, e-mails and consultations about the MPTP mouse model of PD. Dr. Jackson-Lewis has sent her MPTP protocol to a number of investigators, visited a number of institutions and performed experiments at these sites. The expertise of Drs. Jackson-Lewis and Przedborski with this MPTP mouse model has garnered an invitation from Nature Protocol to write their protocol for this model as well as make a video about tissue dissection for tissue samples from this mouse mode of PD. The MPTP protocol for the MPTP mouse model of PD has been accepted for publication by Nature Protocol. The dissection video is now under review by both Nature Protocol and the Institutional Animal Care and Use Committee at Columbia University.

In expanding the role of Core B, during the past year, Dr. Jackson-Lewis has characterized the sub-acute MPTP mouse model of PD which seems to be a model closer to the actual human disease. This model originally established by Tatton and Kish (1997) requires daily dosing of MPTP (30 mg/kg/day) for five days and was thought to demonstrate only the apoptotic form of cell death which is thought to be the form of cell death in PD. However, we performed time course studies with this model using silver staining, fluorojade staining and a number of other stainings and found that this model exhibits three forms of SNpc TH-positive neuron death in the MPTP-treated mice and has a number of features closer to PD than the acute MPTP mouse model. The autophagy part of this work remains to be confirmed in GFP-LC3 mice. This work has not yet been published.

In our continuing efforts to find and examine SOD mimetics that might protect the TH-positive neurons in the SNpc of MPTP-treated mice, we investigated the neuroprotective effects of TEMPOL. TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a stable nitroxyl antioxidant that exhibits SOD mimetic characteristics (Samuni et al, 1990), is thought to be neuroprotective in disorders involving reactive oxygen species. TEMPOL reacts directly with carbon-centered and peroxy radicals (Chateauneuf et al, 1988) and prevents the reduction of hydrogen peroxide to the hydroxyl radical (Samuni et al, 1991). It can oxidize reduced transition metals (Samuni et al, 1991) and decrease the mortality related to intraperitoneal. 6-hydroxydopamine (Purpura et al, 1996; Weinberg et al, 2004). Since recent studies demonstrated its neuroprotective effects against both central and peripheral nervous system damage (Liang et al, 2005), we thought it prudent to examine whether TEMPOL could counteract the damaging effects of MPTP in mice. In our basic studies, we found (1) that TEMPOL does not interfere with MPTP

metabolism; (2) that it protects significantly SNpc TH-positive neurons against MPTP-induced damage; (3) that TEMPOL partially attenuates the MPTP-induced up-regulation of microglia and astrocytes; and (4) that TEMPOL suppresses superoxide radical formation to a significant degree. Although more work needs to be done to clarify the promise of TEMPOL as a possible therapeutic agent against PD, this drug does show some promise. Note: Use of TEMPOL is an attempt to complete studies in DAMD-99-1-9474 for which we could not breed significant numbers of iNOS x SOD and nNOS x SOD mice.

Specific Aim I.
In Vitro Experiments
We have noted that during the MPTP neurotoxic process both microglia and astrocytes are upregulated albeit, not at the same time. Thus, using neuronal/microglial primary ventral midbrain post-natal cultures, we are now in the process of trying to sort out how microglia might contribute to the death of SNpc DA neurons and what is the role of the astrocytes here. Here, we have made astrocyte and microglial conditioned media and are now exposing primary neuronal cultures to these conditioned media in the absence of and in the presence of MPP+.

Specific Aim III.
In our first experiment of this study, we found that nullifying IL-1β and IL-1L1 in mice injected acutely with MPTP offered no neuroprotection to the SNpc DA neurons. In this case, we will repeat this experiment to clarify our results before moving backward to ICE. Furthermore, we may have to move to a subacute dosing schedule since ICE is a recognized effector of apoptosis and not necrosis which results from the acute MPTP dosing schedule.

Specific Aim IV.
In continuing our studies here, we investigated whether COX-2 was also involved in a programmed cell death in SNpc DA neurons induced by sub-acute MPTP intoxication or by a striatal lesion with quinolinic acid (QA), a model of induced apoptotic death in the SNpc. At the peak of apoptotic DA neuron death induced by each of these neurotoxins, ablation of COX-2 significantly reduced the number of SNpc apoptotic cells (40% less in the MPTP model and 50% less in the QA model). To determine whether this reduction in apoptotic cell death resulted in increased survival of DA neurons, we determined in COX-2 deficient mice treated with MPTP, the number of SNpc tyrosine-hydroxylase positive cells compared to their wild-type littermates. The number of surviving SNpc DA neurons was significantly higher in COX-2-deficient mice (75% of saline-injected animals) than in their wild-type littermates (50% of saline injected animals). Knowing that arachidonic acid, the COX-2 substrate, is generated mainly by cytosolic PLA_2 ($cPLA_2$), we determined the localization of $cPLA_2$ in the SN. $cPLA_2$ was present in TH neurons but not in astrocytes or in microglia. MPTP treatment did not affect this localization, and at the peak of toxicity, $cPLA_2$ expression was significantly and specifically decreased in SN (50% less $cPLA_2$ proteins). We also investigated whether ablation of $cPLA_2$ affected the survival of dopaminergic neurons after MPTP

intoxication. We found no differences between $cPLA_2$-deficient mice and their wild-type littermates. Overall, these findings indicate that in the MPTP mouse model, COX-2 but not $cPLA_2$ plays a role in DA neuron death by activating the apoptotic machinery. This work was presented at the Society for Neuroscience Meeting in Atlanta, Georgia.

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Jackson-Lewis, V., Wu, DC., Przedborski, S. Tempol, a stable nitroxide and SOD mimetic, protects SNpc dopaminergic neurons against the damaging effects of MPTP. Society for Neuroscience Annual Meeting, Atlanta, Georgia, October, 2006.

C. Perier, J. Bove, V. Jackson-Lewis, S. Przedborski, M. Vila. AIF deficiency sensitizes dopaminergic neurons to MPTP-induced cell death via a specific alteration in mitochondrial complex I. Society for Neuroscience Meeting, Atlanta, Georgia, 2006.

Discussion

In revisiting the neuropathology of PD, aside from a dramatic loss of DA neurons in the SNpc and a severe decrease in DA in the striatum, there is a marked gliosis in these same brain areas in PD and in the MPTP mouse model of PD. This marked gliosis (glial response) involves the non-neuronal support systems in the nigrostriatal DA pathway. The question is how do the glial cells fit into the neuropathological picture of PD? Astrocytes and microglia make up the support systems of the neurons (Abbott, 1988) supplying them with nutrients necessary for their survival. However, since both are upregulated in PD (Langston et al, 1999) and in the MPTP mouse model of PD during the cell death process (LIberatore et al, 1999; Wu et al, 2002), the question of which set of cells contribute to the progressive nature of PD is germaine to the issue of SNpc DA neurodegeneration. In earlier studies, we found that microglia up-regulate soon after MPTP intoxication (Liberatore et al, 1999) similar to the situation found in human PD. During this up-regulation, both iNOS (Liberatore et al, 1999) and NADPH oxidase (Wu

et al, 2002) contribute to the oxidative stress in the SNpc by producing both NO and the superoxide radical. In this respect, microglia represent a two-edged sword in that they can be supportive (produce pro-survival compounds) as well as destructive produce pro-destructive substances). According to Crow and Beckman (1995), NO and the superoxide radical radical react to produce peroxynitrite which is more damaging than either NO or the superoxide radical. This compound is produced both inside and outside of the neuron in oxidative stress situations (like in MPTP intoxication), thus the structural proteins in the cell wall are altered via nitration and carbonylation (Wu et al, 2003), producing neuronal cell wall damage and contributing to the eventual death of the neuron. Although it has not yet been determined, the initiation of SNpc DA neuron death most likely results from some compound released from the DA neuron which stimulates the microglial response. Also, interestingly, the toxic metabolite of MPTP, MPP+, is released from astrocytes and not from microglia. This seems to suggests that the astrocyte may release a compound similar to MPP+ that could be taken up by the DA transporter to initiate the death of the DA neuron in the SNpc. Thus, it is possible that the astrocytes and not the microglia are the initiators of SNpc DA neuron death. To sort this out, we have made astrocyte, microglia and neuron cultures as well as astrocyte, microglia and neuronal conditioned media for testing in various neuron/glia mixes. Our neuronal/glial culture system should give us some insights into what happens between neurons and glia that contributes to the death of the SNpc DA neuron.

Growing data suggests that inflammation increases the risk of developing PD. Since there are many faces to this process, and increased expression of cyclooxygenase type 2 (COX-2) and production of prostaglandin E(2) (PGE2) have been implicated in neurodegeneration in several pathological settings, we sought to determine the role of COX-2 and PGE2 in the inflammatory process related to PD. In an earlier publication (Teismann et al, 2003), we showed that COX-2, the rate-limiting enzyme in PGE2 synthesis, is up-regulated in brain dopaminergic neurons of both PD and MPTP mice. We noted that this induction occurs through a JNKc-Jun-dependent mechanism after MPTP administration (Hunot et al, 2004). Furthermore, we demonstrated that targeting COX-2 does not protect against MPTP-induced dopaminergic neurodegeneration by mitigating inflammation. This suggests that COX-2 up-regulation in the SNpc DA neuron might not be a compound that contributes to the progressiveness of PD but rather that substance that just might initiate the inflammation process in response to dying SNpc DA neurons. Furthermore, since the death of the SNpc DA neurons is thought to be and reported to be apoptotic (Anglade et al, 1997), this year we investigated the role of COX-2 in programmed cell death in SNpc DA neurons induced by sub-acute MPTP (30 mg/kg/day) intoxication or by a striatal lesion with quinolinic acid (QA), a model of induced apoptotic death in the SNpc (Kelly and Burke, 1996).. Here, we found that at the peak of apoptotic DA neuron death induced by each of these neurotoxins, ablation of COX-2 significantly reduced the number of SNpc apoptotic cells (40% less in the MPTP model and 50% less in the QA model). We also examined in COX-2 deficient mice treated with MPTP whether this reduction in apoptotic cell death resulted in increased survival of DA neurons. SNpc DA neuron numbers were significantly higher in COX-2 deficient mice than in their wild-type littermates which suggests that increased expression of COX-2 in

the SNpc DA neuron may contribute to the demise of these neurons. Again, it is possible that elevated COX-2 in the SNpc DA neuron could be the trigger for its own death.

In keeping with this idea, we backtracked to examine the role of cPLA2 in the SNpc of subacute MPTP-treated mice since arachidonic acid, the COX-2 substrate, is generated mainly by cytosolic PLA_2 ($cPLA_2$) (Kaiei et al, 2005), however, it can translocate to the cell's nucleus (Zhao et al, 2002). We first determined that $cPLA_2$ is localized to the SN, specifically in the TH-positive neurons, the same neurons in which COX-2 is up-regulated following oxidative stress. MPTP treatment did not affect $cPLA_2$ localization, however, at the peak of toxicity in mice which is about two to four days after the last dose of subacute MPTP (Vila et al, 2001), $cPLA_2$ expression was significantly and specifically decreased in SN (50% less $cPLA_2$ proteins) which goes along with the induction and up-regulation of COX-2 in the TH neuron. However, our investigation of whether ablation of $cPLA_2$ affected the survival of dopaminergic neurons after MPTP intoxication found no differences between $cPLA_2$-deficient mice and their wild-type littermates. Thus, COX-2 but not $cPLA_2$ plays a role in DA neuron death by possibly activating the apoptotic machinery. But, since cPLA2 does mediate NADPH oxidase (Zhao et al, 2002) and the NADPH oxidase enzyme was found to be induced in microglia in the SNpc during MPTP intoxication (Wu et al, 2002. Wu et al, 2003), $cPLA_2$ may be that necessary component of the inflammatory response instrumental in microglial activation in the SNpc in response to COX-2 upregulation and release from dying neurons. Insight into the cPLA2 role in inflammation should be obtained from our IL-1β studies in Specific Aim III since IL-1β, $cPLA_2$ and COX-2 all seem involved in the interplay related to the death of SNpc DA neurons.

Both the cause and the mechanisms underlying the death of the nigrostriatal DA neurons remain unknown (Fahn and Przedborski, 2000). Thus, the outstanding questions still remain why and how these specific neurons die in PD. Tissues from PD brains typically provide a fixed image of end-stage PD which is often not suitable to unravel early or dynamic neurodegenerative events. To this end, PD investigators rely heavily on different model systems to explore various aspects of the disease which is a strategy quite popular among PD researchers and which is based on the premise that dopaminergic neurons may have a stereotyped death cascade that can be activated by a range of insults including neurotoxins. These toxins have generated a large body of information regarding the molecular basis that governs the demise of dopaminergic neurons. The MPTP mouse model remains the cornerstone of PD research. As such, it makes sense to take this model as far as we can take it. Many researchers use the acute (4 doses over 8 hours) dosing schedule or some schedule that has not been verified. The majority of our studies have been performed in the acute MPTP mouse model and has indeed provided us with important insights into the death of SNpc DA neurons. Because it has been reported that in humans DA neurons die by apoptosis rather than by necrosis (Anglade et al, 1997), Core B has examined in detail the subacute (30 mg/kg/day x 5 days) MPTP mouse model of PD first introduced by Tatton and Kish, 1997. To determine the different forms of cell death in this model, we used silver staining, Nissl staining, fluorojade staining and staining with monodansylcadaverine. In this model, we found three different kinds of DA neuronal death, necrosis, apoptosis and autophagy. Suppressed silver staining revealed

that following the first dose of MPTP during the subacute dosing schedule, the form of DA neuronal death in the SNpc is necrosis with a number of DA neurons taking up enough of the silver stain to note that these neurons were "sick". If this is the case in human PD, then the initiator of the SNpc DA cell death process is necessarily some sort of toxic insult (Zing et al, 2002; Carvey et al, 2003; Ling et al, 2004). After the necrotic period of DA neuronal death (1 day), the type of death that was noted was apoptosis. Apoptosis began to appear 24 hours after the 3rd MPTP injection and was prolonged as apoptotic bodies were seen as late as 21 days after the last dose of MPTP and were still evident at 42 days after the last dose of drug. This fact ties in well with the fact that Langston et al (1999) found an active ongoing cell death process in humans who had been exposed to MPTP as early as 16 years before death. Thus, a one-time toxic insult could possibly set in motion a self-perpetuating process such as the progressiveness of PD as suggested by these researchers.

Autophagy is a lysosomal pathway responsible for the turnover of organelles and long-lived proteins and has been regarded mainly as an inducible process in neurons and is normally mobilized during oxidative stress and cell injury (Boland and Nixon, 2006). Recent evidence has emerged that shows that the autophagic process is constitutively active in healthy neurons and is vital to cell survival. Constitutive autophagy occurs in healthy neurons in the absence of detectable autophagic vacuole intermediates (Boland and Nixon, 2006) which can accumulate rapidly when the later steps in the autophagic process are blocked. Autophagic vacuoles have been detected in several major neurodegenerative diseases (Marino and Lopez-Otin, 2004) including Parkinson's disease (Anglade et al, 1997), where they have been linked neuronal cell death. The build-up of autophagic vacuoles in neurons during the disease process may reflect some impairment in the autophagic pathway. Determining the basis for autophagic vacuole accumulation and what causes this is critical for our understanding of the pathogenic significance of autophagy in a given pathologic state and for designing possible therapies based on modulating autophagy. In 1997, Anglade et al was the first research group to suggest that some of the cell death seen in PD may be autophagic in nature. In our subacute MPTP study of this Tatton and Kish model of PD, we noted "sick" TH-positive neurons. On H and E staining, these neurons looked strange with dense areas and they were not apoptotic. We tried conventional staining for autophagic granules with no success. Since conventional staining did not work, as we did with hydroethium injection to demonstrate the presence of the superoxide radical (Wu et al, 2002), we injected monodansylcadaverine (Kahn et al, 2004) intraperitoneally and observed fluorescent neurons with areas in the cell dense fleck-like staining. We repeated this process and performed a time-course study as well. Autophagy in SNpc neurons appeared at 7 days after MPTP intoxication and thereafter but not at 4 days after the drug. We are now in the process clarifying the autophagy seen in SNpc TH-positive cells following subacute MPTP administration using GFP-L3C mice. If we document autophagy here, then the subacute MPTP model mirrors PD far more than the more acute model. This model,when clarified, will be a tremendous leap forward in our understanding of PD and opens an avenue more akin to PD for therapeutic strategies.

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Addendum

Publications for this year of the award will be sent separately by FedEx.

Complex I deficiency primes Bax-dependent neuronal apoptosis through mitochondrial oxidative damage

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Dysfunction of mitochondrial complex I is a feature of human neurodegenerative diseases such as Leber hereditary optic neuropathy and Parkinson's disease. This mitochondrial defect is associated with a recruitment of the mitochondrial-dependent apoptotic pathway *in vivo*. However, in isolated brain mitochondria, complex I dysfunction caused by either pharmacological or genetic means fails to directly activate this cell death pathway. Instead, deficits of complex I stimulate intramitochondrial oxidative stress, which, in turn, increase the releasable soluble pool of cytochrome *c* within the mitochondrial intermembrane space. Upon mitochondrial permeabilization by the cell death agonist Bax, more cytochrome *c* is released to the cytosol from brain mitochondria with impaired complex I activity. Given these results, we propose a model in which defects of complex I lower the threshold for activation of mitochondrial-dependent apoptosis by Bax, thereby rendering compromised neurons more prone to degenerate. This molecular scenario may have far-reaching implications for the development of effective neuroprotective therapies for these incurable illnesses.

mitochondria | neurodegeneration | Parkinson's disease

Reduced activity in mitochondrial complex I (NADH/ubiquinone oxidoreductase) is associated with a wide spectrum of neurodegenerative diseases (1). Low complex I activity due to mitochondrial DNA point mutations is found in many cases of Leber hereditary optic neuropathy, which is characterized by a massive retinal ganglion cell degeneration resulting in a rapid loss of central vision (2). Reduced complex I activity has also been reported in both autopsy brain tissues and platelets of patients affected with sporadic Parkinson's disease (PD) (3, 4). The pathogenic role of this mitochondrial dysfunction is supported by demonstrations that natural and synthetic complex I antagonists provoke neuronal death in animals (5–7). The molecular basis of neuronal death mediated by defective complex I activity is just beginning to be deciphered, in part by the utilization of the mitochondrial poisons 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone. For instance, it is now established that inhibition of complex I in rodents leads to degeneration of dopaminergic neurons of the substantia nigra pars compacta, as seen in PD (5), through activation of apoptotic molecular pathways (8–10). Moreover, it is believed that complex I dysfunction and the subsequent impairment of mitochondrial respiration provoke the activation of the mitochondrial-dependent apoptotic machinery by directly triggering the release of the apoptogenic molecule cytochrome *c* from the defective mitochondria (11–14).

Here we show that, contrary to the proposed direct effect of complex I deficit on cytochrome *c* release and consequent cell death, complex I defects do not autonomously recruit the apoptotic machinery. Instead, we show that complex I deficiency sensitizes neurons to mitochondrial-dependent apoptosis in response to the cell death agonist Bax through mitochondrial oxidative damage, by increasing the releasable soluble pool of cytochrome *c* within the mitochondrial intermembrane space. This molecular scenario sheds light into the mechanisms of cell death in chronic diseases linked to complex I deficiency and may have far-reaching implications for the development of new neuroprotective therapies for these incurable illnesses.

Materials and Methods

Animals and Treatment. Eight-week-old wild-type or Bax-deficient male mice received one i.p. injection of MPTP-HCl per day (30 mg/kg per day of free base; Sigma-Aldrich) for 5 consecutive days and were killed at 0, 2, 4, 7, 21, and 42 days after the last injection; control mice received saline injections only ($n = 3$–10 mice per time point, treatment, and genotype).

Subcellular Fractionation. Protein extraction of mitochondrial and cytosolic fractions was performed on fresh ventral midbrain tissue from saline- and MPTP-injected mice, as described (15).

Antibodies. The following primary antibodies were used for Western blot analysis: mouse monoclonal anti-cytochrome *c* (PharMingen); mouse monoclonal anti-cytochrome *c* oxidase-IV (Molecular Probes); rabbit polyclonal anti-cleaved caspase-3 (CM1; Idun Pharmaceuticals, La Jolla, CA); rabbit polyclonal anti-cleaved caspase-9 (Asp-353; Cell Signaling Technology, Beverly, MA); mouse monoclonal anti-β-actin (clone AC15; Sigma); mouse monoclonal anti-Bax (B-9; Santa Cruz Biotechnology); rabbit polyclonal anti-sulfite oxidase (gift from J. L. Johnson, Duke University Medical Center, Durham, NC); and goat polyclonal anti-HSP60 (Santa Cruz Biotechnology).

Immunofluorescence. For double immunofluorescence and confocal microscopy, a mouse monoclonal anti-cytochrome *c* (catalog no. 556432; PharMingen) and a rabbit polyclonal anti-adenine nucleotide translocase-1 (ANT-1; Oncogene, Boston) were used. Distribution of the fluorescent signal for both ANT-1 and cytochrome *c* stainings was analyzed by using the IMAGEJ 1.28U software (National Institutes of Health), similarly as de-

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Conflict of interest statement: No conflicts declared.

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Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine; ROS, reactive oxygen species; ANT-1, adenine nucleotide translocase-1; MPP^+, 1-methyl-4-phenylpyridinum ion; TMPD, *N,N,N',N'*-tetramethyl-1,4-benzenediamine.

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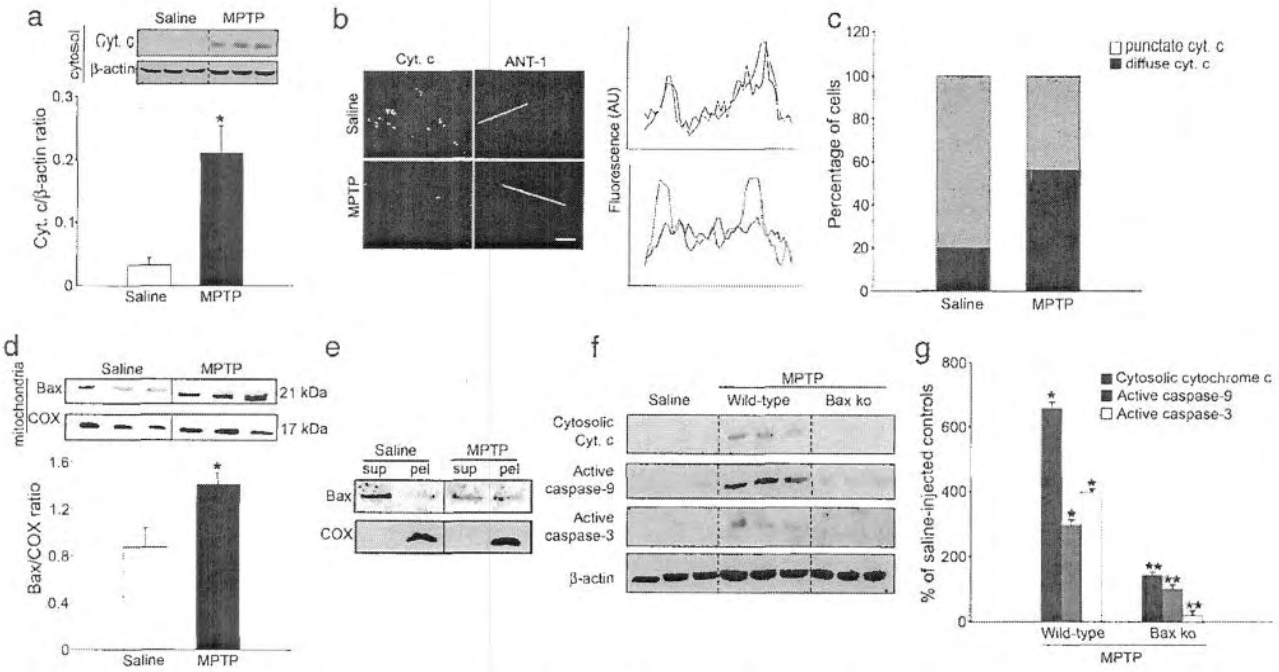

Fig. 1. Bax-dependent recruitment of mitochondrial apoptotic pathway following complex I inhibition in mice. (*a*) Cytochrome *c* levels are increased in ventral midbrain cytosolic fractions of MPTP-intoxicated mice at day 4 after the last MPTP injection, as determined by immunoblot. (*b* and *c*) Double immunofluorescence of substantia nigra pars compacta (SNpc) sections with cytochrome *c* (green) and the mitochondrial marker ANT-1 (red) show that in saline injected animals, 80% of SNpc neurons ($n = 77$) exhibit cytochrome *c* immunostaining colocalized with ANT-1, indicative of its mitochondrial localization. After complex I inhibition by MPTP, ≈60% of SNpc neurons ($n = 206$) show a diffuse cytochrome *c* staining no longer colocalized with ANT-1, indicative of its cytosolic redistribution. The fluorescence intensity profiles reported in the diagram correspond to the lines drawn in the confocal images (see *Materials and Methods* for details) (AU, fluorescence arbitrary units). (*d*) Bax levels are increased in ventral midbrain mitochondrial fractions at day 4 after the last MPTP injection, indicating Bax mitochondrial translocation. (*e*) In saline-injected animals, most of mitochondrial Bax appears in the supernatant fraction (Sup) after alkaline extraction, indicating its loose association with mitochondrial membranes. In contrast, after complex I blockade by MPTP, a significant fraction of mitochondrial Bax remains in the mitochondrial pellet (Pel), indicating its insertion into mitochondrial membranes. The inner mitochondrial membrane protein cytochrome oxidase was not extracted by alkaline treatment. (*f* and *g*) Genetic ablation of Bax in mutant mice (Bax ko) attenuates MPTP-induced cytochrome *c* release and caspase activation. *, $P < 0.05$, compared with saline-injected mice; **, $P < 0.05$, compared with MPTP-injected wild-type mice.

scribed (16, 17). Briefly, the analysis is based on a comparison of the intensity profile of pixels generated by the two fluorochromes. A typical pixel profile generated along a straight line transecting a cell immunostained for ANT-1 will show high-intensity pixels over mitochondria alternating with low-intensity pixels over the cytosol devoid of mitochondria. Thus, in a healthy cell, cytochrome *c* is confined to mitochondria, giving rise to a pixel profile for cytochrome *c* that overlaps with that of the mitochondrial marker (see Fig. 1*b*). Conversely, in a sick cell with cytochrome *c* translocation to the cytosol, the pixel profile for cytochrome *c* is more diffuse and diverges from that of the mitochondrial marker (see Fig. 1*b*).

Alkaline Extraction. Ventral midbrain mitochondrial fractions from saline-injected and MPTP-intoxicated mice were resuspended in 0.1 M Na_2CO_3 (pH 11.5) and incubated for 30 min on ice. The membranes were then centrifuged (75,000 × g, 10 min), and both the pellet and the supernatant were analyzed by Western blot for Bax or cytochrome *c* oxidase.

Isolation of Brain Mitochondria, Polarography, and Cytochrome *c* Release Studies. Isolation of nonsynaptosomal brain mitochondria and monitoring of mitochondrial oxygen consumption were performed as described (18). For cytochrome *c* release experiments, 250 μg of isolated brain mitochondria were incubated for different lengths of time (from 15 to 60 min) with different amounts of recombinant oligomeric Bax (kindly provided by S. J. Korsmeyer, Dana–Farber Cancer Institute, Harvard Medical School, Boston), complex I inhibitors 1-methyl-4-phenylpyridinum ion (MPP^+) or rotenone (Sigma-Aldrich) and/or the antioxidant M40401 or its inactive homologue M40404 (kindly provided by Metaphore Pharmaceuticals, Fort Lee, NJ). The percentage of cytochrome *c* release was estimated by assessing the intensities of the immunoblot bands for the soluble fractions versus total fractions (soluble + particulate). Two different types of buffers were used: low (225 mM mannitol/75 mM sucrose/10 mM KCl/5 mM Hepes/2 mM K_2HPO_4) and high (125 mM KCl/2 mM K_2HPO_4/1 mM $MgCl_2$/5 mM Hepes) ionic strength.

Measurements of Mitochondrial H_2O_2 Production. Samples were prepared as for the polarographical study. H_2O_2, converted from superoxide by manganese-superoxide dismutase, was measured by using 5 μM Amplex red and 5 units/ml horseradish peroxidase, both in presence or absence of ADP, as described (19).

Cybrid Cells. Cybrid cell lines were constructed by using enucleated fibroblasts from Leber hereditary optic neuropathy (LHON) probands as mitochondria donors carrying 3460 LHON primary mutations and the osteosarcoma (143B.TK$^-$)-derived 206 cell line as acceptor rho^0 cell line. Parental and cybrid cell lines were grown in DMEM supplemented with 15% FBS/100 units/ml penicillin/100 μg/ml streptomycin at 37°C in an incubator with a humidified atmosphere of 5% CO_2. Mitochondrial

extraction, polarography, H_2O_2 production and cytochrome *c* release studies were performed as described above.

Ascorbate/*N,N,N′,N′*-Tetramethyl-1,4-Benzenediamine (TMPD) Assay. These experiments were performed as described (20). Briefly, 1 mg/ml mitochondria was incubated in sucrose buffer (0.2 M sucrose/10 mM Trisphosphate-4-morpholinepropanesulfonic acid, pH 7.4/1 mM Pi/5 mM glutamate/2.5 mM malate/10 μM EGTA-phosphate Tris, pH 7.4) and treated as indicated in Fig. 4. After the indicated time, 400 pmol carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and 1 nmol antimycin A per mg of protein $^{-1}$ were added, and the reaction was transferred to a Clark-type oxygen electrode chamber. Final volume was 1 ml at 25°C. After 2 min, 6 mM ascorbate was added, followed by 300 μM TMPD 3 min thereafter. The ascorbate-driven oxygen consumption rate over the total TMPD-driven rate is plotted as a percentage of the ratio in the untreated mitochondria.

MPP$^+$–Cardiolipin Interaction Assay. Different amounts of [^{3}H]-MPP$^+$ were incubated in a cardiolipin-coated ELISA plate (Alpha Diagnostics, San Antonio, TX) for 15 min. Residual radioactivity was measured after washing out radioactive MPP$^+$.

Lipid Extraction and HPLC. Lipids from isolated brain mitochondria were extracted as described (21). Lipid extraction from midbrain, striatal, and cerebellar brain mitochondria was performed by pooling the above-mentioned anatomical regions from five different saline- or MPTP-intoxicated mice. The HPLC measurements were carried out by using an adaptation of a previously described method (21).

Statistical Analysis. All values are expressed as the mean ± SEM. Differences among means were analyzed by using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Student–Newman–Keuls post hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level.

Results

Complex I Inhibition Relies on Bax to Engage Mitochondrial-Dependent Apoptosis. Bax, a proapoptotic member of the Bcl-2 family, plays a critical role in the demise of dopaminergic neurons provoked by complex I inhibition (8). In most circumstances, including the pharmacological blockade of complex I (22), Bax-mediated cell death is accompanied with a mitochondrial release of cytochrome *c* and activation of caspase-9 and -3. We confirmed that mitochondrial release of cytochrome *c* does occur in MPTP-intoxicated mice (Fig. 1). This molecular event is time-dependent and coincides with the induction (8) and relocation of Bax from the cytosol to the mitochondria (Fig. 1). We also show that Bax mitochondrial translocation and cytochrome *c* release parallel the activation of downstream caspases (Fig. 1) and the previously reported time course of neuronal apoptosis caused by MPTP (8). Supporting the pivotal role of Bax in this molecular cascade is the demonstration that the release of cytochrome *c* and the activation of caspases are absent in Bax-deficient mutant mice treated with MPTP (Fig. 1 *f* and *g*); these mice were previously found resistant to MPTP-induced neurodegeneration (8). However, abrogation of Bax in mutant mice did not impair the potency of MPTP to inhibit complex I (Fig. 6, which is published as supporting information on the PNAS web site). Our results thus indicate that complex I deficiency operates together with Bax to engage the mitochondrial-dependent apoptotic pathway *in vivo*.

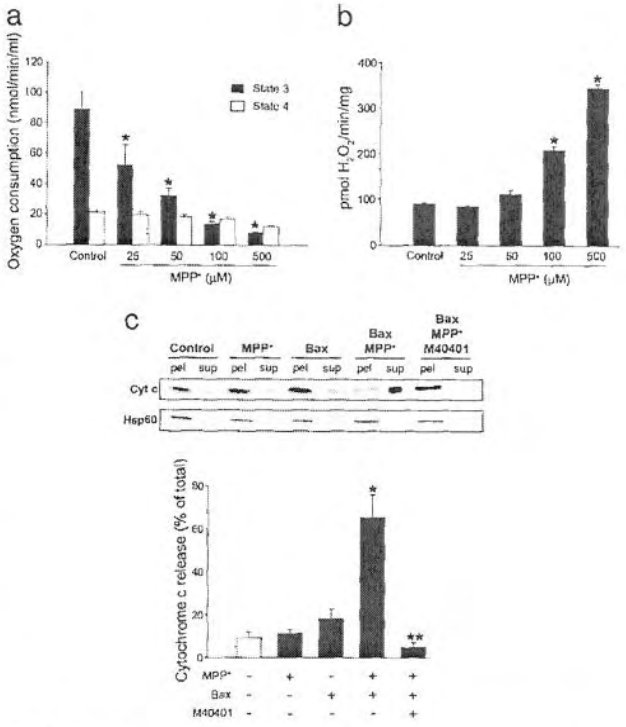

Fig. 2. Complex I inhibition stimulates ROS production and promotes Bax-dependent cytochrome c release in isolated brain mitochondria. (*a*) MPP$^+$ induces a dose-dependent inhibition of complex I-driven mitochondrial respiration, as assessed by monitoring oxygen consumption after addition of ADP, which in normal mitochondria induces a transient mitochondrial depolarization with a subsequent burst of oxygen consumption (state 3 respiration) until the added ADP is converted to ATP (state 4). (*b*) Complex I inhibition by MPP$^+$ induces dose-dependent ROS production in brain mitochondria, as assessed by measuring H_2O_2 using the fluorescent dye Amplex Red. (*c*) Complex I inhibition with 100 μM MPP$^+$ or incubation with ≈100 nM recombinant Bax, alone did not trigger significant release of cytochrome *c* from isolated brain mitochondria. However, combining complex I inhibition with recombinant Bax resulted in a marked release (>60%) of cytochrome *c*. This effect was abolished by 50 μM of the superoxide dismutase mimetic M40401. Matrix mitochondrial protein HSP60 was not mobilized by any of the tested conditions. *, $P < 0.05$, compared with untreated mitochondria; **, $P < 0.05$, compared with mitochondria treated with MPP$^+$ and recombinant Bax.

Inhibition of Complex I Potentiates Bax-Induced Cytochrome *c* Release. We next ascertained in isolated brain mitochondria the respective roles of complex I deficiency and Bax activation in the recruitment of the mitochondrial-dependent neuron death program, as well as the molecular basis for their interaction. First, we incubated purified brain mitochondria with different concentrations of MPTP's active metabolite, MPP$^+$, or rotenone. These experiments confirmed that both MPP$^+$ and rotenone caused, in a dose-dependent manner: (*i*) a reduction of ADP-stimulated oxygen consumption (state 3 respiration) supported by the NADH-linked substrates glutamate/malate (Fig. 2*a* and Fig. 7, which is published as supporting information on the PNAS web site); and (*ii*) an increased production of reactive oxygen species (ROS) (Figs. 2*b* and 7), likely generated by a higher rate of molecular oxygen reduction into superoxide radical in response to the hampered terminal step of electron transfer from the highest potential iron–sulfur cluster of complex I to ubiquinone (23). Contrary to the effect of complex I deficiency on cytochrome *c* release in intact cells *in vivo*, we found no evidence that MPP$^+$- or rotenone-induced complex I inhibition elicited a release of cytochrome *c* from purified brain mitochondria, even

when oxygen consumption was reduced by nearly 100% (Figs. 2*c* and 7). Recombinant oligomeric Bax protein, at concentrations as high as ≈100 nM and in the absence of complex I inhibitors, elicited only a minimal, not statistically significant, cytochrome *c* release (≈18%) from brain mitochondria (Fig. 2*c*). Unexpectedly, when brain mitochondria were incubated with both recombinant Bax and complex I inhibitors, up to 65% of the mitochondrial cytochrome *c* was released (Figs. 2*c* and 7). Thus, neither complex I inhibition nor permeabilization of the outer membrane with Bax, alone, triggers overt release of cytochrome *c*, whereas their combination results in a marked release (>60%) of this proapoptotic molecule.

ROS Are Mandatory for the Interaction Between Complex I Inhibition and Bax. We next explored the molecular basis for the observed mobilization of cytochrome *c* release induced by the combination of complex I inhibition and Bax. None of the tested conditions (complex I inhibition, recombinant Bax, or both) caused a release of the matrix mitochondrial heat-shock protein HSP-60 (Fig. 2*c*), ruling out the possibility that the mobilization of cytochrome *c* observed by combining complex I inhibition and Bax resulted from a mitochondrial structural damage. Because the ionic strength of the mitochondrial buffer may modify the electrostatic attachment of cytochrome *c* to the inner membrane and, therefore, the ability of the mitochondria to release cytochrome *c* (21), it is noteworthy that comparable results were obtained with low (mannitol/sucrose/Hepes) and high (KCl) ionic strength buffers (see *Materials and Methods*). Furthermore, sulfite oxidase, a soluble mitochondrial intermembrane protein, was not released by complex I inhibition alone. Sulfite oxidase was released by recombinant Bax but, in contrast to cytochrome *c*, no enhancement of its release was observed by combining Bax with complex I inhibition (not shown). These results indicate that complex I dysfunction alone is unable to engage the mitochondrial-dependent apoptotic pathway. They also demonstrate that activated oligomeric Bax, while able to permeabilize the outer mitochondrial membrane, stimulates only significant release of cytochrome *c* in conjunction with complex I defect.

Remarkably, the enhanced release of cytochrome *c* obtained by combining Bax and complex I inhibition was abolished by the lipophilic superoxide dismutase mimetic M40401 (24), but not by its inactive homologue M40404 (Figs. 2c and 7), indicating that this interaction relies on the mitochondrial production of ROS. Supporting this assertion is the fact that oxidative stress generated by Fe_3SO_4/ascorbate also enhances the release of cytochrome *c* induced by recombinant Bax in isolated rat liver mitochondria (21).

A Genetic Defect in Complex I Also Potentiates Bax-Induced Cytochrome *c* Release. Because inhibitors of complex I cause an acute loss of mitochondrial respiration, we sought to confirm the above findings in a chronic genetic model of complex I deficiency. Accordingly, we used an osteosarcoma-derived cytoplasmic hybrid (cybrid) cell line harboring the pathogenic point mutation of the mitochondrial DNA 3460G>A in complex I's ND1 subunit gene, which causes a deficit of complex I activity (25). In humans, the 3460/ND1 mutation is associated with massive retinal ganglion cell degeneration and loss of central vision in Leber hereditary optic neuropathy (26). Upon incubation in galactose-enriched medium, 3460/ND1 cybrids die by activating the mitochondrial-dependent apoptotic pathway, as shown by the release of cytochrome *c* (27).

We found that isolated mitochondria from 3460/ND1 cybrids, but not from wild-type cybrids or parental osteosarcoma cells, exhibited reduced complex I-driven mitochondrial respiration (Fig. 3*a*) associated with an increased production of ROS that could be quenched by M40401 (Fig. 3*b*). Similar to what we observed with pharmacological inhibition of complex I, the genetic disruption of mitochondrial respiration in 3460/ND1 cybrids was not associated with any detectable release of cytochrome *c* from isolated mitochondria in the absence of Bax, whereas ≈50% of cytochrome *c* was released in these mutant cybrids in the presence of recombinant oligomeric Bax (Fig. 3*c*). The latter effect was markedly attenuated in mitochondria isolated from 3460/ND1 cybrids grown in the presence of M40401 and was not observed in mitochondria isolated from wild-type cybrids or parental osteosarcoma cells (Fig. 3*c*).

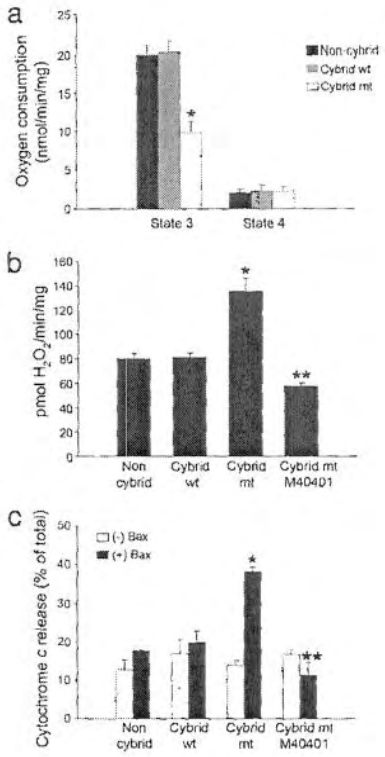

Fig. 3. Genetic disruption of complex I in cybrid cells stimulates ROS production and promotes Bax-dependent cytochrome *c* release. (*a*) Isolated mitochondria from mutant 3460/ND1 cybrids (*cybrid mt*) exhibit reduced complex I-driven mitochondrial respiration, as assessed by monitoring oxygen consumption supported by NADH-linked substrates glutamate/malate. (*b*) Impairment of mitochondrial respiration in mutant 3460/ND1 cybrids is associated with an increased production of ROS that was quenched by 50 μM of M40401. (*c*) Recombinant Bax (≈100 nM) induced a marked release (≈50%) of cytochrome *c* in mitochondria isolated from mutant 3460/ND1 cybrids but not from wild-type cybrids (cybrid wt) or parental osteosarcoma cells. This effect was attenuated by 50 μM M40401. *, $P < 0.05$, compared with noncybrid and cybrid wild-type mitochondria; **, $P < 0.05$, compared with mutant cybrid mitochondria.

Inhibition of Complex I Increases the Soluble Pool of Cytochrome *c* in the Mitochondrial Intermembrane Space in a ROS-Dependent Manner. Under physiological conditions, most cytochrome *c* is bound to the inner mitochondrial membrane by anionic phospholipids, primarily cardiolipin, whereas only a fraction is soluble in the intermembrane space and thus releasable upon outer membrane permeabilization (20). Therefore, we asked whether oxidative damage linked to complex I inhibition could increase the soluble pool of cytochrome *c* in the intermembrane space. Because ascorbate is capable of reducing only soluble cytochrome *c*, whereas the uncharged reductant TMPD is membrane-permeant and reaches all cytochrome *c*, the ratio of ascorbate-driven respiration over the total TMPD-driven respiration provides an index of cytochrome *c* soluble pool (20). Inhibition of complex I by MPP^+ resulted in a dose-dependent increase of

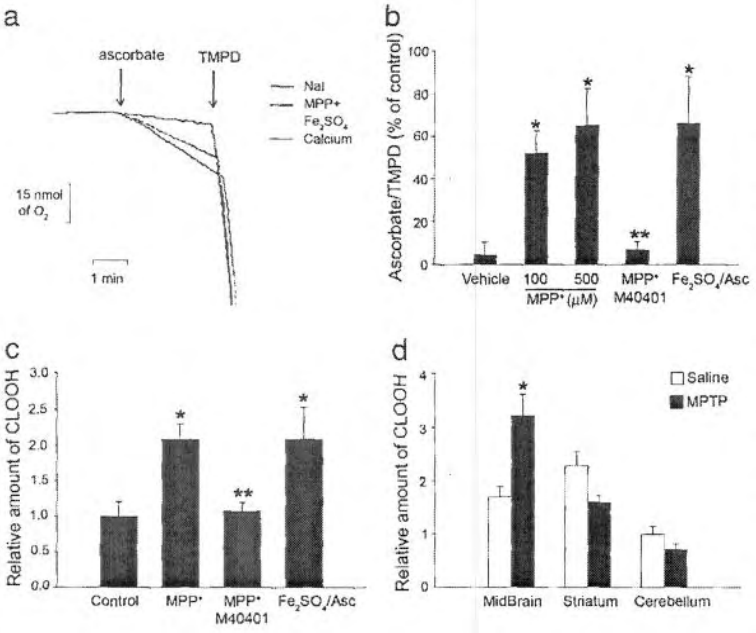

Fig. 4. Complex I inhibition increases the soluble pool of cytochrome *c* in the mitochondrial intermembrane space by oxidizing cardiolipin. (*a* and *b*) Complex I inhibition by MPP^+ induced a dose-dependent increase of mitochondrial ascorbate/TMPD-driven respiration ratio, consistent with an increased intermembrane soluble pool of cytochrome *c*. This effect was prevented by 50 μM M40401 and could be reproduced by the ROS-generating compound, Fe_2SO_4 (60 μM)/ascorbate (500 μM). Sodium iodide (NaI) was used as vehicle, because MPP^+ was used in the form of MPP^+-I. Ca^{2+}-mediated mitochondrial swelling, which increases the soluble pool of cytochrome c (20), was used as a positive control. (*c*) Complex I inhibition by MPP^+ induced oxidation of inner mitochondrial membrane cardiolipin in isolated brain mitochondria, as assessed by determining cardiolipin hydroperoxide (CLOOH) content by HPLC. Oxidation of cardiolipin was also produced by Fe_2SO_4/ascorbate and was attenuated by M40401. (*d*) Oxidized cardiolipin was detected in ventral midbrain samples from MPTP-intoxicated mice but not in regions devoided of MPTP-induced cell loss, such as striatum and cerebellum. *, $P < 0.05$, compared with controls; **, $P < 0.05$, compared with MPP^+-treated mitochondria.

mitochondrial ascorbate/TMPD-driven respiration ratio (Fig. 4 *a* and *b*), consistent with an increased intermembrane space soluble pool of cytochrome *c*. This effect was also prevented by M40401 (Fig. 4*b*) and could be reproduced by Fe_2SO_4/ascorbate (Fig. 4 *a* and *b*), indicating its dependency on ROS production.

ROS Generated by Complex I Inhibition Oxidize the Inner Mitochondrial Lipid Cardiolipin both *in Vitro* and *in Vivo*. It has been shown in isolated rat liver mitochondria that cytochrome *c* can be freed from the inner mitochondrial membrane by oxidative modifications of cardiolipin (21). Therefore, we tested whether cardiolipin peroxidation occurred after complex I blockade. After having excluded the possibility that MPP^+ displaces cytochrome *c* from the negatively charged cardiolipin by simple electrostatic interaction (see *Materials and Methods*), we assessed by HPLC the contents of oxidized cardiolipin in isolated brain mitochondria. Complex I blockade by MPP^+ resulted in a marked increase of oxidized cardiolipin that was attenuated by M40401, indicating its dependency on ROS production (Fig. 4*c*). Furthermore, incubation of isolated brain mitochondria with the ROS-generating system Fe_2SO_4/ascorbate also increased cardiolipin oxidation (Fig. 4*c*). Supporting a role in neurodegeneration induced *in vivo* by complex I inhibition, cardiolipin oxidation was also detected in mitochondria isolated from ventral midbrain of mice intoxicated with MPTP (Fig. 4*d*) in a time-dependent manner that preceded activation of Bax and apoptotic neuron death in this model of complex I deficiency (8).

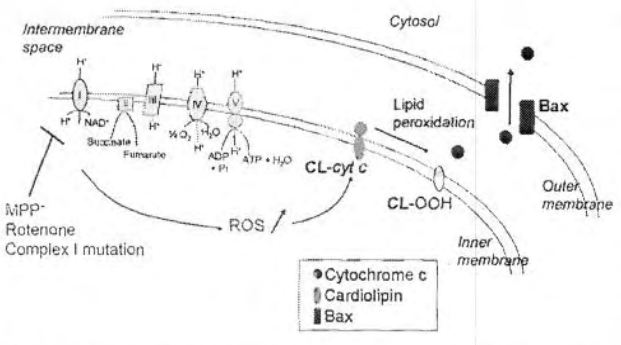

Fig. 5. Proposed pathogenic scenario induced by complex I deficiency. Pharmacological or genetic inhibition of complex I disrupts mitochondrial respiration and stimulates the mitochondrial production of ROS. As a consequence, an array of molecules is likely oxidatively modified in response to complex I defect, including the inner mitochondrial membrane lipid cardiolipin. Cardiolipin peroxidation, in turn, affects the binding of cytochrome c to the mitochondrial inner membrane, leading to an increased soluble pool of cytochrome *c* in the intermembrane space. Consequently, upon permeabilization of the outer mitochondrial membrane by activated Bax, a larger amount of mitochondrial cytochrome c can be released, making it more likely for a compromised neuron to undergo apoptosis (see *Discussion* for more details).

Discussion

The mitochondrial-dependent apoptotic pathway has been shown to be instrumental in the neuronal degeneration associated with disruption of mitochondrial respiration caused by complex I deficiency, as demonstrated by targeting molecules of this pathway such as Bax, caspase-9, or Apaf-1 (8, 22, 28). Our study, while confirming that complex I defects lead to a recruitment of the mitochondrial-dependent apoptotic pathway *in vivo*, sheds light onto the molecular mechanisms linking these two events. For instance, after MPTP administration, there is indeed a time-dependent and region-specific mitochondrial release of cytochrome *c* that occurs in association with activation of both caspase-9 and -3. All of these molecular alterations appear to be regulated by the death agonist Bax, because they coincide with Bax up-regulation and translocation to the mitochondria and are prevented by genetic ablation of Bax. Although Bax induction was previously shown to rely on p53 activation after complex I inhibition (29), the mechanism driving Bax mitochondrial translocation after complex I inhibition remains to be determined (10). Both Bid and Bak are known for cooperating with Bax to initiate mitochondrial-dependent apoptosis in response to the ligation of cell-surface death receptors. However, in contrast to the pivotal role of Bax, both Bid and Bak have been shown to be dispensable in complex I deficiency-mediated neuronal death (22, 30).

Our study also clarifies the process by which complex I defects contribute to the actual recruitment of the mitochondrial-

dependent cell death pathway. It has been widely assumed that complex I inhibition and the subsequent impairment of mitochondrial respiration directly trigger the release of cytochrome *c* from the affected mitochondria (11–14). Contrary to this view, our data demonstrate that complex I inhibition does not directly induce cytochrome *c* release from isolated brain mitochondria but, instead, it increases the "releasable" soluble pool of cytochrome *c* in the mitochondrial intermembrane space that can be subsequently released to the cytosol by some cell death agonists, such as Bax.

In both the pharmacologic and genetic models used here, the initiating event is complex I dysfunction, which, in turn, stimulates the mitochondrial production of ROS. Because the latter can be abated by M40401, it can be asserted that the endogenous antioxidant arsenal of the mitochondria is not sufficient to scavenge the excess of ROS generated by the complex I defect. We can also state that, because M40401 is a superoxide dismutase mimetic devoid of catalase activity (24), superoxide is the main reactive species produced. Yet, superoxide poorly penetrates membranes, hence its damaging effects are likely to be confined to the mitochondrial matrix and inner membrane. Although a broad range of molecules is likely modified by ROS in response to complex I dysfunction, our HPLC data indicate that cardiolipin is among the targets. Therefore, our data argue that complex I inhibition produces a mitochondrial oxidative damage that includes cardiolipin peroxidation. The latter alteration affects the binding of cytochrome *c* to the mitochondrial inner membrane, leading to an increased soluble pool of cytochrome *c* in the intermembrane space. Consequently, upon permeabilization of the outer mitochondrial membrane by activated Bax, a larger amount of mitochondrial cytochrome *c* can be released, making compromised neurons more likely to undergo apoptosis (Fig. 5).

Mobilization of the cytochrome *c* stores has been previously associated to a remodeling of mitochondrial cristae structure by Bid (20). Because Bid is not required for cytochrome *c* release in the context of complex I inhibition (22), and rotenone does not induce ultrastructural changes in mitochondrial morphology (11), it is unlikely that such a remodeling accounts for the observed ROS-related mobilization of cytochrome *c* associated with complex I inhibition. Instead, our results support the concept that oxidative modifications of cardiolipin may be responsible for the increased intermembrane stores of soluble cytochrome *c* following complex I deficiency.

Conclusion

Our study supports a pathogenic scenario in which complex I deficiency, which occurs in numerous neurodegenerative situations, does not autonomously kill cells, but rather sensitizes neurons to the action of death agonists such as Bax, through mitochondrial oxidative damage. Our results also provide a molecular basis to the observation of strong allometric correlations between mitochondrial ROS production and rates of brain and retinal neurodegeneration (31) and support the concept that steady-state ROS production, acting on the mitochondrial stress integration machinery, may be pivotal in setting the threshold for the occurrence of apoptosis for a given species and cell type in response to a cellular stress (31). Finally, our study shows that membrane-permeant antioxidants block the neuronal death-promoting interaction between mitochondrial oxidative damage and Bax-induced permeabilization of the outer mitochondrial membrane, suggesting that these compounds, of which a few are near approval for human use, may prove effective in mitigating neurodegeneration in complex I cytopathies.

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Toxin-Induced Models of Parkinson's Disease

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Summary: Parkinson's disease (PD) is a common neurodegenerative disease that appears essentially as a sporadic condition. It results mainly from the death of dopaminergic neurons in the substantia nigra. PD etiology remains mysterious, whereas its pathogenesis begins to be understood as a multifactorial cascade of deleterious factors. Most insights into PD pathogenesis come from investigations performed in experimental models of PD, especially those produced by neurotoxins. Although a host of natural and synthetic molecules do exert deleterious effects on dopaminergic neurons, only a handful are used in living laboratory animals to recapitulate some of the hallmarks of PD. In this review, we discuss what we believe are the four most popular parkinsonian neurotoxins, namely 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat. The main goal is to provide an updated summary of the main characteristics of each of these four neurotoxins. However, we also try to provide the reader with an idea about the various strengths and the weaknesses of these neurotoxic models. **Key Words:** Parkinson's disease, experimental models, neurodegeneration, pathogenesis, 6-hydroxydopamine, MPTP, rotenone, paraquat.

INTRODUCTION

Parkinson's disease (PD) is currently regarded as the most common degenerative disorder of the aging brain after the Alzheimer's dementia. Most epidemiological studies estimate that over one million individuals in the United States are carrying the diagnosis of PD and that roughly 50,000 new cases arise each year.[1] Clinically, PD is characterized by the tetrad of tremor at rest, slowness of voluntary movements, rigidity, and postural instability.[1] The cardinal biochemical abnormality in PD is the profound deficit in brain dopamine level, primarily, but not exclusively, attributed to the loss of neurons of the nigrostriatal dopaminergic pathway.[2] This pathway is made of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta and whose axons and nerve terminals project to the striatum.[2] However, the neuropathology of PD is far from being restricted to the nigrostriatal pathway, and histological abnormalities are also found in many other dopaminergic and nondopaminergic cell groups.[2] Aside from the loss of neurons, other prominent neuropathological features of PD include gliosis[3] and the presence of intraneuronal proteinaceous inclusions called Lewy bodies (LBs) in the few remaining substantia nigra dopaminergic neurons.[2]

Until now, very little is known about why and how the PD neurodegenerative process begins and progresses. Yet over the last two decades, tremendous strides toward acquiring a better knowledge of both the etiology and pathogenesis of PD have been achieved, thanks to numerous elegant clinical studies and investigations performed in autopsy materials and *in vitro* and *in vivo* experimental models of PD.[2] Despite these unquestionable advances, we still have major gaps in our understanding of the molecular and cellular biology of PD. Consequently, investigators still rely heavily on experimental models of PD to obtain greater insights into its cause, but more particularly into its pathogenesis. Whereas recent genetic discoveries have lead to a number of different genetic models of PD, none of these shows the typical degeneration of dopaminergic neurons. Thus far, among the various accepted experimental models of PD, neurotoxins have remained the most popular tools to produce selective neuronal death in both *in vitro* and *in vivo* systems.

In this paper, we will review the key neurotoxic models of PD, namely those produced by the toxins 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat.

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Other less often used neurotoxins such as isoquinoline derivatives and methamphetamine will not be discussed here, but information regarding those toxins can be found (see Ref. 4). Finally, two points must be emphatically stressed at the outset of this review paper. First, *in vitro* data will only be mentioned whenever necessary as this paper will focus on *in vivo* studies. This should not be taken as evidence undermining the significance of *in vitro* studies, but merely as a deliberate choice made by these authors. Second, some parts of this review represent shortened and updated pieces from previous reviews written by these authors. Should the reader be interested in these other reviews, see Refs. 2 and 4–6.

6-OHDA, OR THE PROTOTYPIC OXIDATIVE STRESS NEUROTOXIN

On an historical note, it must be remembered that among all the selected techniques developed to study specific structures of the nervous system, the noradrenergic analog 6-OHDA and several other synthesized analogs have been introduced as catecholaminergic neurotoxins over 30 years ago.[7] Ever since, these neurotoxin compounds, especially 6-OHDA, have remained extensively used for both *in vitro* and *in vivo* investigations. Because of practical considerations, in living animals 6-OHDA has been used essentially in small animals such as rodents. In some instances, however, it has also been administered in nonhuman primates[8–11] and, in particular for studies geared toward investigating the cardiovascular system, in dogs.[12–14]

6-OHDA shares some structural similarities with dopamine and norepinephrine, exhibiting a high affinity for several catecholaminergic plasma membrane transporters such as the dopamine (DAT) and norepinephrine transporters (NET). Consequently, 6-OHDA can enter both dopaminergic and noradrenergic neurons and inflict damage to the catecholaminergic pathways of both the peripheral and the central nervous systems. Therefore, should the goal be the production of a model of PD, with a specific lesion of the nigrostriatal dopaminergic pathway, it is imperative that attention be paid to the mode of administration of 6-OHDA (see below) as well as to several important technical details which have been reviewed by Jonsson.[7,15]

With respect to its mode of action, it is well accepted that 6-OHDA destroys catecholaminergic structures by a combined effect of reactive oxygen species (ROS) and quinones.[16] This view stems primarily from the demonstration that 6-OHDA, once dissolved in an aerobic and alkaline milieu, readily oxidizes, yielding hydrogen peroxide (H_2O_2) and *para*-quinone[17,18] as depicted in Figure 1. Although the chemical reaction that underlies 6-OHDA-induced neurotoxicity appears straightforward, it is in fact a remarkably complicated reaction that does not occur as a spontaneous oxidation by molecular oxygen. This chemical reaction has been reviewed elsewhere and will thus not be discussed herein, but any reader interested in acquiring further information regarding this aspect of 6-OHDA biology is encouraged to refer to Przedborski and Ischiropoulos.[6]

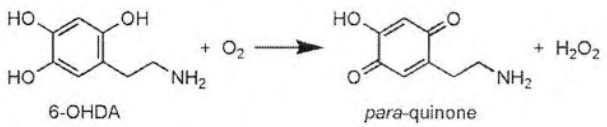

FIG. 1. Oxidation of 6-OHDA. Used with permission from Przedborski and Ischiropoulos. Reactive oxygen and nitrogen species: weapons of neuronal destruction in models of Parkinson's disease. *Antioxid Redox Signal* 7:685–693. Copyright © 2005, Mary Ann Liebert, Inc. All rights reserved.[6]

Like other parkinsonian neurotoxins to be discussed here, 6-OHDA can be administrated by systemic injection. However, contrary to MPTP, rotenone, or paraquat, this route of administration will not produce the desired nigrostriatal lesion. Instead, this is the preferred route of 6-OHDA administration to cause a chemical sympathectomy by damaging the peripheral nervous system.[7] Indeed, 6-OHDA poorly crosses the blood-brain barrier (BBB), hence failing to accumulate within the brain parenchyma to meaningful neurotoxic concentrations following systemic injections. To circumvent this problem, 6-OHDA has to be injected directly into the brain either free-hand or by stereotaxic means. As such, over the years several local sites of injection have been used to damage the central dopaminergic pathways including intraventricular, intracisternal, and intracerebral.[7] Although these models of lesioning have been primarily utilized in rats, sometimes mice and even monkeys have also been subjected to 6-OHDA lesioning.[9–11,19–22]

As far as the intraventricular and intracisternal administration of 6-OHDA is concerned, both produce a bilateral catecholaminergic lesion, observed within a few hours of the injection of the toxin, with generally very limited re-growth of affected nerve fibers.[7] However, when a bilateral 6-OHDA lesion is severe, animals often die primarily due to the occurrence of marked aphagia, adipsia, and seizures.[23,24] Accordingly, a much more popular and practical model of 6-OHDA is the unilateral intracerebral injection. The latter can be successfully used to target a particular catecholaminergic pathway of the brain.[25,26] To specifically damage the nigrostriatal dopaminergic pathway, 6-OHDA is injected stereotaxically into the substantia nigra, the medial forebrain bundle (that comprises the nigrostriatal tract), or the striatum.[7,27] After 6-OHDA injections into substantia nigra or the medial forebrain bundle, dopaminergic neurons start to die within the first 24 h and show a nonapoptotic morphology.[28] Maximal reduction of striatal dopamine level is reached within 3–4 d after lesion,[29] and, in most studies, residual striatal dopamine content is less than

20% of controls. Interestingly, despite the dramatic loss of dopaminergic neurons in the substantia nigra after a medial forebrain bundle injection of a high dose of 6-OHDA, levels of extracellular dopamine are still close to normal.[30] Perhaps this can be explained by a somatodendritic release of dopamine from the few spared neurons in the substantia nigra. When injected into the striatum, 6-OHDA produces a more protracted retrograde degeneration of the nigrostriatal system which can last from 1–3 wk after lesion,[31,32] and the dying neurons exhibit a varied morphology including some features reminiscent of apoptosis.[33] In addition to the lesion of the dopaminergic system, gliosis is also a prominent feature of the 6-OHDA model.[34] Many data support the idea that the glial response in experimental models of PD, especially of microglia, exacerbates the degeneration of dopaminergic neurons.[3] However, other studies also indicate that, under specific circumstances, the activation of astrocytes, produced in rats by the administration of interleukine-1β before a 6-OHDA-induced lesion, mitigates rather than enhances toxicity on dopaminergic neurons.[35] If confirmed, this observation would argue that the temporal relationship between the initiation of glial activation and dopaminergic neuronal death is critical in defining the role of the different kinds of glial cells in the neurodegenerative process. Finally, LB formation has never been convincingly demonstrated in the brain of 6-OHDA-lesioned rats, a lack that will be regarded by some as a major shortcoming of this model.

In terms of behavioral abnormalities, Rodriguez and collaborators[36] have reported that the few rats that survive and recover normal ingestion and weight following bilateral 6-OHDA lesion exhibit motor abnormalities that are partially corrected by drugs that stimulate dopaminergic receptors. Further description of the motor abnormalities of rats with bilateral 6-OHDA lesion can be found in Cenci and co-authors.[37] In contrast, unilateral injections cause a typical asymmetric circling motor behavior whose magnitude in rodents depends on the degree of nigrostriatal lesion.[31,38,39] This specific behavioral abnormality is most prominent after administration of drugs that stimulate dopaminergic receptors, such as apomorphine (rotation away from the lesion), or drugs that stimulate the release of dopamine, such as amphetamine (rotation toward the lesion), due to physiologic imbalance between the lesioned and the unlesioned striatum. Quantification of this turning behavior has been used extensively to assess the antiparkinsonian potency of new drugs,[40] transplantation, and gene therapies[41,42] and to study the motor fluctuations in the chronic treatment with levodopa.[43,44] Moreover, Olsson et al.[45] developed a very useful stepping test for unilateral-lesioned-rats that shows a forelimb akinesia that is improved by dopaminergic stimulation and which is reminiscent of the slowness of movements seen in PD patients. Several additional motor tests have also been developed and validated in 6-OHDA rats, including a model of L-DOPA-induced dyskinesia; all of these are discussed and evaluated in details by Cenci and co-authors elsewhere.[37]

The 6-OHDA model has also been used successfully to demonstrate the importance of dopamine stimulation for the proliferation of precursor cells in both the subependymal and the subgranular zones of the adult brain in rats.[46] It also allowed to demonstrate that, contrary to earlier reports, there is no evidence for *de novo* generated dopaminergic neurons in the adult rat substantia nigra.[47] In conclusion, although technically this specific model may be more challenging to use than some others discussed below, the huge body of work based on its utilization represents a significant impetus for using it in a variety of investigations. In keeping with this, the reader must remember that the unilateral 6-OHDA rat model has been and continues to be one of the most popular experimental models of PD when it comes to the preclinical testing of new symptomatic therapies, neuroprotective strategies (e.g., trophic factor delivery), and transplantation approaches.[20,44,48–55] Also important to remember is the instrumental role played by the unilateral 6-OHDA rat model in the identification of key neurotransmitter pathways governing the functional neuroanatomy of the basal ganglia.[56–60] Yet, for studying the fine molecular basis of dopaminergic neuronal death, the stereotaxic injection of 6-OHDA, especially in the substantia nigra, may be problematic as the insult, and the molecular mechanisms of cytotoxicity recruited by it may differ among cells depending if they are located near or far from the site of injection. However, despite this caveat, many data show that 6-OHDA neurotoxicity provokes molecular alterations comparable to those seen in PD,[61] thus supporting the meaningfulness of this model to explore the mechanisms of neurodegeneration in PD.

THE HUMAN PARKINSONIAN NEUROTOXIN, 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE

In the early 1980s, several drug users from Northern California developed an acute state of akinesia (initially confused with catatonia) following the intravenous injection of a street preparation of 1-methyl-4-phenyl-4-propionpiperidine (MPPP), an analog of the narcotic meperidine.[62] After fine detective work, it was found that MPTP, which was inadvertently produced during the illicit synthesis of MPPP, was the culprit behind this dramatic clinical picture.[62] The chemical structures of MPPP and MPTP are shown in Figure 2.

Since this discovery, the mitochondrial electron transport chain complex I inhibitor MPTP has been used in a

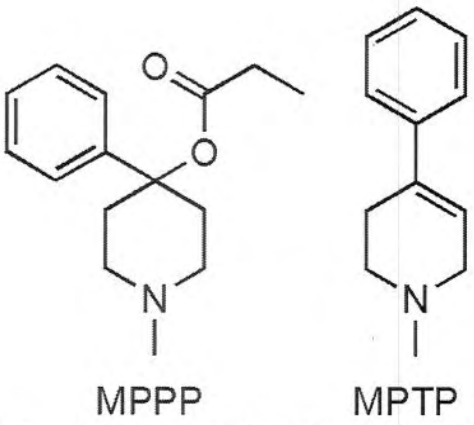

FIG. 2. Comparison of chemical structures of MPPP and MPTP.

variety of mammalian species to model PD ranging from nonhuman primates to invertebrates such as worms.[63,64] During the past years, several reviews dedicated to the MPTP model have been published, of which most focused on the effects of MPTP in small vertebrate animals such as mice.[65,66] It is to note that dopaminergic neurons in rats are relatively resistant to MPTP-induced neurotoxicity for reasons remaining unclear. As the reader will see in these previous reviews, an enormous body of work regarding the elucidation of the mechanisms of dopaminergic neuron death and the development of experimental neuroprotective therapies has been achieved thanks to the use of the MPTP mouse model of PD. In that sense, a large variety of MPTP administration procedures have been developed; for more details, interested readers can refer to Przedborski et al.[65] Yet, rarely and often briefly is the question of MPTP in humans and monkeys discussed in these papers. Thus, this review will rather summarize this aspect of the MPTP story, which after all represents a striking competitive advantage over any other model of PD that either lacks or seldom utilizes primates.

It is now well established that MPTP produces, in both humans and monkeys, an irreversible and severe parkinsonian syndrome, characterized by all of the cardinal features of PD, including tremor, rigidity, slowness of movement, postural instability, and even freezing. Yet, in nonhuman primates, the typical 4-Hz resting tremor of PD has only been demonstrated convincingly in the African green monkey[67]; other species of monkeys rather exhibit a postural/action tremor. Cognitive impairments evidenced by poor performances on constructive, verbal fluency and executive function tests were demonstrated in MPTP patients.[68,69] Apparently, not in humans[68,69] but in monkeys intoxicated with MPTP,[70] deficits in maintenance of a response set and difficulties in shifting attentional sets were also found. Impaired ability to sustain spatial attention or to focus attention, deficit in motor readiness and planning, and impaired time estimation were observed in these animals.[70] Collectively, these cognitive alterations are consistent with attention and executive functional deficits following MPTP intoxication, which is very similar to some of the cognitive alterations seen in PD patients. As emphasized by Stern,[68] it is also remarkable to note that none of the symptomatic individuals who were intoxicated with MPTP showed neurological signs others than those expected for PD; a similar statement may be true for MPTP-injected monkeys.

On a therapeutic point of view, both humans and monkeys intoxicated with MPTP respond very well to anti-PD treatments such as L-DOPA/carbidopa. However, as in PD patients,[71] long-term treatment with L-DOPA leads to hyperkinetic motor complications called dyskinesia, which can be as disabling as the parkinsonian symptoms themselves. For instance, among the seven indexed MPTP human cases, five developed dyskinesia within the first year of treatment with L-DOPA/carbidopa.[72] As of yet, the occurrence of L-DOPA-induced motor complications remains a major impediment to the proper management of PD patients. Here, the MPTP monkey model has emerged as an invaluable tool to investigate the molecular basis of these drug-induced abnormal movements and to test therapeutic strategies to control them.[73] This fact is elegantly illustrated in the recent study by Bézard and collaborators[74] in which the administration of a D3-dopamine partial agonist was shown to markedly improve L-DOPA-induced dyskinesia in MPTP monkeys, without exacerbating the parkinsonian symptoms.

Neuropathological data in both humans and monkeys[75,76] indicate that MPTP causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD.[77] Moreover, like PD, MPTP causes a greater loss of dopaminergic neurons in the substantia nigra than in the ventral tegmental area[78,79] and, in monkeys intoxicated with low doses of MPTP (but not in humans), a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus.[80,81] However, as for 6-OHDA, LBs have thus far not been convincingly observed in MPTP-induced parkinsonism[76]; however, in older MPTP-injected monkeys, intraneuronal proteinaceous inclusions reminiscent of LBs have been described.[82] At this point, it is still unknown whether the lack of definite LB formation in the MPTP model is due to the actual molecular mechanism by which MPTP kills dopaminergic neurons or rather the rate by which this neurotoxin destroys dopaminergic neurons. Indeed, all regimens of MPTP intoxication, even if achieved through several low dose injections, provoke in reality a unique or recurrent acute insult.

One other unsettled issue surrounding the neurotoxicity of MPTP is whether an acute exposure to this parkinsonian neurotoxin causes a progressive neurodegen-

eration. On the one hand, Burns and collaborators[83] reported the case of a young chemist who developed parkinsonism after substantial laboratory exposure to MPTP and who failed to show any evidence of worsening of his neurological condition over several years. On the other hand, positron emission tomography performed twice, 7 years apart, on 10 individuals exposed to MPTP, revealed worsening of striatal [^{18}F]fluorodopa uptake in these patients.[84] Moreover, postmortem studies in three individuals who survived 3–16 years after exposure to MPTP[85] and in six monkeys who survived 5–14 years after exposure to MPTP[86] showed evidence of extracellular neuromelanin accumulation and activated microglia in the substantia nigra, two neuropathological features consistent with an ongoing degenerative process. As previously speculated,[66] these findings suggest that a single acute MPTP insult can set in motion a self-sustained cascade of cellular and molecular events with long-lasting deleterious effects.

Although a large body of work with MPTP has been accomplished in monkeys, nonhuman primates have not generally been used to study the molecular mechanisms of dopaminergic neurodegeneration; instead, the MPTP administration to mice (essentially by systemic injections), and to a lesser extend to rats (essentially by intracerebral injection) are typically used for such studies.[2,66] Conversely, the monkey MPTP model remains the gold standard for the assessment of novel strategies and agents for the treatment of PD symptoms. For example, electrophysiological studies in MPTP monkeys have led to the demonstration that hyperactivity of the subthalamic nucleus is a key factor in the development of bradykinesia and rigidity.[87] This finding prompted investigators to consider targeting the subthalamic nucleus by using high-frequency electric stimulation in an attempt to ameliorate the motor function of PD patients with intractable symptoms.[88] MPTP-injected monkeys[89,90] were also used to demonstrate that the delivery of glial-derived neurotrophic factor (GDNF) can limit nigrostriatal dopaminergic neurodegeneration and promote behavioral recovery when given before lesioning animals,[90] both of which are important advances in the treatment and understanding of PD over the last decade.

ROTENONE: FROM PESTICIDE TO MODELING PD

Among the toxic animal models of PD, rotenone represents one of the most recently used approaches.[91] Rotenone is the most potent member of the rotenoids, a family of natural cytotoxic compounds extracted from various parts of *Leguminosa* plants. Rotenone's chemical structure is presented in Figure 3.

Rotenone is widely used around the world as insecticide and piscicide.[92] In the United States, treated areas

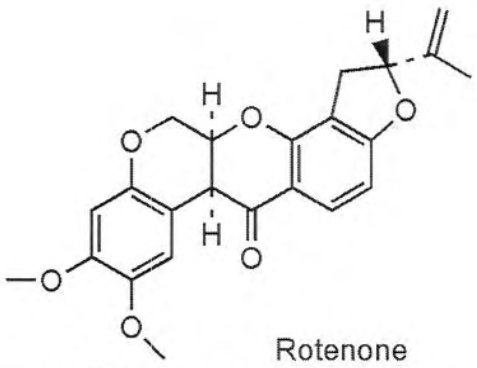

FIG. 3. Chemical structure of rotenone.

are restricted, and treatment dates are selected outside the periods of crop irrigation and swimming.[92] Rotenone readily breaks down by exposure to sunlight. Nearly all of the toxicity of the compound is lost in 5–6 days of spring sunlight or 2–3 days of summer sunlight. Rotenone is also rapidly broken down in soil and in water. The half-life in both of these environments is between 1 and 3 days.[92] Because of its short half-life and because it does not readily leach from soil, it is not expected to be a groundwater pollutant. Consequently, the likelihood of PD being caused by an environmental exposure to rotenone is low, to not say null. Conversely, it must be remembered that many environmental neurotoxins other than rotenone have a long half-life. Thus, if rotenone appears an unlikely culprit, several others could account for the epidemiological data showing that the risk of PD increases with exposure to pesticides.[93,94] The most common way that rotenone exposure to humans would take place is through ingestion. However, absorption in the stomach and intestines is slow and incomplete, and the liver breaks down the compound effectively. These facts make it unlikely that meaningful amounts of rotenone could enter the general circulation, unless enormous quantities are ingested. Consistent with this view is the fact that chronic ingestion of rotenone to rats for 24 months at doses 30 times greater that used to model PD by systemic infusion[91] failed to cause any behavioral or neuropathological features of the disease.[95] Nevertheless, there is one case of fatal rotenone poisoning after its acute ingestion.[96] At autopsy, rotenone was found in the blood, liver, and kidney, but not in brain.

Like MPTP, rotenone is highly lipophilic and thus readily gains access to all organs including the brain. After a single intravenous injection, rotenone reaches maximal concentration in the CNS within 15 min and decays to about half of this level in less than 2 h.[97] Its brain distribution is heterogeneous,[97] paralleling regional differences in oxidative metabolism.[97] Rotenone also freely crosses all cellular membranes and can accumulate in subcellular organelles such as mitochondria. In mitochondria, rotenone impairs oxidative phosphorylation by

inhibiting reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase activity through its binding to the PSST subunit of the multipolypeptide enzyme complex I of the electron transport chain.[98] Aside from its action on mitochondrial respiration, rotenone also inhibits the formation of microtubules from tubulin.[99,100] This effect may be quite relevant to the mechanism of dopaminergic neurodegeneration because excess of tubulin monomers may be toxic to cells.[101,102] Interestingly, a protein implicated in some familial forms of PD, parkin, appears to bind to tubulin, thereby enhancing the ubiquitination and degradation of misfolded tubulins, an effect that is lacking with the PD-linked parkin mutants.[103]

Rotenone has been used extensively as a prototypic mitochondrial poison in cell cultures, but less frequently in living animals. Exposure of embryonic ventral midbrain cultures to rotenone causes major neurotoxicity,[104] especially in the presence of microglial cells.[105] In these two studies, markers of dopaminergic neurons were more altered than those of γ-aminobutyric acid (GABA) neurons, suggesting greater susceptibility of dopaminergic neurons to such an insult. In animals, rotenone has been administered by different routes. As stated above, oral delivery of rotenone appears to cause little neurotoxicity in animals.[95] Systemic administration, on the other hand, often causes toxicity and lethality, the degree of which is related to the dose used. Stereotaxic injection of rotenone into the median forebrain bundle depletes striatal dopamine and serotonin.[106] Rats treated for a week with 10-18 mg/kg · day of rotenone by intravenous infusion show bilateral lesions of the striatum and the globus pallidus, characterized by neuronal loss and gliosis.[107] In that study, the nigrostriatal dopaminergic pathway remained unaffected.[107] Similarly, subcutaneous injection of either 15 mg/kg of rotenone once or 1.5 mg/kg multiple times, although causing fatality, failed to affect striatal dopaminergic contents in mice.[108] Conversely, Greenamyre and collaborators[91,109] have found that intravenous and subcutaneous infusion of 2-3 mg/kg · day of rotenone for about 3 weeks to rats does produce nigrostriatal dopaminergic neurodegeneration. By quantitative analysis, it appears that substantia nigra dopaminergic neuron numbers are reduced by about 30% in rotenone-infused rats compared with vehicle controls.[110] This study also shows that the numbers of mesolimbic dopaminergic neurons, the cell bodies of which reside adjacent to the substantia nigra in the ventral tegmental area (VTA), are unchanged by rotenone administration.[110] In the striatum, the average loss of dopaminergic fibers is estimated to be 55% after rotenone infusion in rats,[110] that, like in PD, is greater than the loss of substantia nigra dopaminergic neurons. Despite the use of the exact same regimen of rotenone, the severity of the striatal dopaminergic damage in rats within a given experiment appears highly variable, ranging from none to near complete.[91,109–112] After the infusion of rotenone, the loss of tyrosine hydroxylase-positive fibers in the striatum is either focal, showing a zone of maximal loss at the center, or diffuse[91,109–112]; whether the latter represents a more severe lesion of the striatal dopaminergic fiber network than the former remains to be demonstrated. Of note, the focal loss in the center of the striatum seen in some of the lesioned rats is a pattern that differs from that of PD in which the dorsolateral quadrant of the striatum is typically the most affected.

In contrast to the 6-OHDA and MPTP models, in rotenone-infused rats, some of the remaining substantia nigra dopaminergic neurons contain proteinaceous inclusions.[91,109,110] Like LBs in PD, these inclusions are immunoreactive for both ubiquitin and α-synuclein,[91] and by electron microscopy they appear composed of a dense core with fibrillar peripheral elements.[91] Likewise in PD in which neurodegeneration extends beyond the dopaminergic system,[77] rotenone infusion is associated with 35% reduction in serotonin transporter density in the striatum, 26% reduction of noradrenergic neurons in the locus coeruleus, and 29% reduction in cholinergic neurons in the pedunculopontine nucleus.[110]

Although the initial descriptive studies did not report any striatal lesion,[91] the number of dopamine-regulated phosphoprotein-32 projecting neurons, cholinergic interneurons and reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase-positive neurons in the striatum were all found significantly reduced by the infusion of rotenone in rats.[110,111] Unexpectedly, even at doses of rotenone that did not damage the nigrostriatal dopaminergic pathway in rats, Höglinger and collaborators[110] still found significant loss of intrinsic striatal neurons. Remarkably, Zhu and collaborators[112] found that the rotenone-induced intrinsic striatal neuronal loss occurs especially in those rats exhibiting the central striatal loss of tyrosine hydroxylase immunoreactivity mentioned above. These results indicate that rotenone exerts a much more widespread neurotoxicity than initially thought and, contrary to the initial contention, it does not consistently spare striatal postsynaptic dopaminergic neurons. Nor do the nigrostriatal dopaminergic neurons appear preferentially sensitive to rotenone intoxication.

Behaviorally, rotenone-infused rats exhibit reduced mobility, flexed posture, and in some cases rigidity[109] and even catalepsy.[113] Four weeks after the infusion of rotenone, rats show more than 70% reduction in spontaneous motor activity.[110] Although this required independent confirmation, these motor abnormalities appear to be reversed by L-DOPA administration.[114] However, some rotenone-infused rats without nigrostriatal dopaminergic lesion have been reported to exhibit a similar set of motor abnormalities.[109] In addition, indices of dopaminergic damage across different doses of rotenone did

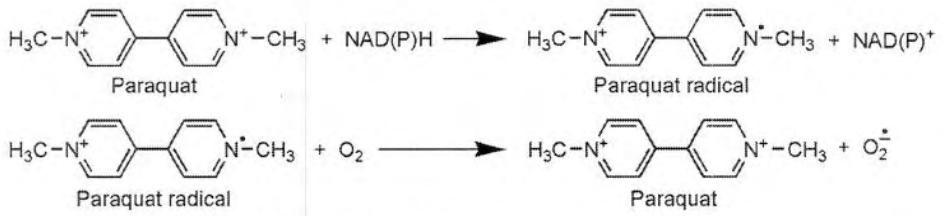

FIG. 4. Reduction-oxidation cycling reaction of paraquat.

not correlate with motor behavior in individual rats.[115] Therefore, whereas the rotenone-related motor abnormalities are dramatic, it is still questionable that they result from a loss of nigrostriatal dopaminergic neurons and thus the use of these behavioral alterations as an experimental correlate of PD symptoms must be done with caution.

Based on this review, it may be concluded that the chronic administration of rotenone has still a long way to go to become a routine PD model, due to its inconsistent and unpredictable effect on the nigrostriatal pathway. Unless these problems are resolved, it is unlikely that preclinical neuroprotection studies could be carried out successfully in such a model.

THE NEUROTOXIC HERBICIDE PARAQUAT

The potent herbicide paraquat (*N,N'*-dimethyl-4-4'-bipiridinium) is another prototypic toxin known to exert deleterious effects through oxidative stress. Indeed, as reviewed elsewhere,[6] paraquat toxicity is mediated by redox cycling with cellular diaphorase such as nitric oxide synthase,[116] yielding ROS. As detailed,[6] the actual reduction-oxidation cycling reaction of paraquat can thus be depicted in Figure 4. Thus far, there have been several cases of lethal poisoning resulting from ingestion or dermal exposure.[117] For many years, experimental studies using paraquat were focusing on its effects on lung, liver, and kidney probably because the toxicity induced by this herbicide in these organs is responsible for death after acute exposure. However, significant damage to the brain is seen in individuals who died from paraquat intoxication[118,119] despite the fact that paraquat poorly crosses the BBB spontaneously.[120] Furthermore, epidemiological studies have suggested an increased risk for PD due to paraquat exposure,[121] raising the possibility that paraquat could be an environmental parkinsonian toxin. In keeping with this, it is relevant to point out that paraquat exhibits a striking structural similarity to MPTP toxic metabolite 1-methyl-4-pheylpyridinium (MPP^+) (FIG. 5).

Upon its systemic injection to mice, confusing results have been reported. Some investigators have published reduced motor activity and dose-dependent losses of striatal dopaminergic nerve fibers and substantia nigra neuronal cell bodies in paraquat-treated mice.[122] It seems quite clear that, in this case, paraquat did enter the brain via the assistance of L-neutral amino acid transports, as pretreatment of animals with L-valine or L-phenylalanine completely prevented neurodegeneration.[123] Other investigators, however, have initially failed to see any behavioral abnormality or nigrostriatal dopaminergic pathway damage in similarly treated mice,[124,125] but then[125] they also found selective nigral dopaminergic cell loss in mice injected with paraquat.[126] Aside from this bewildering aspect, it must be stressed that levels of α-synuclein were reported as elevated in both the frontal cortex and ventral midbrain as well as α-synuclein-positive inclusions in substantia nigra neurons of mice treated with paraquat.[127] As far as the observed α-synuclein upregulation is concerned, it may not be a noxious mediator of paraquat-induced neurotoxicity because dopaminergic neurons from transgenic mice expressing high levels of either wild-type or mutant α-synuclein appear more resistant to paraquat than those from their nontransgenic counterparts.[128] Still, we cannot rule out that compensatory mechanisms may have occurred in these constitutive transgenic animals, hence confounding the interpretation of the role that high levels of α-synuclein may have on dopaminergic neurons. This cautionary note is particularly relevant in light of the fact that increased expression of α-synuclein is noxious in its own right to dopaminergic neurons in humans.[129] The association of dopaminergic neuron death with α-synuclein up-regulation and aggregation suggests that the paraquat model could be quite valuable for reproduc-

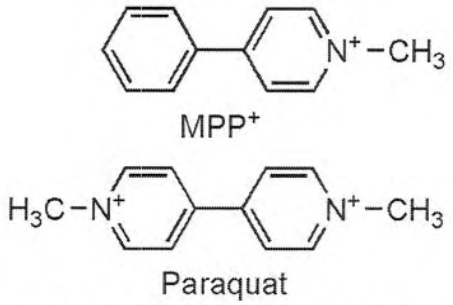

FIG. 5. Comparison of chemical structures of MPP^+ and paraquat. Used with permission from Przedborski and Ischiropoulos. Reactive oxygen and nitrogen species: weapons of neuronal destruction in models of Parkinson's disease. *Antioxid Redox Signal* 7:685–693. Copyright © 2005, Mary Ann Liebert, Inc. All rights reserved.[6]

ing a PD-like pathology (e.g., nigral cell loss and synuclein pathology). Although ROS are incontestably involved in the deleterious mechanism by which paraquat kills dopaminergic neurons, the molecular link between oxidative stress and cell death in this model remains unknown. It appears, however, that paraquat can trigger the sequential activation of c-Jun N-terminal kinase (JNK), c-Jun, and caspase-3 both *in vitro* and *in vivo*,[130] suggesting that JNK signaling pathways could mediate paraquat-induced neurodegeneration.

It is worth mentioning that the fungicide manganese ethylenebisdithiocarbamate, or Maneb, which is used in overlapping geographical areas with paraquat, has been shown to decrease locomotor activity and potentiate paraquat effects on the nigrostriatal pathway in mice.[124,125] In these studies, combined paraquat and Maneb exposures produce greater effects on the dopaminergic system than either of the chemicals alone.[124,125] However, in none of these studies did the authors assess phenotypic markers for neuronal populations other than the dopaminergic neurons. Thus, whether GABAergic neurons, cholinergic neurons, or even astrocytes are also affected after paraquat injection, which is likely, is unknown. Therefore, at this point it remains unclear whether the observed paraquat/Maneb cytotoxicity is really specific to the dopaminergic systems and could thus be regarded as a reliable experimental model of PD. Interestingly, Maneb has also been used in several studies to ascertain whether a pre- or postnatal insult by an environmental toxicant to the developing brain could give rise to an adult-onset neurodegenerative process involving the nigrostriatal dopaminergic pathway.[131,132]

CONCLUSION

This review summarized the salient aspects characterizing the four most popular toxic models of PD. Although all four neurotoxins reviewed here are thought to kill dopaminergic neurons, they all produce specific clinical or neuropathological abnormalities that make them different from each other. As we have stressed herein, each model has advantages and shortcomings, and none should be regarded as suitable to represent all aspects or to address all questions that pertain to PD. Thus, the take home message is that none of the presented models is perfect, and the selection of one over the other must be governed solely by the question and the type of investigations to be undertaken. In other words, we must always ask ourselves before embarking on a study using a model of PD: which model may be best suited to address the question to be investigated?

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Fractalkine: moving from chemotaxis to neuroprotection

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Microglia are thought to contribute to neurodegeneration. Now ablating the receptor for the chemokine fractalkine is shown to increase microglial inflammatory response and neuronal death *in vivo* in several models of CNS insult.

Chronic neurodegenerative disorders, including Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis (ALS, also called Lou Gehrig's disease), involve neuron death and inflammation in specific brain regions. Areas of degeneration are populated by resident inflammatory cells of the CNS, such as microglia and astrocytes, and, to a much lesser extent, by infiltrating T-cells. Inflammation, which is traditionally thought to eliminate cellular debris, was considered until recently to be a trivial secondary response to neuronal death. However, *in vitro* evidence indicates that, upon activation, microglia themselves promote neurodegeneration by producing a barrage of cytotoxic molecules, including proteases, reactive oxygen species, nitric oxide, prostaglandins and cytokines[1].

Microglia in the brain can behave differently from microglia in a dish, and it has remained unclear how microglia could promote neurotoxicity in *vivo*. In this issue, Cardona and collaborators[2] take a major step in this direction by making a compelling case that chemokines are critical for regulating the function of microglia and for mediating microglia-dependent neurotoxicity *in vivo*. The authors use three different *in vivo* models of CNS insult to show that without the receptor for a chemokine called fractalkine, excessive microglial activation occurs in response to both inflammatory and neurotoxic stimuli. The authors also show that ablation of the fractalkine receptor exacerbates neuronal loss in mouse models of Parkinson disease and ALS.

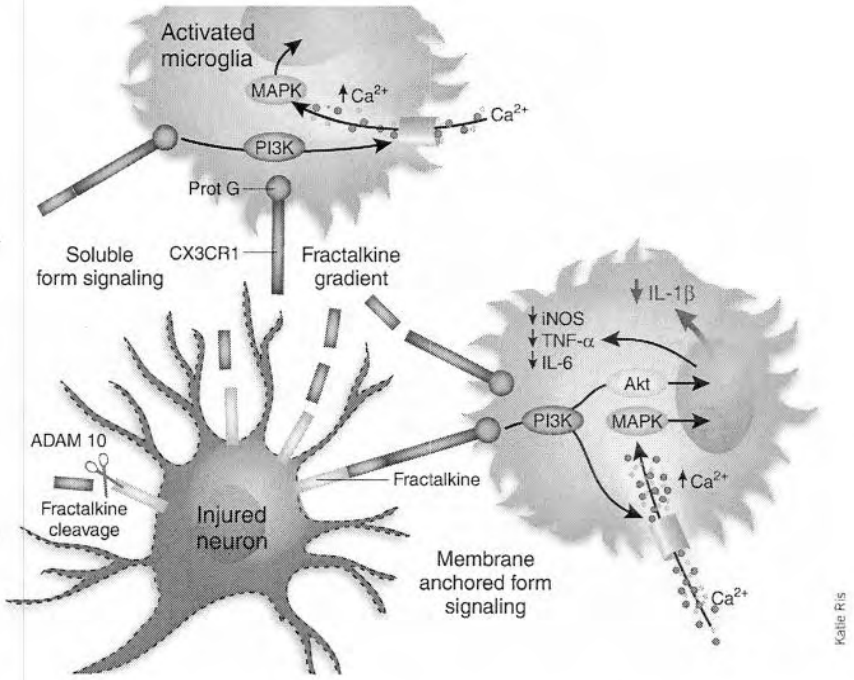

Figure 1 Fractalkine is primarily expressed by neurons, whereas its G protein–coupled receptor (CX_3CR1) is mainly expressed by microglia. Fractalkine is either membrane bound or soluble; the latter results from the cleavage of membrane-bound fractalkine by the metalloproteinase ADAM10. CX_3CR1 ligation triggers phosphatidylinositol-3 kinase (PI3K)-dependent Ca^{2+} influx, which is greater with membrane-bound than soluble fractalkine. Downstream to PI3K, CX_3CR1 signaling activates the mitogen-activated protein kinase (MAPK, important for chemotaxis) and Akt pathways (important for cell survival). Both fractalkine forms mediate chemotaxis, whereas the membrane-bound form also captures microglia. Fractalkine acts as an anti-inflammatory molecule *in vitro* by attenuating the secretion of interleukin-6 and tumor necrosis factor-α (TNF-α) and the upregulation of inducible nitric oxide synthase (iNOS) in LPS-activated microglia. The new data from Cardona *et al.* indicate that fractalkine controls the degree of microglia activation and ensuing neurotoxicity by attenuating the production of interleukin-1β (IL-1β).

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Chemokines (short for 'chemotactic cytokines') induce chemotaxis of neighboring cells. They are produced by a wide variety of cell types. So far, we know of ~50 distinct human chemokines that, despite a relatively low sequence homology, share common tertiary structures. They also have conserved cysteine residues near the N terminus whose relative positions define four chemokine subfamilies. The biological effects of all chemokines are mediated by specific receptors that belong to the large family of

G protein–coupled receptors with seven transmembrane regions. Of the chemokines, fractalkine has several singular features that make it particularly appealing in the context of neuroinflammation and neuroprotection.

Fractalkine is one of only two chemokines that are constitutively expressed in neurons throughout the CNS (ref. 3). Its receptor CX_3CR1 is also highly expressed in the CNS, primarily by microglia[3] but also by cultured astrocytes[4] and neurons[5]. Fractalkine is also unique in that its chemokine domain is tethered to the cell surface by a long, extensively glycosylated stem (a mucin-like stalk) attached to a transmembrane and intracellular domain[6]. It can be secreted when cleaved from the mucin-like stalk by metalloproteinases such as ADAM10 (ref. 7; **Fig. 1**). Although both the soluble and membrane-bound forms mediate chemotaxis and stimulate an influx of calcium into the cell, the membrane-anchored form of fractalkine also captures CX_3CR1-bearing cells and induces a greater calcium influx than the soluble fractalkine[3]; whether these two forms of calcium influx trigger distinct transduction events is unknown.

In vitro, fractalkine seems to be important for neuroprotection as well. Primary microglial cultures from newborn mice, when activated by lipopolysaccharide (LPS), produce proinflammatory molecules, such as interleukin-6 and tumor necrosis factor-α (TNF-α), and show increased inducible nitric oxide synthase (iNOS) activity[8]. Adding recombinant fractalkine protein before LPS treatment, however, reduces all these effects. In neuron-microglia cocultures, fractalkine reduces the neuronal death induced by LPS-activated microglia[8] and blocks apoptosis induced by the HIV-1 envelope protein gp120 in hippocampal neurons[9]. In the new paper, Cardona and collaborators examined the outstanding question of whether fractalkine has similar neuroprotective properties *in vivo*.

The authors first showed that CX_3CR1 is expressed exclusively by microglia *in vivo*, at least in mice. They used knock-in mice in which the reporter gene encoding GFP was inserted into the CX_3CR1 locus[10]. In these $CX_3CR1^{+/GFP}$ mice, fluorescently labeled CX_3CR1-expressing cells were immunopositive for markers of microglia, but not of astrocytes or neurons. Together with the previous demonstration that fractalkine is present on neurons[3], the finding that CX_3CR1 expression *in vivo* is restricted to microglia positions fractalkine well to act as a molecular messenger in neuron-microglia communication.

The authors then assessed the role of fractalkine in three different *in vivo* models of CNS insult. First, they injected (intraperitoneally) the gram-negative bacterial endotoxin LPS into mice, which is a popular model for generic inflammatory response in the CNS but of uncertain relevance to any specific neurological disease. Despite this caveat, the comparison of microglial behavior and effects following LPS injection in $CX_3CR1^{+/GFP}$ and $CX_3CR1^{GFP/GFP}$ mice generated fascinating data. In contrast to heterozygote $CX_3CR1^{+/GFP}$ mice (which retain receptor function and whose microglia behave like wild-type microglia[10]), homozygote $CX_3CR1^{GFP/GFP}$ mice (which lack CX_3CR1 expression) showed more intense and widespread microglial activation after LPS injection, along with greater neuronal apoptosis in frontal cortex and hippocampus. These data suggest that the fractalkine receptor is essential in restraining the CNS inflammatory response and the ensuing parenchymal damage in response to pathological stimuli such as LPS.

Like microglia, blood-borne monocytes and natural killer lymphocytes respond to fractalkine. Does the enhanced neurotoxicity in response to LPS in $CX_3CR1^{GFP/GFP}$ mice involve peripheral cells that could have invaded the CNS? To answer this, the authors transferred purified, GFP-positive microglia from LPS- and vehicle-injected $CX_3CR1^{GFP/GFP}$ and $CX_3CR1^{+/GFP}$ mice into the frontal cortex of wild-type recipient mice. By 36 hours later, tissue sections near the site of injection revealed striking genotypic and treatment differences. LPS-treated $CX_3CR1^{GFP/GFP}$ microglia remained at the injection site and were surrounded by many apoptotic neurons. In contrast, both LPS-treated $CX_3CR1^{+/GFP}$ microglia and vehicle-treated microglia of either genotype migrated away from the injection site and were not associated with any increase in neuronal apoptosis. These findings confirm that activated CX_3CR1-deficient microglia are neurotoxic in the CNS of wild-type mice, but do not rule out the possibility that other cell types may contribute to this adverse effect. Because only LPS-treated $CX_3CR1^{GFP/GFP}$ microglia accumulated at the site of injury, the data also suggest that the fractalkine receptor in activated microglia may regulate microglia-directed migration, local retention or both.

Suspecting that a soluble factor secreted by activated $CX_3CR1^{GFP/GFP}$ microglia may account for this enhanced neurotoxicity, the authors then used an RNAse protection assay to compare the inflammatory cytokine transcripts produced by these cells with those of activated $CX_3CR1^{+/GFP}$ microglia. They found markedly different expression of the proinflammatory molecule interleukin-1β (IL-1β) by the two genotypes, but, in contrast to a previous report[8], not of TNF-α or interleukin-6 (**Fig. 1**). They then demonstrated that IL-1β mediates neurotoxicity induced by activated CX_3CR1-deficient microglia. If an IL-1 receptor antagonist (IL-1ra) was included in the adoptive transfer along with activated $CX_3CR1^{GFP/GFP}$ microglia, this reversed the homing phenotype, allowing the CX_3CR1-deficient microglia to migrate throughout the CNS of wild-type recipients. Including IL-1ra also decreased the number of apoptotic neurons. Moreover, adoptive transfer of activated $CX_3CR1^{GFP/GFP}$ microglia into mice lacking the IL-1 receptor did not cause neurodegeneration.

Microglial neurotoxicity is thought to increase the severity of several neurodegenerative disorders, including Parkinson disease and ALS. Does CX_3CR1 mediate the microglial toxicity in these disorders? The authors found that after systemic administration of the parkinsonian toxin MPTP to mice[11], the severity of dopaminergic neuronal loss and microglial activation was far more profound in animals lacking the fractalkine receptor than in $CX_3CR1^{+/GFP}$ littermates. A similar result was found when MPTP was given to mice deficient in fractalkine itself. The authors also crossed mutant mice deficient in CX_3CR1 with transgenic $SOD1^{G93A}$ mice[12], the most extensively used mouse model of ALS. Compared to their transgenic $SOD1^{G93A}/CX_3CR1^{+/GFP}$ littermates, transgenic $SOD1^{G93A}/CX_3CR1^{GFP/GFP}$ mice had more severe loss of spinal motor neurons, more rapid decline in grip strength, earlier loss of weight and shorter life span.

Although these findings indicate that the lack of the fractalkine receptor exacerbates neurotoxicity in these models of Parkinson disease and ALS, they provide no information about whether the mechanism potentiating neuronal death after LPS injection applies to these diseases as well. Indeed, considering the horde of inflammatory factors produced by activated microglia, it would be surprising if IL-1β alone orchestrates the observed inflammation-related worsening of the Parkinson disease and ALS phenotype, as after MPTP injection other microglial mediators, including iNOS (ref. 13) and NADPH-oxidase[14], also contribute to neurodegeneration. Although future work will be required to determine whether removal of the fractalkine receptor potentiates LPS-, MPTP- and mutant SOD1-related neurotoxicity via identical mechanisms, the results are nevertheless consistent about one key point—fractalkine is important in the demise of several different groups of neurons provoked by different causes. An important next step is to determine fractalkine levels in cerebrospinal fluid or tissue samples from Parkinson disease and ALS patients.

This study raises the exciting possibility that mimicking the effects of fractalkine may control microglia activation and provide neuroprotection in a variety of neurological diseases featuring neuroinflammation. Some success has already been achieved in preclinical models of neurodegenerative diseases by targeting neuroinflammation through the inhibition of microglial activation with agents like minocycline, dextromethorphan or vasoactive intestinal peptide, or by suppression of specific microglial toxic effectors using iNOS antagonists or nonsteroidal anti-inflammatory drugs[1]. Among these, several (for example, minocycline) are currently being tested in clinical trials for Parkinson disease and ALS. We may envision that fractalkine agonists that can permeate the blood-brain barrier, should they become available, would be prime candidates in neuroprotective clinical trials for these incurable neurodegenerative diseases. However, stimulation of the fractalkine pathway may be a double-edged sword if not finely tuned, as it may also aggravate atherosclerosis[15]. Thus, fractalkine-based human clinical trials will need to begin by determining how these drugs may affect susceptibility to cardio- and cerebrovascular accident.

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When is enough enough?

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How does the decision-making process stop? Lo and Wang propose that a large-scale interconnected network encompassing parietal cortex, basal ganglia and motor structures controls the balance between speed and accuracy.

A hallmark of higher brain function is the ability to contemplate the world rather than to respond reflexively to it. Indeed, the roots of cognition rest in the brain's ability to process information in a time frame that is not governed by immediate changes in the environment or the need to move. Of course, with freedom comes responsibility. Detachment from sensory and motor time implies that the brain must determine when its own contemplative processes should start and stop. As we gather information and weigh possible options in everyday decisions, eventually we must commit one way or another, or move on. It may be prudent to take time to reach a carefully reasoned position, but if time is at a premium, the benefits of speed may trump accuracy or at least permit commitment even when uncertainty remains.

Over the past decade, the neurobiology of decision making has begun to yield insights into how the brain integrates information and expected value to make simple choices. However, an important piece of the puzzle has been missing: how does the process stop? What controls the balance between speed and accuracy? In this issue, Lo and Wang[1] suggest that the basal ganglia may hold the key.

Decisions are proposed to be based on the accumulation of evidence to a subjective criterion level or 'threshold'[2]. When the collected evidence in favor of one choice reaches this threshold, the brain makes a commitment; decision making is terminated. A high threshold means more evidence is collected before a decision is made, thereby reducing the probability of mistakes but increasing the response time. This framework explains the speed-accuracy tradeoff and suggests that the brain might adjust this threshold to optimize its rate of reward[3]. Although there is considerable behavioral and electrophysiological support for the accumulation of evidence in some tasks[4], a neural substrate for the adjustable threshold remains elusive. Lo and Wang propose a biophysically plausible candidate: detection of threshold crossing and tuning of the threshold take place in a large-scale interconnected network encompassing parietal cortex, basal ganglia and motor structures. Their multimodule computational model simulates all steps of decision formation in a reaction-time task.

The model was developed to address a simple kind of perceptual decision (**Fig. 1a**)[4]. In one version of this task, subjects (humans and monkeys) indicate their judgment of the direction of a random dot motion stimulus by making an eye movement (saccade) as soon as they make a decision. On easy trials, there is overwhelming evidence for one direction, leading to accurate, fast decisions. On difficult trials, when the motion direction is ambiguous, the evidence trickles in over time and accrues slowly and inconsistently. The choices are less accurate and slower[4,5]. Some of the key neurons responsible for motion processing and evidence accumulation have been identified using neural recording and stimulation in the monkey (see ref. 6 for citations).

The model of Lo and Wang begins by mimicking the responses of neurons in the lateral intraparietal area (LIP), which represent the accumulating evidence for one of the direction choices[6]. During decision making, LIP neurons respond with ramp-like changes in their firing rates. The activity rises when the monkey chooses the target in the cell's response field (**Fig. 1b**), and it declines for the opposite choice. Furthermore, the slope of this ramping activity is steeper for stronger motion, consistent with the accumulation of sensory information about direction of motion. Finally, for all strengths of motion, the buildup in the neuron's activity reaches a fixed level just before the saccade, in accordance with a threshold-crossing termination process[7] (**Fig. 1c**). Lo and Wang implement this cortical module using a neural network with fast and slow reverberatory dynamics (mediated by AMPA and NMDA conductances). Wang showed previously that this implementation

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Neurobiology of Disease

Ablation of the Inflammatory Enzyme Myeloperoxidase Mitigates Features of Parkinson's Disease in Mice

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Parkinson's disease (PD) is characterized by a loss of ventral midbrain dopaminergic neurons, which can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Inflammatory oxidants have emerged as key contributors to PD- and MPTP-related neurodegeneration. Here, we show that myeloperoxidase (MPO), a key oxidant-producing enzyme during inflammation, is upregulated in the ventral midbrain of human PD and MPTP mice. We also show that ventral midbrain dopaminergic neurons of mutant mice deficient in MPO are more resistant to MPTP-induced cytotoxicity than their wild-type littermates. Supporting the oxidative damaging role of MPO in this PD model are the demonstrations that MPO-specific biomarkers 3-chlorotyrosine and hypochlorous acid-modified proteins increase in the brains of MPTP-injected mice. This study demonstrates that MPO participates in the MPTP neurotoxic process and suggests that inhibitors of MPO may provide a protective benefit in PD.

Key words: MPTP; Parkinson's disease; oxidative stress; inflammation; neuroprotection; nitrotyrosine

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (Dauer and Przedborski, 2003). Its main neuropathological feature is the loss of the nigrostriatal dopaminergic neurons, the cell bodies of which reside in the substantia nigra pars compacta (SNpc) and nerve terminals of which extend to the striatum (Dauer and Przedborski, 2003). Except for a handful of inherited cases related to known gene defects (Vila and Przedborski, 2004), PD is a sporadic condition of unknown pathogenesis (Dauer and Przedborski, 2003). However, epidemiological studies suggest that inflammation increases the risk of developing PD (Chen et al., 2003), and experimental models of PD show that inflammatory oxidants modulate SNpc dopaminergic neuronal death (Liberatore et al., 1999; Gao et al., 2002; Wu et al., 2002, 2003). For instance, NADPH oxidase and inducible nitric oxide synthase (iNOS), which are major sources of inflammatory oxidants, are upregulated in damaged areas in both PD and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD (Hunot et al., 1996; Liberatore et al., 1999; Wu et al., 2003). Studies of mice deficient in NADPH oxidase or iNOS indicate that superoxide radical (O_2^-) and NO contribute to the MPTP-induced neurodegenerative process (Liberatore et al., 1999; Wu et al., 2003). However, both O_2^- and NO are relatively unreactive, and a variety of secondary oxidants, such as peroxynitrite ($ONOO^-$), are more likely to account for the injurious capacity of inflammation in PD. Supporting this view are the demonstrations that levels of 3-nitrotyrosine, a major product of $ONOO^-$ oxidation of proteins, are elevated in affected brain areas after MPTP injections to mice (Pennathur et al., 1999), for the most part in an iNOS-dependent manner (Liberatore et al., 1999).

Levels of *O,O'*-dityrosine also increase markedly in the SNpc of MPTP-intoxicated animals (Pennathur et al., 1999). This is an intriguing finding because *O,O'*-dityrosine is a relatively minor product of $ONOO^-$ (Pennathur et al., 1999). Conversely, myeloperoxidase (MPO), and not $ONOO^-$, seems to promote *O,O'*-dityrosine formation in this model of PD (Pennathur et al., 1999). Moreover, MPO can use the NO degradation product NO_2^- to generate reactive nitrogen species (RNS) (van der Vliet et al., 1997), and studies of mice deficient in MPO demonstrate that this enzyme is one of the major sources of 3-nitrotyrosine during acute inflammation (Gaut et al., 2002). Thus, these results raise the unanticipated possibility that MPO, a heme enzyme expressed in abundance in a variety of phagocytic cells (Hampton et al., 1998), would contribute to the MPTP-induced neurodegenerative process and would represent a previously unrecognized culprit in the inflammatory-mediated oxidative insult associated with diseases such as PD. Consistent with this hypothesis, we

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show here not only that MPO is detected in affected brain areas of MPTP-injected mice and PD patients, specifically in glial cells, but also that mutant mice deficient in MPO are more resistant to MPTP-induced dopaminergic neurotoxicity. These findings indicate that MPO does participate in the MPTP neurotoxic process and suggest that inhibitors of MPO may provide protective benefit in PD.

Materials and Methods

Animals and treatment. Procedures using laboratory animals were in accordance with the National Institutes of Health (NIH) guidelines for the use of live animals and approved by the Institutional Animal Care and Use Committee of Columbia University. The mice used in this study were 10-week-old male C57BL/6J mice (Charles River Laboratories, Wilmington, MA) and MPO-deficient mice that had been backcrossed >10 times into the C57BL/6J background (Brennan et al., 1985) and their wild-type (WT) littermates, all weighing 22–25 g. For MPTP intoxication, 4–10 mice per group received four intraperitoneal injections every 2 h of MPTP–HCl (18–20 mg/kg of free base; Sigma-Aldrich, St. Louis, MO) dissolved in saline. Mice were killed from 0–7 d after the last injection, and their brains were used for morphological and biochemical analyses. Control mice received saline only. MPTP handling and safety measures were in accordance with published guidelines (Przedborski et al., 2001).

RNA extraction and reverse transcription-PCR. Total RNA was extracted from selected brain regions and at selected time points after MPTP and used for reverse transcription-PCR analysis as described previously (Wu et al., 2003). The primers used for mouse MPO and β-actin were as follows: MPO, 5′-AGGATAGGACTGGATTGCCTG-3′ (forward) and 5′-GTGGTGATGCCAGTGTTGTCA-3′ (reverse); β-actin, 5′-CTTTGATGTCACGCACGATTTC-3′ (forward) and 5′-GGGCCGCTCTAGGCACCAA-3′ (reverse). The thermal cycling conditions of the PCR were 94°C for 3 min, followed by 23–35 cycles for 20 s at 94°C, 1 min at 60°C, 1 min at 72°C, and a final extension at 72°C for 5 min. After amplification, products were separated on a 5% PAGE and quantified by a FluorChem 8800 digital image system (Alpha Innotech, San Leandro, CA). PCR products were of expected sizes, and sequences were confirmed by direct cycle sequencing.

Immunoblots. Mouse brain protein extracts from selected regions were prepared and used for Western blot analysis as described previously (Wu et al., 2003). The primary antibodies used were as follows: a rabbit polyclonal antibody raised against a 14 aa peptide representing the C terminus of the mouse MPO (NTLPKLNLTSWKET; 1:1000 dilution; generated by J.W.H.'s laboratory) and a mouse monoclonal anti-β-actin antibody (1:10,000; Sigma, St. Louis, MO). A horseradish-conjugated secondary antibody (1:500–1:25,000; Amersham Biosciences, Piscataway, NJ) and a chemiluminescent substrate (SuperSignal Ultra; Pierce, Rockford, IL) were used for detection. Bands were quantified using the FluorChem 8800.

MPO isolation and activity. The methods used to prepare brain samples and to measure MPO activity are slight modifications of those described previously by Daugherty et al. (1994). In brief, fresh mouse tissues from selected brain regions were homogenized in a 100 mM sodium phosphate buffer, pH 7.0, containing 1% (wt/vol) cetyltrimethylammonium bromide (CTAB) and centrifuged (6000 × *g*, 4°C, 10 min). Then 1 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$ (final concentration) were added to each sample before being incubated overnight at 4°C with 0.3 ml of concanavalin A-Sepharose B (Sigma). The gel was then pelleted by centrifugation and washed three times with a 0.1 M sodium acetate buffer, pH 6.0, containing 0.1 M NaCl and 0.05% CTAB. Then samples were centrifuged (6000 × *g*, 5 min) to remove residual washing buffer. The glycoprotein bound to the lectin gel was then eluted by incubation with 0.15 ml elution buffer (0.5 M methyl α-D-mannoside in washing buffer) for 30 min. After the last centrifugation, final supernatants were collected and used immediately to assess MPO activity by monitoring the oxidation of tetramethylbenzidine as described previously (Andrews and Krinsky, 1982). The absorbance was read at 655 nm with a microplate reader (Bio-Rad, Hercules, CA).

Mouse MPO, glial fibrillary acidic protein, β_2 integrin MAC-1 (CD11b/CD18), neutrophil, and tyrosine hydroxylase immunohistochemistry. At selected time points after MPTP, mice were killed, and their brains were processed for immunohistochemical studies following our standard protocol for single or double immunostaining (Wu et al., 2003). The primary antibodies used were rabbit polyclonal anti-MPO (1:500; Lab Vision, Fremont, CA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:500; Chemicon, Temecula, CA), mouse monoclonal anti-MAC-1 (1:1000; Serotec, Raleigh, NC), and the monoclonal rat anti-mouse neutrophil antibody MCA771GA (1:100; Serotec). Immunostaining was visualized by 3,3′-diaminobenzidine (DAB) or fluorescein and Texas Red (Vector Laboratories, Burlingame, CA) and examined by regular or confocal microscopy. Colocalization studies were performed on doubly immunofluorescent stained sections, which were analyzed with an LSM 510 META laser-scanning microscope (Zeiss, Thornwood, NY).

For quantitative tyrosine hydroxylase (TH) immunostaining, mice were killed 7 d after MPTP. Both striatal and nigral sections (30 μm), spanning the entire extent of the structures, were incubated with a polyclonal anti-TH antibody (1:1000; Calbiochem, San Diego, CA) for 48 h at 4°C. Immunoreactivity was visualized by incubation in DAB, glucose, and glucose oxidase, and sections were counterstained with thionin. The total numbers of TH- and Nissl-positive neurons in the SNpc were counted stereologically using the optical fractionator method (West, 1993) as used previously (Tieu et al., 2003). Striatal OD of TH immunostaining, determined by the Scion (Frederick, MD) Image program, was used as an index of striatal density of TH innervation (Tieu et al., 2003). The concentration of anti-TH antibody and DAB used here and the length of time striatal sections were incubated in DAB were the same as reported previously (Tieu et al., 2003).

Human samples. All human samples were obtained from the New York Brain Bank at Columbia University (http://cumc.columbia.edu/research/equip/eq-tb_bb.htm). Procedures using this autopsy material were in accordance with the NIH guidelines for human studies and approved by the Institutional Review Board of Columbia University. Samples used in this work included the cerebellum, striatum, and ventral midbrain (for PD and controls); the caudate nucleus [for Huntington's disease (HD) and controls]; and the frontal motor cortex [for amyotrophic lateral sclerosis (ALS) or motor neuron disease and controls]. All of these cases were selected on the basis of neuropathological diagnoses using the criteria for definite PD, HD, and ALS outlined in the supplemental material (available at www.jneurosci.org). Relevant clinical and neuropathological information regarding all of the cases used here are presented in supplemental Table 2 (available at www.jneurosci.org as supplemental material). The procedures for Western blot analysis and immunohistochemistry in human tissues were identical to those described above in mouse tissues; the primary anti-MPO antibody was a rabbit anti-human MPO antibody (DakoCytomation, Carpinteria, CA) used at 1:1000 for Western blot and 1:200 for immunohistochemistry, as well as a rabbit polyclonal anti-GFAP antibody (1:10,000; DAKO, Carpinteria, CA). Visualization of the bound antibody was achieved using chromogenes SG (blue/gray) and 3-amino-9-ethylcarbazole (red) from Vector Laboratories.

MPTP metabolism. Striatal 1-methyl-4-phenylpyridinium (MPP^+) levels were determined by HPLC with UV detection (λ = 295 nm) in WT and MPO-deficient mice at 90 min after the last injection of 20 mg/kg MPTP. Striatal tissue lactate production induced by MPP^+ and synaptosomal uptake of [^{3}H]MPP^+ were performed as described previously (Wu et al., 2003). The assays were repeated three times, each time using duplicate samples.

Mass spectrometric analysis. At selected time points, anesthetized mice were perfused with ice-cold 50 mM sodium phosphate, pH 7.4, containing an antioxidant mixture made of 100 μM diethylenetriaminepentaacetic acid, 1 mM butylated hydroxytoluene, 10 mM 3-amino-1,2,4-triazole, and 1% ethanol (v/v) to minimize *ex vivo* oxidation. The ventral midbrain and cerebellum were then dissected and pulverized in liquid N_2, delipidated, dialyzed to remove low-molecular weight compounds, and hydrolyzed using HBr instead of HCl to prevent artifactual chlorination. [$^{13}C_6$]-Ring-labeled internal standards were added before hydrolysis. The amino acids were isolated using a C-18 solid-phase extraction col-

umn and subjected to derivatization and analysis by isotope dilution gas chromatography/mass spectroscopy (GC/MS) (Heinecke et al., 1999).

Detection of hypochlorous acid-modified protein. Immunochemical detection of hypochlorous (HOCl)-modified proteins was performed with the antibody HOP-1 (clone 2D10G9; dilution 1:500; provided by E. Malle, Medical University of Graz, Graz, Austria). HOP-1 is specific for HOCl-modified epitopes/proteins and does not cross-react with other oxidative modifications (Malle et al., 1995; Hazell et al., 1996). Immunostaining was visualized by using DAB, and sections were counterstained with methylgreen (Vector Laboratories).

Statistical analysis. All values are expressed as mean ± SEM. Differences among means were analyzed using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls *post hoc* testing. In all analyses, the null hypothesis was rejected at the $p \leq 0.05$ level.

Results

MPO is induced in the mouse ventral midbrain during MPTP-induced dopaminergic neurodegeneration

To examine the possibility that MPO is a component of the inflammatory response seen in the MPTP model of PD (Liberatore et al., 1999; Wu et al., 2002), we first assessed MPO mRNA and protein content in the ventral midbrain (i.e., brain region containing the SNpc dopaminergic neurons) over the entire active phase of neurodegeneration and gliosis provoked by this neurotoxin (Liberatore et al., 1999; Przedborski and Vila, 2001). In saline-injected control mice, the ventral midbrain contained low levels of MPO mRNA and protein (Fig. 1*A–C*). In contrast, in MPTP-injected mice, ventral midbrain levels of both MPO mRNA and protein increased in a time-dependent manner (Fig. 1*A–C*). Ventral midbrain MPO mRNA and protein expression levels peaked at 1 and 2 d after MPTP exposure, respectively (Fig. 1*C*), which is contemporaneous to the most-intense phase of SNpc dopaminergic neuronal death in this PD model (Przedborski and Vila, 2001). We next asked whether the observed changes in MPO ventral midbrain content in MPTP-injected animals paralleled an alteration of MPO enzymatic activity by monitoring oxidation of tetramethylbenzidine. Consistent with the protein data, we found that ventral midbrain MPO activity also rose during MPTP neurotoxicity in a time-dependent manner (Fig. 1*D*). In contrast, in mutant mice deficient in MPO ($MPO^{-/-}$; $n = 2$), the ventral midbrain did not show higher oxidation of tetramethylbenzidine after MPTP administration (data not shown). Unlike in the ventral midbrain, levels of MPO mRNA, proteins, and catalytic activity in the cerebellum (brain region resistant to MPTP) were unaffected by MPTP administration. However, more unexpected was the finding that no MPO alteration could be detected in the striatum (where dopaminergic fibers degenerate after MPTP administration), as illustrated by the lack of change in striatal MPO activity: saline, 14.0 ± 4.1 ($n = 7$), versus MPTP (at 2 d), 16.2 ± 1.5 ($n = 11$; $p > 0.05$). Thus, both protein levels and activity of MPO increase in the MPTP mouse model of PD, specifically in ventral midbrain where the demise of the nigrostriatal dopaminergic neurons is taking place.

MPO is expressed in reactive astrocytes after MPTP injection

To elucidate the cellular origin of MPO in the ventral midbrain of MPTP-treated mice, immunohistochemical studies were performed. In saline controls, diffuse MPO immunoreactivity was seen in the neuropil (Fig. 2*A*,*C*). In MPTP-treated mice 2 d after the last injection, ventral midbrain MPO immunostaining was stronger, especially at the level of the substantia nigra, and cells with a glial morphology appeared labeled (Fig. 2*B*,*D*). These MPO-positive cells showed punctate immunoreactivity over both the cell bodies and proximal processes (Fig. 2*D*). To corroborate the bright-field microscopy results, we performed double-immunofluorescence confocal microscopy on ventral midbrain sections from mice 2 d after MPTP. This analysis confirmed that MPO colocalized with the astrocytic marker GFAP as shown by the merged image from the two fluorochromes (Fig. 2*E–G*) and

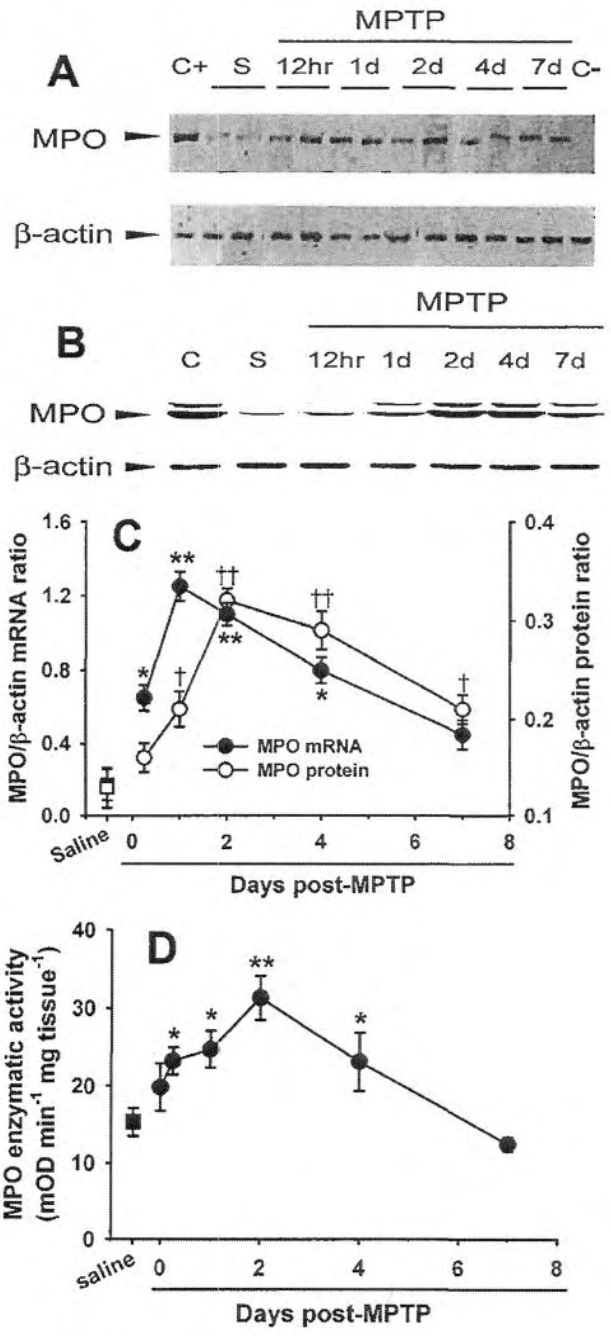

Figure 1. MPTP injections are associated with a time-dependent increase in ventral midbrain MPO mRNA (*A*, *C*), protein expression (*B*, *C*), and enzymatic activity (*D*) relative to saline injections. Data are means ± SEM for 3–11 mice per group. $^{*,\dagger}p < 0.05$, $^{**,\dagger\dagger}p < 0.01$ compared (Newman–Keuls *post hoc* test) with saline-injected control animals. S, Saline; C+, positive control (bone marrow); C−, negative control (absence of reverse transcriptase); mOD, millioptical density.

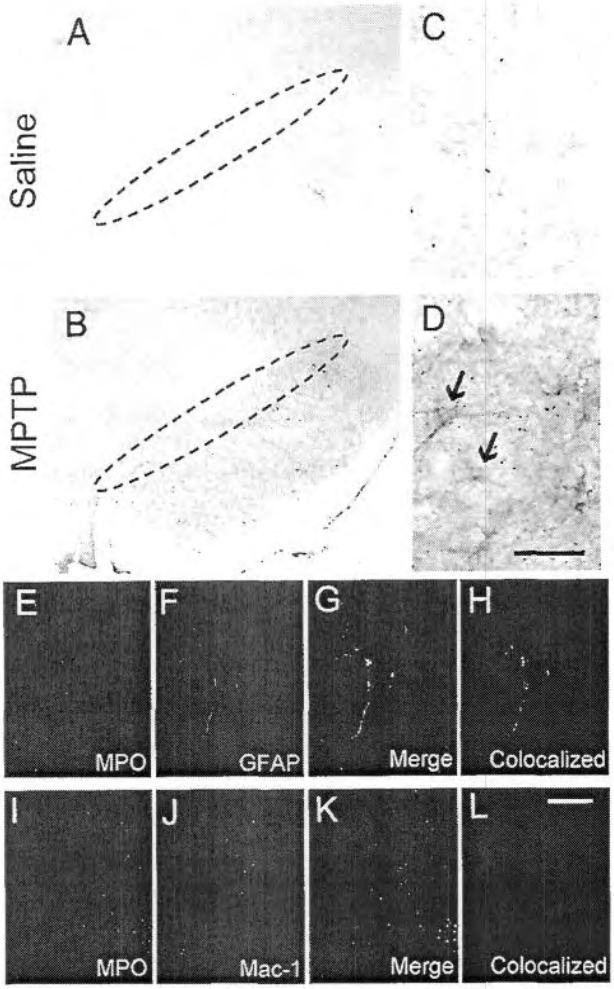

Figure 2. *A, C,* Immunochemical studies revealed no specific MPO immunoreactivity in the ventral midbrain of saline-injected control mice. The dashed oval delineates the SNpc. *B, D,* However, a dense network of fibers and scattered cell bodies positive for MPO are seen at the level of the SNpc after MPTP injections. Black arrows in *D* show the MPO-positive cellular elements. *E–H,* Confocal microscopy demonstrates that ventral midbrain MPO-positive structures (*E*, red) are also GFAP positive (*F*, green), as evidenced by the overlay of the two fluorochromes (*G*) and by the computed mask of the colocalized pixels (*H*). *J–L,* In contrast, ventral midbrain MPO-positive structures (*I*, red) are not MAC-1 positive (*J*, green), as evidenced by the overlay (*K*) and the mask of colocalized pixels (*L*). Tissue sections are from mice at 24 and 48 h after saline or MPTP injections. Scale bars: (in *D*) *A, B,* 250 μm; *C, D,* 25 μm; (in *L*) *E–L,* 10 μm.

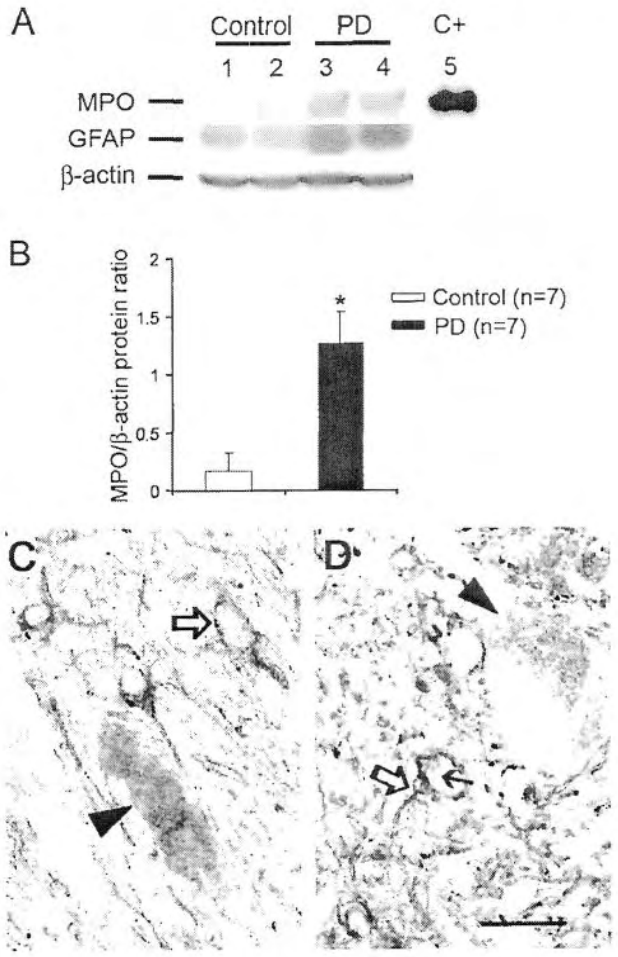

Figure 3. *A, B,* Ventral midbrain MPO tissue content is increased in postmortem tissue from PD patients compared with controls, as well as GFAP tissue content. C+, Positive control (purified MPO). *C,* In ventral midbrain sections, MPO (blue) is not detected in control tissues, neither in GFAP-positive cells (open arrow) nor in or around neuromelanized dopaminergic neurons (arrowhead). *D,* Conversely, MPO immunoreactivity (blue, small black arrow) is found in GFAP-positive cells (open arrow) in PD tissue but not in the rare remaining neuromelanized dopaminergic neurons (arrowhead). Scale bar, 20 μm. Data are means ± SEM for seven samples per group. $^*p < 0.05$ compared with normal controls (Newman–Keuls *post hoc* test).

the computed mask of the colocalized pixels (Fig. 2*H*). Conversely, no evidence of MPO expression in microglial cells could be documented by using the same techniques (Fig. 2*I–L*). Although abundant neutrophils were seen in our mouse bone marrow preparations (positive controls) using the anti-mouse neutrophil antibody MCA771GA, none were detected within the brain parenchyma (data not shown). No noticeable cellular MPO immunoreactivity was observed in the striatum or cerebellum of either saline- or MPTP-treated mice (data not shown). These results demonstrate that MPO is primarily expressed in ventral midbrain astrocytes during the demise of dopaminergic neurons caused by MPTP.

Expression of MPO is increased in PD midbrain

To determine whether the changes in MPO observed in the MPTP mouse model of PD were present in the human condition, we assessed MPO protein levels in postmortem ventral midbrain samples from sporadic PD patients. Consistent with the mouse data, PD samples had significantly higher MPO protein contents compared with controls (Fig. 3*A,B*). Like in mice, there was no significant difference in MPO to β-actin ratios in the striatum (PD, 1.1 ± 0.8, vs controls, 1.4 ± 0.8; $p > 0.05$; $n = 7$) or cerebellum (PD, 0.8 ± 0.2, vs controls, 1.0 ± 0.3; $p > 0.05$; $n = 7$) between the PD and control samples. Histologically, cellular MPO immunoreactivity was not detected in the control ventral midbrain parenchyma per se (Fig. 3*C*) but only in small cells within blood vessels. However, MPO immunoreactivity was seen in ventral midbrain sections from PD patients (Fig. 3*D*), where it was identified in SNpc glial cells in the vicinity of neuromelanin-containing neurons (Fig. 3*D*). The similarity of the MPO alterations between the MPTP mice and the PD postmortem specimens strengthens the relevance of using this experimental model to study the role of MPO in the PD neurodegenerative process.

Because gliosis is a common pathological feature of many neurodegenerative diseases, we wondered whether increases in

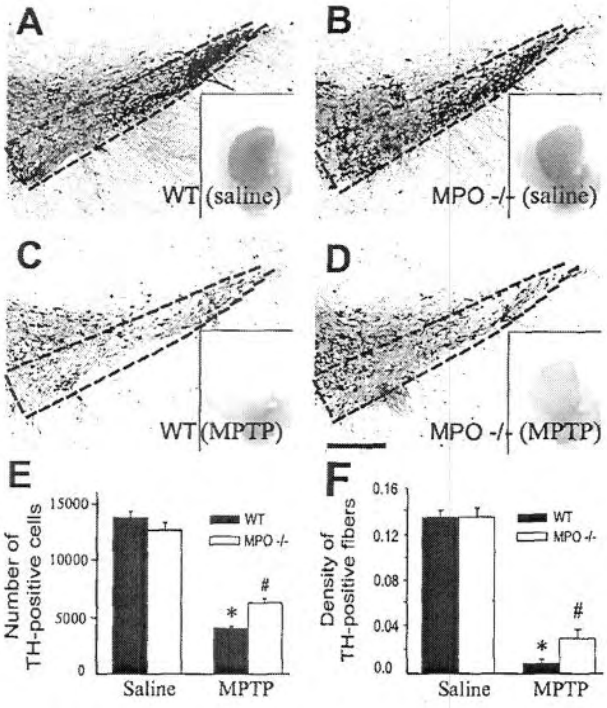

Figure 4. ***A–D***, Ablation of MPO in mutant mice attenuates MPTP-induced striatal TH fibers and SNpc TH neuronal loss, as assessed 7 d after either saline or MPTP injections. ***E***, ***F***, Quantification of neuronal (***E***) and fiber (***F***) loss. Data are means ± SEM for four to six mice per group. $^{*}p < 0.05$ compared with saline-injected animals; $^{\#}p < 0.05$ compared with saline- and MPTP-injected MPO $^{+/+}$ mice.

the expression of MPO within areas of neurodegeneration can be found in neurodegenerative disorders other than PD. Compared with controls, the motor cortex from ALS patients did not exhibit higher GFAP or MPO values (data not shown). Conversely, we found that caudate nucleus tissues from stage 4 HD patients had higher GFAP to β-actin ratios (HD, 0.7 ± 0.1, vs controls, 0.1 ± 0.1; $p < 0.01$; $n = 3$–4) as well as MPO to β-actin ratios (HD, 0.8 ± 0.2, vs controls, 0.2 ± 0.1; $p < 0.05$; $n = 5$–6). This suggests that brain MPO expression is not specific to PD but rather generic to neurodegenerative diseases in which areas of neuronal loss are accompanied with gliosis.

MPO deficiency protects against MPTP-induced neurodegeneration

Next we compared the effects of MPTP on the nigrostriatal pathway of mutant mice deficient in MPO (MPO $^{-/-}$) and their WT littermates (MPO $^{+/+}$). Seven days after the last injection of saline or MPTP, the brains of these animals were processed for quantification of dopaminergic cell bodies in the SNpc and of projecting dopaminergic fibers in the striatum using TH immunostaining. In saline-injected MPO $^{-/-}$ and MPO $^{+/+}$ mice, stereological counts of SNpc dopaminergic neurons and striatal TH-positive OD (Fig. 4*A*,*B*,*E*,*F*) were comparable. In MPTP-injected MPO $^{+/+}$ mice, there was a ~70% loss of SNpc TH-positive neurons (Fig. 4*C*,*E*) and ~92% reduction of striatal TH OD (Fig. 4*C*,*F*) compared with saline-injected controls (Fig. 4*A*,*E*,*F*). In contrast, in MPTP-injected MPO $^{-/-}$ mice, there was only ~50% loss of SNpc TH-positive neurons (Fig. 4*D*,*E*) and ~70% reduction of striatal TH OD (Fig. 4*D*,*F*) compared with saline-injected controls (Fig. 4*B*,*E*,*F*). The TH/Nissl ratio of neuronal counts did not differ between saline- and MPTP-injected WT mice (saline, 1.68 ± 0.15, vs MPTP, 1.46 ± 0.20; $n = 5$ per group), supporting the assertion that the reduction in dopaminergic neuron numbers corresponds to an actual cell loss and not to a downregulation of TH.

To examine whether MPO ablation protects not only against structural damage but also against functional deficits caused by MPTP, we compared the levels of dopamine and its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in the striatum as well as locomotor activity between MPO $^{-/-}$ and MPO $^{+/+}$ mice, after MPTP injections. Contrasting with the protection afforded by the lack of MPO on the nigrostriatal dopaminergic neurons, the loss of striatal dopamine and the deficit in motor performance caused by MPTP were as severe in MPO $^{-/-}$ as in MPO $^{+/+}$ mice (supplemental material, available at www.jneurosci.org).

MPTP metabolism

Major factors controlling MPTP neurotoxicity are its conversion in the brain to MPP $^{+}$, followed by MPP $^{+}$ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (Przedborski and Vila, 2001). To ascertain that the resistance of MPO $^{-/-}$ mice was not attributable to alterations in MPTP toxicokinetics, we assessed its three key neurotoxic steps (Tieu et al., 2003). Results show that striatal levels of MPP $^{+}$, striatal uptake of [^{3}H]MPP $^{+}$, and MPP $^{+}$-induced lactate production (a measure of mitochondrial function) did not differ between MPO $^{-/-}$ mice and their WT littermates (Table 1).

MPO damages ventral midbrain proteins

MPO is the only known mammalian source of HOCl at plasma concentrations of halide ion (Gaut et al., 2001). HOCl reacts with tyrosine to form 3-chlorotyrosine, a specific and stable biomarker of protein damage by MPO (Heinecke et al., 1999). To determine whether MPTP promotes oxidative damage to brain proteins, we used isotope dilution GC/MS (Heinecke et al., 1999), a sensitive and specific method, to quantify 3-chlorotyrosine levels in samples from eight saline-injected controls and eight MPTP-injected mice 24 h after injection. We compared levels of 3-chlorotyrosine in the ventral midbrain and cerebellum. In MPTP-treated mice, 3-chlorotyrosine levels in the ventral midbrain were markedly increased ($p < 0.05$) compared with saline-injected controls: MPTP, 30.8 ± 5.7 nmol of 3-chlorotyrosine per molar of tyrosine ($n = 8$) versus saline controls, 4.8 ± 2.1 nmol of 3-chlorotyrosine per molar of tyrosine ($n = 8$). 3-Chlorotyrosine was undetectable in the cerebellum of mice injected with either saline or MPTP. In contrast, in MPTP-treated MPO $^{-/-}$ mice ($n = 3$), ventral midbrain 3-chlorotyrosine was undetectable. The identification of chlorinated tyrosine in tissues therefore supports the hypothesis that reactive intermediates produced by MPO damage brain proteins in MPTP-intoxicated mice.

To localize MPO-damaged proteins, tissue sections were immunostained with HOP-1, a mouse antibody that specifically recognizes HOCl-modified proteins (Malle et al., 1995); the chlorotyrosine antibody was not available to us. Intense HOP-1 immunoreactivity was observed in the SNpc of MPTP-injected mice (Fig. 5*A*–*C*). HOP-1-positive material was seen in the neuropil within beaded-appearing fibers and in cells with both neuronal and non-neuronal morphology within vesicular elements (Fig. 5*A*–*C*). No HOP-1 immunostaining was detected in the SNpc of saline-injected mice or MPTP-injected MPO $^{-/-}$ mice (data not shown).

Table 1. Striatal MPTP metabolism in MPO-deficient mice

	MPP^+ level (μg/g striatum)	MPP^+ uptake (IC_{50}, nM)	MPP^+-induced lactate (μM/100 mg protein)
$MPO^{+/+}$ mice	4.46 ± 0.24	113.7 ± 1.2	57.6 ± 7.5
$MPO^{-/-}$ mice	5.54 ± 0.71	114.3 ± 1.7	66.8 ± 4.4

Striatal MPP^+ levels in WT ($MPO^{+/+}$) and MPO-deficient mice ($MPO^{-/-}$) were determined 90 min after the last injection of MPTP (20 mg/kg). Values are means ± SEM of either six mice per group (MPP^+ level) or three independent experiments each performed in duplicate ([^{3}H]MPP^+ uptake and lactate level). None of the presented values differ significantly ($p > 0.05$) between $MPO^{+/+}$ and $MPO^{-/-}$ mice.

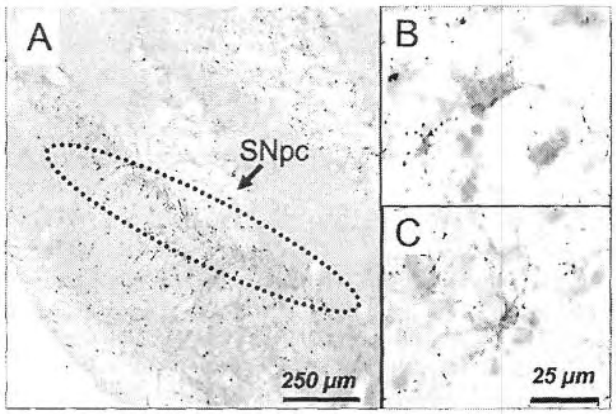

Figure 5. Immunohistochemical localization of HOCl-modified proteins with the HOP-1 antibody in ventral midbrain sections. Twenty-four hours after MPTP injections, HOP-1-positive immunoreactive material is seen mainly at the level of the SNpc (*A*) and within or around cellular elements (*B*, *C*). Scale bars: *A*, 250 μm; *B*, *C*, 25 μm.

Discussion

The present study shows that the level of MPO expression increases markedly in diseased SNpc from both mice exposed to MPTP (Figs. 1, 2) and human PD (Fig. 3). This work also demonstrates that changes of MPO protein content and enzymatic activity in MPTP-intoxicated mice parallel (Fig. 1) the degeneration of SNpc dopaminergic neurons (Przedborski and Vila, 2001). Moreover, MPO is found primarily in SNpc-reactive astrocytes (Figs. 2, 3), which are major cellular components of the PD- and MPTP-associated inflammatory response (Przedborski and Goldman, 2004). Conversely, we failed to detect any of the well established cellular sources of MPO (neutrophils, monocytes, or macrophages) (Hampton et al., 1998) within the ventral midbrain parenchyma of PD patients and MPTP-injected mice. The presence of MPO in damaged SNpc thus appears to derive essentially from a resident, not a blood-borne, inflammatory response associated with the degeneration of dopaminergic neurons. Based on assessments performed in two other neurodegenerative diseases, namely HD and ALS, it appears that MPO upregulation in the brain is not pathognomonic of PD. Instead, we believe that the occurrence of MPO in diseased brains is likely indicative of a disease process associated with chronic gliosis rather than a particular etiology. That said, our results are surprising because phagocytic white blood cells are generally believed to be the only cellular sources of MPO. However, neuronal expression of MPO is also increased in Alzheimer's disease (Green et al., 2004), raising the possibility that this enzyme may contribute to oxidative damage in a variety of chronic neurodegenerative disorders.

Contrary to ventral midbrain, the striatum, which is also a site of a strong inflammatory reaction after MPTP administration and sometimes in PD, did not show any alteration in MPO expression or enzymatic activity as illustrated in Figure 3. Remarkably, detectable changes in iNOS expression and enzymatic activity are also confined to ventral midbrains of MPTP-injected mice (Liberatore et al., 1999), whereas activation of NADPH oxidase is observed in both ventral midbrains and striata of these animals (Wu et al., 2003). Collectively, these observations suggest that the molecular composition of the inflammatory response to injury may be, to a certain extent, regionally specific. Supporting this view is the finding that a stereotaxic injection of 5 μg/μl bacterial endotoxin lipopolysaccharide into the hippocampus or cortex of adult rats produces no apparent neuronal loss, whereas an identical administration into the substantia nigra dramatically reduces the number of neurons (Kim et al., 2000). Although this distinct regional susceptibility has been linked to differences in microglial densities, its molecular basis might well be related to differences in the quantity or variety of the inflammatory mediators produced.

After MPTP injections, mutant mice deficient in MPO showed more spared SNpc dopaminergic neurons and striatal dopaminergic fibers than their WT littermates (Fig. 4). We also found that the lack of MPO did not alter key aspects of MPTP toxicokinetics (Table 1). Together, these findings indicate that MPO contributes to the pathogenic cascade of deleterious events responsible for the demise of dopaminergic neurons in the MPTP model and perhaps in PD as well. Surprisingly, although alterations in MPO protein and enzymatic activity were only detected in the ventral midbrain (Fig. 1), both cell bodies and fibers of nigrostriatal dopaminergic neurons were preserved in MPTP-injected $MPO^{-/-}$ mice (Fig. 4). This observation implies that an entire neuron may be salvaged by mitigating deleterious factors that specifically injure cell bodies and that nigrostriatal dopaminergic neurons are not degenerating solely via a dying-back process, as one may have thought based on previous observations (Herkenham et al., 1991; Wu et al., 2003).

The relative resistance of dopaminergic neurons to MPTP-induced neurotoxicity in $MPO^{-/-}$ mice was, however, not accompanied by a preservation of striatal dopamine levels or attenuation of motor deficits caused by this parkinsonian neurotoxin (supplemental material, available at www.jneurosci.org). This discrepancy may be explained by the fact that TH (the rate-limiting enzyme in the synthesis of dopamine) can be inactivated by injury, such as that inflicted by MPTP (Ara et al., 1998). It is thus conceivable that although ablation of MPO attenuates the loss of TH protein (as evidenced by immunostaining), this beneficial effect may not be enough to prevent the loss of TH catalytic activity (as evidenced by the dopamine levels). Targeting MPO alone may thus suffice to provide observable structural, but not functional, neuroprotection in this experimental model of PD. Accordingly, optimal therapeutic interventions for PD may rely on the combination of strategies capable of providing structural protection such as MPO inhibition, with other strategies capable of protecting/stimulating dopaminergic function. Yet, given the relentless nature of PD, it can be surmised that the death signal in this illness may not be as harsh as that provoked by MPTP. Therefore, whether MPO inhibition in PD can succeed, not only in slowing neuronal death but also in sustaining dopamine synthesis, is a possibility that should not readily be excluded.

As to how MPO neurotoxic actions on dopaminergic neurons are mediated, two distinct and not mutually exclusive mechanisms may be invoked. First and foremost, MPO is known for its production of cytotoxic reactive oxygen species and RNS (Harrison and Schultz, 1976; Eiserich et al., 1996; Hampton et al., 1998).

Therefore, neurons located in the vicinity of MPO-containing cells may have their plasma membrane proteins and lipids subjected to the deleterious effects of MPO-derived oxidants such as HOCl. In keeping with this scenario, we found high levels of 3-chlorotyrosine, a specific oxidative modification of tyrosine residues mediated by HOCl in the MPTP-susceptible brain region, the ventral midbrain. Also supporting the oxidative role of MPO in the MPTP model is our immunohistochemical demonstration of HOCl-modified protein in the ventral midbrain of intoxicated mice (Fig. 5*A–C*). Aside from this oxidative effect, MPO can be secreted and bind CD11b/CD18 integrins to the cell surface (Lau et al., 2005). In the case of neutrophils, ligation of CD11b/CD18 by MPO stimulates signaling pathways implicated in the activation of these cells (Lau et al., 2005). Because brain microglia do express CD11b/CD18 integrins and seem to participate in the neurodegenerative process in the MPTP model and in PD, this cytokine-like effect of MPO may represent an additional mechanism by which dopaminergic neurons are affected by this enzyme.

As raised previously (Wu et al., 2003), a key issue is the selective damage to dopaminergic neurons observed during inflammation in MPTP-treated mice and humans suffering from PD. Many lines of evidence suggest that dopaminergic neurons are particularly vulnerable to oxidative stress compared with the other cells in the brain (Dauer and Przedborski, 2003). Alternatively, it is likely that in the MPTP model and in PD, the magnitude of the inflammatory response and resulting oxidative stress is mild and only inflicts sublethal lesions. Thus, inflammation-mediated oxidative stress would succeed in killing only neurons already compromised, as dopaminergic neurons probably are in PD and after MPTP injections.

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Forum Review

Reactive Oxygen and Nitrogen Species: Weapons of Neuronal Destruction in Models of Parkinson's Disease

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ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disease whose etiology and pathogenesis remain mainly unknown. To investigate its cause and, more particularly, its mechanism of neuronal death, numerous *in vivo* experimental models have been developed. Currently, both genetic and toxic models of PD are available, but the use of neurotoxins such as 6-hydroxydopamine, paraquat, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and rotenone are still the most popular means for modeling the destruction of the nigrostriatal dopaminergic neurons seen in PD. These four neurotoxins, although distinct in their intimate cytotoxic mechanisms, kill dopaminergic neurons via a cascade of deleterious events that consistently involves oxidative stress. Herein, we review and compare the molecular mechanisms of 6-hydroxydopamine, paraquat, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and rotenone, placing the emphasis of our discussion on how reactive oxygen and nitrogen species contribute to the neurotoxic properties of these four molecules. As the reader will discover, to achieve the above stated goal, we had to not only appraise recent findings, but also revisit earlier landmark studies to provide a comprehensive view on this topic. This approach also enabled us to describe how our understanding of the mechanism of actions of certain toxins has evolved over time, which is particularly striking in the case of the quatrogenarian neurotoxin, 6-hydroxydopamine. *Antioxid. Redox Signal.* 7, 685–693.

INTRODUCTION

PARKINSON'S DISEASE (PD) affects ~1% of the population over the age of 50 in the United States alone, and it is the second most frequent neurodegenerative disorder after Alzheimer's disease (15). This common neurodegenerative disorder is essentially a sporadic disease, meaning that it presents itself with no apparent genetic linkage (15). Yet in rare instances, as in several other neurodegenerative diseases (70), PD can be inherited (16). Whether it is sporadic or familial, PD is a slow, progressive disease characterized mainly by resting tremor, slowness of movement (bradykinesia), stiffness (rigidity), and poor balance (postural instability) (25). Most, if not all, of these clinical abnormalities are attributed to the severe loss of the nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc), which leads to a profound deficit in brain dopamine (15). Another pathological hallmark of PD is the eosinophilic intraneuronal proteinaceous inclusion called the Lewy body (27), whose pathogenic significance remains controversial.

There is no evidence that PD patients must be treated upon emergence of the clinical symptoms. However, at some point, the motor disability becomes so severe that treatment aimed at either replenishing dopaminergic stores in the brain (*e.g.*, levodopa) or stimulating dopamine receptors (*e.g.*, dopamine agonists), or both, is required to maintain the patient's autonomy and quality of life. Several of the approved drugs for PD are quite potent in alleviating symptoms, but their chronic administration often causes serious motor and psychiatric side effects (24).

Regardless of the nature of the etiologic factor that initially provokes neurodegeneration, two major hypotheses regarding

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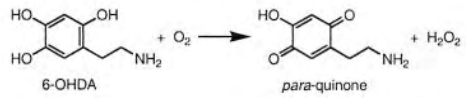
the pathogenesis of the disease have emerged from studies probing the functions of genes implicated in inherited forms of PD and from animal and cellular model systems of PD. One hypothesis postulates that inappropriate aggregation of proteins is instrumental in the death of SNpc dopaminergic neurons, whereas the other, which is the focus of this review, suggests that the offender is oxidative stress, including potentially toxic intermediates of oxidized dopamine. This latter hypothesis posits that the fine-tuned balance between the production and destruction of oxidants is altered in such a way that oxidative damage arises, leading to cellular dysfunction and, ultimately, to cell death. Unquestionably, support for the "oxidative stress hypothesis" of PD comes from descriptive investigations performed on fluids and tissue samples of PD patients (64). However, in our opinion, the most compelling evidence for a role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the death of SNpc dopaminergic neurons in PD originates, not from human studies, but rather from investigations in animal models of PD generated by various neurotoxins. What these neurotoxins are and how they engender oxidative stress are the topics that we will discuss in this review. Conversely, how faithfully these neurotoxins model PD and how they should be used to achieve this goal will not be discussed. Readers interested in these latter aspects are encouraged to review other references (65, 66).

ROS-PRODUCING "PARKINSONIAN" NEUROTOXINS

Toxic models of PD are numerous, but thus far only a handful of such models have been thoroughly characterized with respect to their biochemical and molecular modes of action and neurodegenerative effects. Relatively well characterized models of PD include 6-hydroxydopamine (6-OHDA), paraquat, rotenone, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (15). In principle, these toxins all share the same function, namely, the killing of SNpc dopaminergic neurons by a process in which oxidative stress is instrumental. Yet, as discussed below and depending on the neurotoxin, the molecular basis of the generated oxidative stress is quite different, and in broad terms 6-OHDA toxicity is dependent on its oxidation. The toxic action of paraquat is due to its reduction–oxidation cycling, whereas, at least in part, inhibition of the mitochondrial electron transport chain is responsible for the neurotoxicity of both rotenone and MPTP.

THE 6-OHDA MODEL: A PATRIARCH STILL IN THE RACE

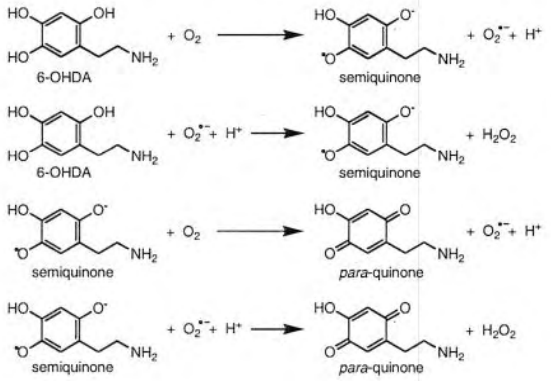
6-OHDA was introduced as a catecholaminergic toxin >30 years ago (46) and, ever since, it has remained an extensively tested model both *in vitro* and *in vivo*. The effects of 6-OHDA on both the central and peripheral catecholaminergic pathways in rodents and in a variety of cultured cell types have been reviewed elsewhere, as well as the molecular basis for its specificity (45, 46, 65). 6-OHDA can be administered to rodents via a variety of different routes, but its proper utilization *in vivo* and *in vitro* relies on one's knowledge of a series of technical points that have been discussed in detail (45, 46, 65). Because of the emphasis of this special *Forum* of *Antioxidants & Redox Signaling* on oxidative stress in PD and experimental models of the disease and as 6-OHDA is a prototypical "oxidative-stress neurotoxin," we will focus the discussion on the 6-OHDA-induced neurotoxic mechanism. From the outset, it can be said that most experts agree on the concept that 6-OHDA destroys catecholaminergic structures by a combined effect of ROS and quinones (10). This popular view is based on the evidence that 6-OHDA, once dissolved in an alkaline solution, readily oxidizes in the presence of oxygen, yielding, in a stoichiometric fashion, hydrogen peroxide (H_2O_2) and *para*-quinone (37, 72) as depicted by the following reaction:



Although the chemical reaction that underlies 6-OHDA-induced neurotoxicity appears quite straightforward, it is in fact a remarkably complicated reaction that does not occur as a spontaneous oxidation by molecular oxygen. Still, molecular oxygen is mandatory for the reaction or, in anaerobic conditions, no conversion of 6-OHDA into quinones is detectable (30). If oxygen is necessary for the reaction, it is not, however, sufficient to drive 6-OHDA oxidation alone because desferrioxamine, a potent metal chelator, does inhibit the aerobic formation of 6-OHDA quinones to a dramatic extent (29–31, 80). This observation implies that 6-OHDA oxidation requires the presence of redox-capable transitional metals such as iron or copper to catalyze the transfer of electrons from 6-OHDA to molecular oxygen. It is now well accepted that even the presence of trace amounts of transitional metal contaminants, brought into the reaction mixture by the reagents and glassware, suffice to set this aerobic reaction in motion.

Aside from quinones, the oxidation of 6-OHDA also generates H_2O_2, as illustrated above. In biological systems, the production of H_2O_2 results from a two-electron reduction of oxygen. Thus, it can be surmised that during 6-OHDA oxidation a pair of electrons is transferred from 6-OHDA to molecular oxygen to produce H_2O_2. Yet it appears that the reaction of molecular oxygen with 6-OHDA is primarily a two-electron process only in the presence of excess oxygen, but it is a four-electron process in the presence of excess 6-OHDA (30). Accordingly, H_2O_2 is an end product of the reaction merely if 6-OHDA is limiting. Furthermore, even if the experimental conditions favor an overall exchange of a pair of electrons, the fact that oxygen has two unpaired electrons on its outermost orbital with a same spin quantum number makes it more likely that the reduction of oxygen proceeds by one electron at the time forming superoxide ($O_2^{\bullet -}$) and semiquinone radicals as the intermediary species. This interpretation is consistent with the demonstration that superoxide dismutase (SOD), by scavenging superoxide radicals, dramatically inhibits the oxidation of 6-OHDA (39). Subsequent studies have confirmed the production of superoxide radicals, and have moreover demonstrated that superoxide radicals generated by the first step of 6-OHDA oxidation are critical in propagating the oxidation of 6-OHDA (11, 30, 31, 80). As de-

tailed elsewhere (39), the progressive oxidation of 6-OHDA can be schematized as follows:



This shows that the oxidation of 2 moles of 6-OHDA leads to the formation of 2 moles of quinone and 2 moles of H_2O_2. In addition to the H_2O_2 and superoxide radicals, 6-OHDA oxidation is also associated with the production of hydroxyl radicals as demonstrated by using spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide (26) and methional as spin traps (11). In this system, hydroxyl radicals can arise from the Fenton reaction by which the breakdown of H_2O_2 is catalyzed by transitional metals such as iron.

The above studies indicate that 6-OHDA oxidation generates not only *para*-quinone and H_2O_2, but also the superoxide and hydroxyl radicals. As stressed by many authors throughout this *Forum*, ROS such as H_2O_2, superoxide radical, and hydroxyl radical can either directly or indirectly inflict an array of cellular oxidations that can ultimately lead to cell death. Given this, the reader may encounter no difficulty envisioning how ROS generated by the oxidation of 6-OHDA could contribute to the neurotoxicity of this compound. On the other hand, how the quinone of 6-OHDA may exert deleterious effects may be less obvious. Early on in the characterization of the 6-OHDA mode of action, it was recognized that *para*-quinone formed though the oxidation of 6-OHDA undergoes covalent binding with sulfhydryl and other biological macromolecules with nucleophilic centers (32, 72). Accordingly, *para*-quinone is thus likely to react with glutathione and protein amino acid residues such as cysteine, tyrosine, and lysine. The deleterious consequences of the *para*-quinone of 6-OHDA may thus range from depletion of vital antioxidants such as glutathione, whose concentration is diminished in PD (64), to inactivation of critical enzymes such as catechol-*O*-methyltransferase (4) and tyrosine hydroxylase (49) and, more importantly, to an accumulation of potentially neurotoxic α-synuclein protofibrils, a proposed key event in PD pathogenesis (12).

Although the above-cited studies would argue that both the produced ROS and *para*-quinone are probably equally instrumental in the 6-OHDA neurotoxic processes, available evidence appears to favor the view that ROS are the dominant noxious mediators. For example, the addition of ascorbic acid to tissue slices, which is known to recycle *para*-quinone into 6-OHDA with a net formation of H_2O_2 (38, 80), prevents the appearance of colored quinones, but enhances neurotoxicity (38).

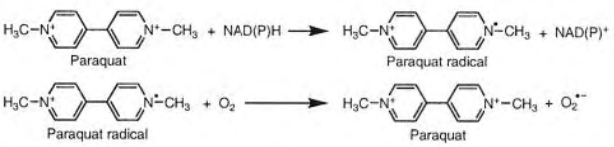
Finally, it should be emphasized that, like other monoamines, 6-OHDA can be metabolized by monoamine oxidase (MAO), a reaction that also generates ROS. This observation raises the possibility that the oxidative domination of 6-OHDA contributes to the neurotoxic process. Yet the finding that pretreatment with MAO inhibitors such as pargyline, rather than mitigating 6-OHDA toxicity, enhances it (45), argues against a MAO-dependent source of ROS as being contributive to the 6-OHDA neurotoxic process. It should also be stressed that, as long as the environmental conditions are favorable, oxidation of 6-OHDA can occur *in vivo* both intra- and extraneuronally. Consistent with this view is the demonstration that, in mesencephalic cultures, 6-OHDA toxicity is not restricted to dopaminergic neurons (55), and that several cell types devoid of transporters allowing 6-OHDA to be translocated inside the cell—such as C6 glioma, NIH-3T3, and CHO cells—can be damaged by this neurotoxin (3).

THE HERBICIDE PARAQUAT

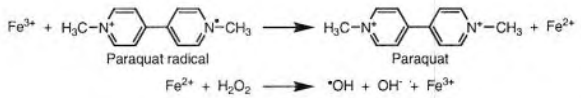
The potent herbicide paraquat, whose chemical name is *N,N'*-dimethyl-4-4'-bipyridinium ion, is another prototypic toxin known to exert deleterious effects through oxidative mechanisms. Structurally, paraquat comprises two pyridine rings, *i.e.*, aromatic rings in which one carbon atom is replaced by a nitrogen atom, joined covalently by their number-4 carbon and with a methyl group attached to each nitrogen. The overall biochemical reaction governing the neurotoxic mechanism of paraquat was reported by Bus and collaborators roughly 30 years ago (6, 7). According to these authors, paraquat undergoes a single electron, reduction-oxidation cycling with subsequent formation of superoxide radicals:

The first of the two steps of this biochemical reaction requires that paraquat go through a single-electron reduction to the blue-colored cation radical, paraquat$^{\bullet+}$ (28, 59). This initial step is not dependent on oxygen, as it can proceed under anaerobic conditions, but it does depend on the presence of diaphorase activity (28), *i.e.*, an enzyme that transfers an electron from a NAD(P)H molecule. Paraquat diaphorases are usually oxidoreductase enzymes containing flavin groups and using NADPH and, presumably, NADH as electron donors (9, 19, 22, 52, 77, 89). Relevant to the brain toxicity of paraquat, it should be noted that nitric oxide synthase (NOS) has been identified as one of the diaphorases capable of reacting with paraquat (19).

The second step of the paraquat toxic reaction is the reoxidation of this compound by oxygen that occurs through a transfer of a single electron from the paraquat radical to molecular oxygen, yielding oxidized paraquat (*i.e.*, the parent compound) and superoxide radicals. The actual reduction-oxidation cycling reaction of paraquat can thus be depicted as follow:



Of note, paraquat$^{\bullet+}$ is a powerful reducing radical capable not only of reacting with molecular oxygen to generate superoxide radicals as shown above, but also of reacting with transitional metals such as iron. Paraquat$^{\bullet+}$ can readily reduce iron(III) and most iron(III)chelates (9) into iron(II) or iron(II) chelates, which in turn could catalyze the formation of hydroxyl radicals via the Fenton reaction:



Presumably, whether paraquat$^{\bullet+}$ reacts with oxygen or iron depends on the concentration of oxygen. Accordingly, it can be speculated that in the brain the high oxygen content should favor the reaction of paraquat$^{\bullet+}$ with molecular oxygen over that with iron(III). Yet hydroxyl radicals can also be produced by an alternative mechanism in which paraquat$^{\bullet+}$ simply supplies the metal needed for the hydroxyl radical formation by reductively mobilizing iron from ferritin (83). Thus far, there is not an unequivocal demonstration that hydroxyl radicals are implicated in the deleterious effects caused by paraquat intoxication. Conversely, there are countless demonstrations that SOD overexpression and SOD mimetics confer resistance against paraquat (17, 18, 61), thus supporting the concept that superoxide radicals are pivotal in paraquat cytotoxicity.

Aside from oxidative stress, it has also been suggested that depletion of the intracellular stores of NAD(P)H, due to its increased oxidation during the reduction-oxidation cycling of paraquat stores, contributes to the overall cytotoxic process. Indeed, loss of NAD(P)H or change in the NAD(P)H over NAD(P)$^+$ ratio may have serious harmful consequences by impairing vital metabolic pathways such as fatty acid synthesis. Increased oxidation of glucose by the pentose phosphate shunt and inhibition of fatty acid synthesis in paraquat-intoxicated animals (79) are consistent with this view.

Most experimental studies of paraquat are related to its effects on the lungs, liver, and kidneys, probably because the toxicity induced by this herbicide in these organs is responsible for death after acute exposure. However, less known is the fact that significant damage to the brain is also seen in individuals who died from paraquat intoxication (33, 41) in spite of the fact that paraquat poorly crosses the blood–brain barrier (78). Thus far, the use of paraquat as a model of PD has been performed mainly in mice, perhaps because previous studies in rats have shown that oral administration of paraquat to these rodents was ineffective in damaging the brain (86) and that its intraventricular or intracerebral injection produces diffuse neurodegeneration (20, 53). Conversely, several authors have now reported reduced motor activity and dose-dependent losses of striatal dopaminergic nerve fibers and substantia nigra neuronal cell bodies in mice that received systemic injections of paraquat (5, 58, 82). Aside from killing dopaminergic neurons, paraquat induces α-synuclein up-regulation and aggregation (56).

Quite surprising is the finding that dopaminergic but not other subpopulations of neurons, such as GABAergic cells in the substantia nigra and the striatum, appeared to be affected by paraquat injection (58). Neuronal subpopulations expressing high activity of NADPH-diaphorase in the brain are apparently not the dopaminergic neurons (63). Given the mechanism of action of paraquat, it is thus fascinating to note that dopaminergic neurons are specifically affected in this model.

THE MITOCHONDRIAL POISONS MPTP AND ROTENONE

Both MPTP and rotenone are well-known mitochondrial toxins used to recapitulate hallmarks of PD in laboratory animals (15). Although MPTP has been regarded for the past 20 years as the parkinsonian toxin *par excellence*, rotenone has recently received major attention for its capacity to produce previously unattainable PD features such as intraneuronal proteinaceous inclusions in rats (2). However, studies about the detailed mechanisms by which rotenone kills dopaminergic neurons are thus far few, which is in striking contrast with the large body of literature available on this topic for MPTP. Because it can be assumed that these two neurotoxins share many of the key molecular mechanisms responsible for their neurotoxicity, we will focus the rest of our discussion on MPTP and only refer to rotenone whenever published data permit.

A distinct feature of MPTP is that this neurotoxin is highly lipophilic and thus readily capable of crossing the blood–brain barrier after its systemic administration (57). Yet, once in the brain, MPTP as such is unable to provoke any neurotoxicity unless it is converted to 1-methyl-4-phenylpyridinium (MPP$^+$) (40, 57). The activation of the protoxin MPTP is a two-step process: first, MPTP is oxidatively deaminated by enzyme MAO-B to form the intermediate, unstable compound 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP$^+$); and second, MPDP$^+$ spontaneously oxidizes, yielding MPP$^+$ (Fig. 1). This process takes place not in dopaminergic neurons, which are devoid of MAO-B, but primarily in glial cells and secon-

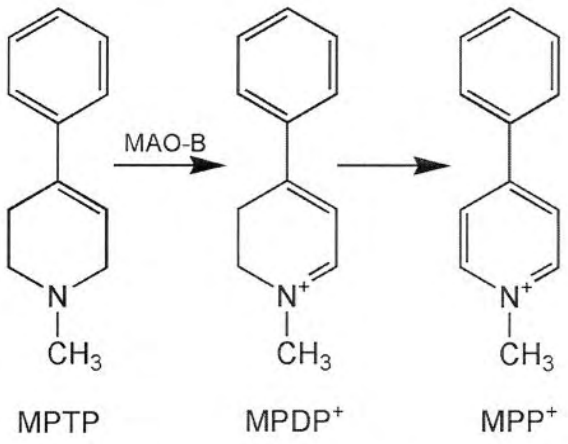


FIG. 1. Bioactivation of MPTP to MPP$^+$. The entire process of MPTP bioactivation takes place in serotonergic neurons and in astrocytes. The transformation of MPTP to MPDP$^+$ is catalyzed by MAO-B; the subsequent transformation of MPDP$^+$ to MPP$^+$ is spontaneous.

darily in serotonergic structures scarcely present in the vicinity of the nigrostriatal dopaminergic neurons. Once formed in these nondopaminergic cells, MPP^+ is released to the extracellular space, and through its binding to the plasma membrane dopamine transporter (44) it is translocated in dopaminergic neurons. Soon after its entry into dopaminergic neurons, MPP^+ participates in a variety of deleterious biochemical processes, among which many could generate oxidants. Although most of these oxidative reactions are taking place within the dopaminergic neuron itself, some meaningful reactive pathways originate from the surrounding glial cells. The current consensus in the field is that both the intrinsic and extrinsic oxidative stresses participate in the demise of nigrostriatal dopaminergic neurons in the MPTP model.

With respect to the intrinsic oxidative stress in the MPTP model, one of the main sources of the oxidant presumably emanates from the mitochondria. MPP^+, like rotenone, can accumulate within the mitochondria and bind to complex I of the electron transport chain (60). In doing so, MPP^+ interrupts the natural flow of electrons along this chain of cytochromes, which leads not only to an acute deficit in ATP formation, but also to an increased production of ROS, especially of superoxide (8, 35, 71). Because of the high amounts of Mn-SOD (SOD2) in the inner compartment of the mitochondria, it is likely that most, if not all, of the superoxide radicals produced by the blockade of complex I are immediately converted into H_2O_2. The latter, in contrast to the superoxide radical, could permeate through the mitochondrial membranes and thus can readily gain access to the cytosol. Accordingly, it is likely that mitochondrially generated superoxide may contribute to oxidative damage inside the mitochondria, whereas H_2O_2 may contribute to oxidative damage both inside and outside the mitochondria. These ROS may also engage in producing secondary and strong oxidants such as the hydroxyl radical, by reacting with an iron released from the destruction of mitochondrial aconitase (36), as well as with nitric oxide to generate peroxynitrite (43). Although there is little evidence that any of the reactive species cited above actually do inflict structural or functional mitochondrial damage in the MPTP model, the demonstration that transgenic mice with increased SOD2 activity are resistant to MPTP toxicity (47) argues that some type of MPP^+-mediated mitochondrial oxidative event has to be instrumental in the neurodegenerative process.

Presumably, ROS production can also occur in the MPTP model from the autooxidation of dopamine (54) resulting from an MPP^+-induced massive release of vesicular dopamine to the cytosol. Furthermore, the induction of cyclooxygenase-2 (COX-2) within the dopaminergic neurons after MPTP injection (42, 81) can also serve as a source of ROS. Indeed, via the peroxidase activity of COX-2, this enzyme can use catecholamines such as dopamine as an electron donor needed to catalyze the formation of dopamine-quinones. The latter may modify proteins by forming dopamine-cysteinyl adducts, which may have major consequences on the structure and function of modified proteins. In support of this scenario, we have found that, following MPTP injections to mice, contents of dopamine-cysteinyl in proteins increase markedly in a COX-2-dependent manner in affected brain regions (81).

The striking structural similarity between MPP^+ and paraquat (Fig. 2) has prompted several investigators to test the idea that MPP^+, like paraquat, could inflict oxidative stress via a reduction-oxidation cycling mechanism. Compared with paraquat, MPP^+ is an extremely stable species unlikely to undergo reduction–oxidation cycling (50). The reason paraquat is more reactive than MPP^+ relates to the double-positive charge on the paraquat, whereas MPP^+ has only one such charge (Fig. 2). For example, the one-electron reduction potential, which reflects the energy required to form the free radical, is -0.446 V for paraquat, well within the range of biological systems. In contrast, MPP^+ has a one-electron reduction potential of -1.18 V or greater, which is outside the range of known biological systems that might be involved in this reaction. Therefore, it seems unlikely that MPP^+ could participate in paraquat-like reduction–oxidation cycling unless an enzyme catalyzes it.

Although fierce discussions are still ongoing about which of these different sources of ROS, or combinations thereof, are implicated in MPTP neurotoxicity, there is compelling evidence that oxidative stress does play a critical role in the neurodegenerative process seen in this PD model. For instance, reduction of Cu/Zn-SOD (SOD1) activity by diethyl dithiocarbamate, which chelates copper and inhibits SOD1, or by genetic ablation of SOD1, potentiates MPTP-induced toxicity in mice (13, 90). The mirror opposite picture is found upon overexpressing human SOD1, in that transgenic mice with increased SOD1 activity are more resistant to MPTP (67). Although similar studies have not yet been done in rotenone, the toxicity of this other poison on dopaminergic cells appears also to implicate oxidative stress (74, 76).

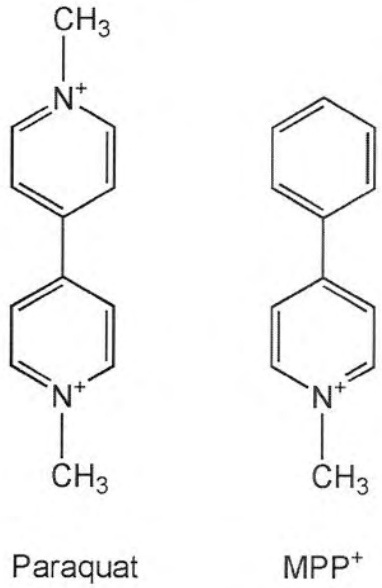


FIG. 2. Comparison of paraquat and MPP^+ chemical structures. Note the striking resemblance of the two compounds.

As already referred to above, ROS exert many or most of their toxic effects in the MPTP model in conjunction with other reactive species such as nitric oxide (1, 62, 69, 73) produced in the brain by both the neuronal and the inducible isoforms of the enzyme NOS (51, 68). A comprehensive review of the source and the role of nitric oxide in the MPTP model can be found in other references (63, 84).

Before discussing the extrinsic oxidative stress in the MPTP model, we should first emphasize the fact that the loss of dopaminergic neurons caused by both MPTP and rotenone is associated with a glial response (75, 85). Activation of microglia, which is quite pronounced in the MPTP and rotenone mouse models (14, 21, 48, 51, 75), reaches a maximum before the peak of dopaminergic neurodegeneration following the last MPTP injection (51). This observation has led to the idea that the MPTP- and rotenone-associated glial response may participate in the demise of dopaminergic neurons in these models. Studies showing that the blockade of microglial activation mitigates nigrostriatal damage caused by MPTP supports the notion that activated microglia participate in the neurodegenerative process (23, 87).

Activated microglial cells can produce a variety of noxious compounds, including ROS and RNS, proinflammatory cytokines, and prostaglandins. In many pathological settings, including MPTP injections, microglia activation involves the up-regulation of inducible NOS (21, 51) and the activation of NADPH oxidase (34). The former produces large amounts of nitric oxide in a calcium-independent manner, whereas the latter reduces oxygen to form superoxide radicals. Targeting inducible NOS by genetic interventions has shown that ablation of this enzyme, which reduces the production of nitric oxide, attenuates MPTP-induced neurotoxicity (21, 51). Similarly, mice defective in NADPH oxidase—and thus having reduced levels of extracellular superoxide—show less dopaminergic neuronal loss and protein oxidation than their wild-type littermates after MPTP injections (88). Further supporting the involvement of extracellular superoxide radicals in MPTP neurotoxicity is the finding that stereotaxic injection in the striatum of purified SOD1, which remains in the extracellular compartment, mitigates MPTP dopaminergic neurotoxicity on the infused side as compared with the noninfused side (88). Together, these findings indicate that the levels of extracellular nitric oxide and superoxide radicals are important components in the MPTP neurotoxic process.

CONCLUSIONS

This review summarized the molecular mechanisms underlying key neurotoxins used to model PD with a specific emphasis on oxidative stress. Whereas all four neurotoxins reviewed undoubtedly kill dopaminergic neurons, they all achieve this goal through different oxidative processes. By far the most complex of all appears to be that engendered by MPTP and, by analogy, probably by rotenone as well. If one is thus interested in the molecular biology behind dopaminergic neurotoxicity, it seems that MPTP, and by extension rotenone, may affect a greater variety of cellular pathways, perhaps making their study more appealing, but also more challenging. Nevertheless, whether the complexity of MPTP and rotenone oxidative processes more closely mimics the actual pathogenic cascade occurring in PD than the simpler oxidative processes engendered by 6-OHDA and paraquat is essentially unknown. Thus, if one is interested in testing new antioxidants for the treatment of PD, it may be necessary to preclinically ascertain the effectiveness of this putative neuroprotective intervention in more than one toxic model of PD.

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ABBREVIATIONS

COX-2, cyclooxygenase-2; H_2O_2, hydrogen peroxide; MAO, monoamine oxidase; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NOS, nitric oxide synthase; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; SOD, superoxide dismutase; SOD1, Cu/Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase.

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Proteasome Inhibition and Parkinson's Disease Modeling

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Impaired proteasome function is a potential mechanism for dopaminergic neuron degeneration. To model this molecular defect, we administered systemically the reversible lipophilic proteasome inhibitor, carbobenzoxy-L-isoleucyl-γ-t-butyl-L-glutamyl-L-alanyl-L-leucinal (PSI), to rodents. In contrast to a previous report, this approach failed to cause any detectable behavioral or neuropathological abnormality in either rats or mice. Although theoretically appealing, this specific model of Parkinson's disease appears to exhibit poor reproducibility.

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Parkinson's disease (PD) is the second most frequent neurodegenerative disorder after Alzheimer's dementia. The main clinical features of PD include resting tremor, bradykinesia, rigidity, and postural instability.[1] Emerging data indicate that PD neurodegeneration is multifactorial.[1] Although its core symptomatology is attributed to a striatal dopaminergic deficit due to a loss of dopaminergic neurons in the substantia nigra pars compacta (SNc),[1] neurodegenerative changes occur in several other brain regions.[2] The Lewy body, an eosinophilic proteinaceous inclusion, is another hallmark of the neuropathology of PD.[1] Recently, McNaught and coworkers[3] showed that systemic administration of the proteasome inhibitor PSI (carbobenzoxy-L-isoleucyl-γ-t-butyl-L-glutamyl-L-alanyl-L-leucinal) to rats emulated many of the aspects of the multisystem degeneration seen in PD and induced the formation of Lewy body–like inclusions. This study attempted to replicate this model in rats and to extend it to model PD in mice.

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Materials and Methods

Animals and Treatment

Both adult Sprague–Dawley rats (Taconic Farms, Germantown, NY) and C57/bl mice (Charles River Laboratories, Wilmington, MA) were housed in temperature-controlled, light/dark cycle–regulated rooms with food and water ad libitum. Animals were injected subcutaneously on the back with either 3.0 or 6.0mg/kg PSI (Calbiochem, San Diego, CA, lot#B64579) in 70% ethanol.[3] The PSI solution was prepared immediately before each injection by dissolving PSI in 100% ethanol at room temperature, vortexing the solution for 2 minutes to ensure that no particles remained, then diluting the PSI-100% ethanol solution to 70% with water. A total of six injections were made over 2 weeks in a manner identical to that described by McNaught and collaborators.[3] Control animals received vehicle only.

Immunostaining and Quantitative Morphology

At selected time points, animals were killed and their brains were processed for immunohistochemistry.[4] Anatomical landmarks for striatum and SNc in rats and mice as were in Przedborski and colleagues'[5] and Muthane and coworkers'[6] and serial cryostat-cut sections of 30μm, encompassing the entire striatum and SNc, were collected. Every eighth striatal and every seventh nigral section in rats, as well as every eighth striatal and fourth nigral section in mice, were immunostained free floating using a polyclonal anti–tyrosine hydroxylase (TH) antibody (1:1,000; Calbiochem, San Diego, CA[4]). Total numbers of SNc TH-positive neurons were counted by the stereological optical fractionator method[7] using the StereoInvestigator 6.5 software (MicroBrightField, Colchester, VT). Striatal optical density of TH fibers[4] was determined using the Scion Image program (Scion Corporation, Frederick, MD). Alternate sections of the SNc were also immunostained for the astrocyte marker glial fibrillary acidic protein,[7] the microglial marker Iba-1, and for ubiquitin[8] and α-synuclein.[9] Hematoxylin and eosin staining was done for anatomical reference.

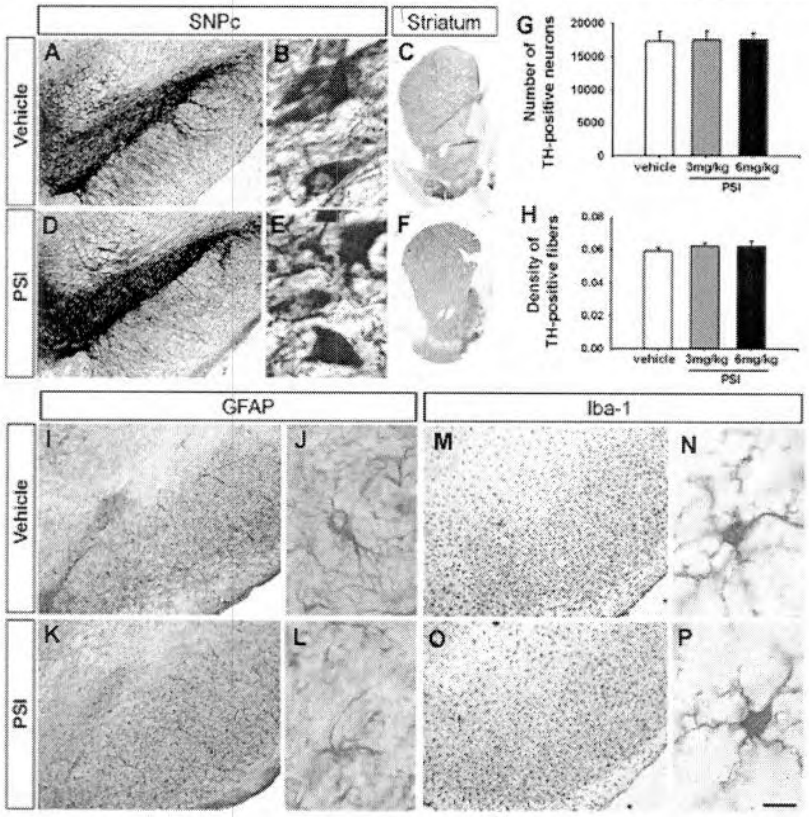

Fig 1. (A–F) Tyrosine hydroxylase (TH) immunostaining of rat tissue sections 12 weeks after the last injections of proteasome inhibitor PSI (D–F) or vehicle (A–C). Bar graphs showing TH-positive nigral neuronal counts (G) and striatal optical density (H). No difference was found between PSI and vehicle (one-way analysis of variance, $p > 0.05$). Data are means ± standard error of the mean. Glial fibrillary acidic protein– (GFAP; I–L) and Iba-1–immunostained (M–P) ventral midbrain sections showing comparable labeling between two groups. Scale bar = 200μm (A, D, I, K, M, O); 10μm (B, E, J, L, N, P); 2mm (C, F). SNPc = substantia nigra pars compacta.

SH-SY5Y Cell Culture and Flow Cytometry

Human neuroblastoma SH-SY5Y cells (kindly provided by Dr. D. Yamashiro, Columbia University, New York, NY) were stably transfected to express a reporter consisting of a short degron, CL1 fused to the C-terminus of green fluorescent protein (GFP^u; kindly provided by Dr. R. Kopito, Stanford University, Stanford, CA[10]). Cells were grown in Dulbecco's minimum essential medium/Ham's F12 medium (Invitrogen Corporation, La Jolla, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum. The proteasome inhibitor solution was made fresh by dissolving 1mg PSI in 100% ethanol and adjusting the concentration with water to obtain a solution of 10μM in 70% ethanol. This solution of PSI was then kept at room temperature for 0 to 6 hours, before being added to the culture medium (final concentration, 0.1nM). After a 24-hour incubation, cells were harvested and analyzed by flow cytometry for a GFP signal, as described previously.[11]

Generation of Transgenic GFP^u Mice and Primary Neuronal Culture

Transgenic mice were generated (L. Petrucelli's laboratory, Mayo Clinic Jacksonville, Jacksonville, FL) with the same GFP^u construct as above cloned into a murine prion protein vector.[12] The linearized construct was injected into the pronuclei of single-celled embryos harvested from B6/D2/SW mice and backcrossed to C57/bl mice. Postnatal cortical neurons from these mice were prepared[13] and plated onto cortical glia monolayers at a density of 400,000 (Western blot) or 80,000 (colocalization) neurons per well. Cultures were treated with MG-132 (5μM) or vehicle for 24 hours. Then cultures were fixed and immunostained for microtubule-associated protein 2 (MAP2). The secondary antibody was goat anti–mouse conjugated to Alexa Fluor 568.

Results

Four independent experiments with PSI (three in rats and one in mice) were performed and all yielded the same results. In each experiment, at least four animals for each PSI dose and for vehicle administration were used. Both the 3 and 6mg/kg doses of PSI were well tolerated. No animal died prematurely or experienced serious distress requiring euthanasia. However, the site of the subcutaneous injection had to be changed slightly with each injection to avoid hardening of the

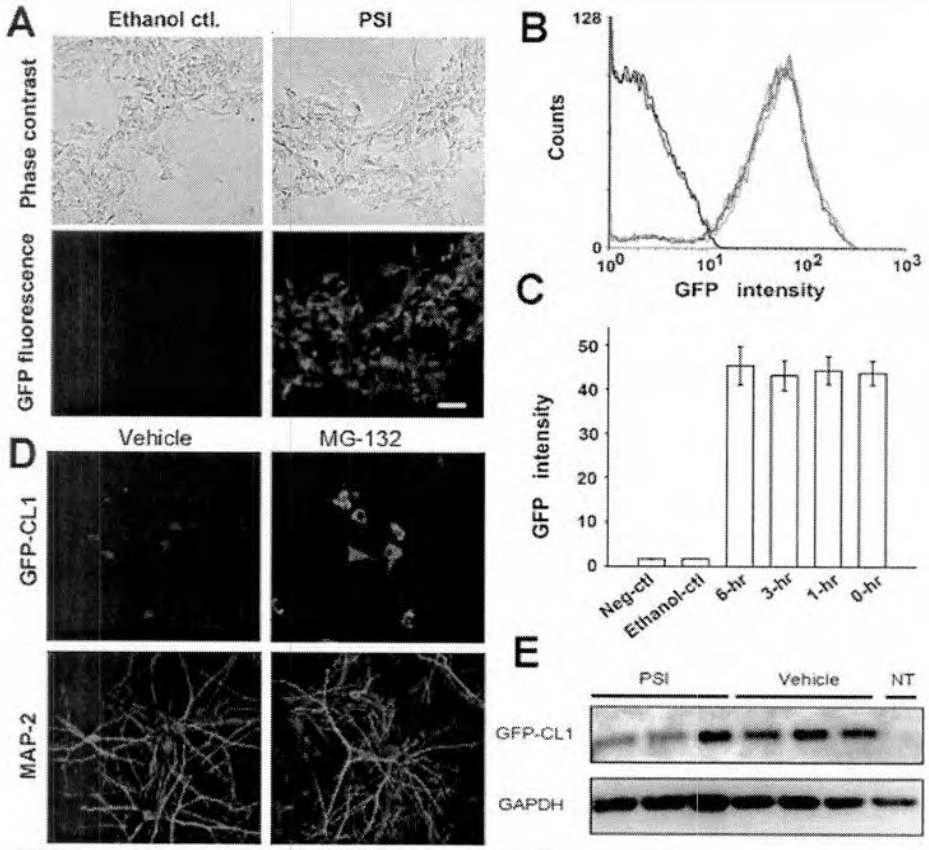

Fig 2. (A) Cultured SH-SY5Y cells expressing GFP^u. (B) Representative flow cytometry analysis of three independent experiments: red, negative control; black, 70% ethanol control; green, 6-hour-old proteasome inhibitor PSI solution; blue, 3-hour-old PSI solution; purple, 1-hour-old PSI solution; cyan, 0-hour-old PSI solution. (C) Bar graph showing green fluorescent protein (GFP) fluorescence intensity (geometric mean) for each condition. No differences were found among the different conditions (one-way analysis of variance, $p > 0.05$). Data are means ± standard error of the mean. (D) Increased expression in GFP-CL1 neurons with proteasomal inhibition. Seven-day-old neurons treated with MG-132 (5μM, 24 hours) showing significant accumulation of GFP-CL1 protein in both cytoplasm and nucleus. (E) Western blot of whole-brain homogenates from PSI-treated mice (9mg/kg) shows no increase in accumulation of GFP^u reporter compared with control. Scale bar = 20μm (A). MAP-2 = microtubule-associated protein 2.

derma, which we noted in our pilot studies, which was caused by the subcutaneous administration of 70% ethanol.

From 2 to 12 weeks after the final injections of PSI, animals were inspected daily for behavioral abnormalities[3] by two investigators blinded to the treatment assignments. Neither rats nor mice developed any apparent alteration in spontaneous motor activity, exploratory behavior, or grooming behavior up to 12 weeks after PSI injections compared with vehicle control animals. Food intake and weight curves were similar between PSI and control animals.

Macroscopically, neither striatal TH optical density nor SNc TH-positive neuronal counts differed between PSI and control animals (Fig 1A–H). Stereological counts of TH-positive neurons were performed independently on the same sets of tissue sections in two of our (S.P.'s and J.H.K.'s) laboratories by investigators blinded to the treatment assignments. A statistical analysis of the two sets of counts yielded a positive linear correlation ($r = 0.89$; $p = 0.0016$), supporting strong agreement between the counts performed by the two laboratories. We also failed to detect any evidence of SNc intraneuronal proteinaceous inclusions in midbrain sections immunostained for ubiquitin or α-synuclein (data not shown). Also absent was SNc glial alteration in midbrain sections (see Fig 1I–P).

To test the potency of PSI over time once in solution, we assessed by flow cytometry the intensity of GFP^u as an index of proteasomal inhibition in living SH-SY5Y cells. In our pilot studies, we found that 0.1nM PSI consistently produced a robust proteasomal inhibition, in concert with less than 10% cell death, by 24 hours. Under these experimental conditions, we found that 0.1nM PSI caused a similar magnitude of proteasomal inhibition whether used immediately after being put into solution or only after the solution was kept at room temperature for 6 hours (Fig 2A–C).

To examine the effect of systemic PSI administration on brain proteasomal function, we used transgenic mice expressing GFP^u. Cultured transgenic neurons treated with 10μM MG-132 for 24 hours exhibited a pronounced accumulation of GFP^u (see Fig 2D), thus confirming the responsiveness of the GFP^u transgene system to proteasomal inhibition. However, Western blot analyses of brain homogenates from transgenic GFP^u mice that received 6mg/kg and even 9mg/kg PSI, following the aforementioned injection schedule, did not demonstrate any evidence of brain GFP^u accumulation (Fig 2E).

Discussion

The above data indicate that, after several attempts, we were unable to induce any behavioral or basal ganglia histological alterations in rodents with systemic administration of the proteasome inhibitor PSI. Our failure is in striking contrast to the overt PD-like motor abnormalities and nigrostriatal pathway damage accompanied by α-synuclein–positive intraneuronal inclusions that McNaught and collaborators[3] reported in rats using apparently the exact same experimental protocol. Other attempts to replicate McNaught and colleagues'[3] findings have met with inconsistent success.[14–19] In our hands, this regimen of PSI also failed to enhance (or attenuate) MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) dopaminergic neurotoxicity in mice (data not shown). Could it be that PSI did not enter the brain or lost its potency before injection? The profound dopaminergic neurotoxicity that McNaught and collaborators[3] reported would argue that PSI accumulates in the brain. However, using the McNaught and colleagues[3] protocol of PSI administration, even at higher doses, we were unable to detect any alteration in brain proteasomal function by utilizing transgenic GFP^u mice. Thus, our data challenge the notion that, under the present systemic regimen, PSI reaches effective inhibitory brain concentrations. It is also unlikely that the discrepancy rests with a problem in our PSI solution as we followed strictly these authors' recommendations for PSI preparation and injections and have used the same lot of drug from the same vendor. Allegedly, PSI, once in 70% ethanol solution, rapidly loses its biological potency. Contrary to this belief, we show here that the inhibitory effects of PSI on proteasomal function remain unimpaired for at least 6 hours at room temperature. Under strict experimental conditions, neurotoxins such as rotenone or MPTP do not damage the nigrostriatal pathway in all injected animals. For instance, with rotenone, about 40% of the treated rats are lost because of severe toxicity, and among those that survive, only 50% develop nigrostriatal damage, and only Lewis rats show a consistent lesion.[20] This fact cannot account for the discrepancy either, because none of the 42 rats exhibited any alteration, whereas virtually all of McNaught and colleagues'[3] animals did. In one additional experiment, we also used dimethylsulfoxide instead of ethanol as vehicle (data not shown), but this also failed to produce any effect on the nigrostriatal pathway in rats.

Based on our data in rodents, it may be concluded that the systemic PSI model of PD cannot readily be reproduced by following the published protocol.[3] Perhaps, some amendments to the original protocol are required for this approach to be successful. Alternatively, it is also plausible that some unidentified technical or biochemical factors may be responsible for this glaring discrepancy. In any event, it is our belief that, currently, the enthusiasm for this new model be tempered until the critical issue of reproducibility of this model is elucidated. That being said, the failure by investigators other than McNaught and colleagues[3] to reproduce their results in rats should by no means undermine the enthusiasm and efforts put into characterizing the actual role of protein degradation pathways in dopaminergic neuron degeneration and in the pathogenesis of PD.

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Failure of Proteasome Inhibitor Administration to Provide a Model of Parkinson's Disease in Rats and Monkeys

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McNaught and colleagues[1] reported recently that systemic administration of proteasome inhibitors PSI (Z-Ileu-Glu(O*t*Bu)-Ala-Leu-CHO) or epoxomicin recapitulated many of the degenerative changes seen in Parkinson's disease including loss of striatal dopamine and cell loss in the substantia nigra, locus ceruleus, dorsal motor nucleus of the X cranial nerve, and nucleus basalis of Meynert. Intracytoplasmic inclusions resembling Lewy bodies were also described. All experiments administering PSI to rats using identical procedures and multiple attempts failed to induce any of the previously described changes. Furthermore, administration of PSI or epoxomicin to monkeys in an attempt to extend the model to a primate species failed. Currently, systemic proteasome inhibition is not a reliable model for Parkinson's disease.

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Parkinson's disease (PD) is defined clinically by the presence of bradykinesia, rigidity, resting tremor, and postural instability.[2] Degeneration of the dopaminergic nigral neurons and the presence of α-synuclein–containing Lewy bodies define the disease pathologically.[3,4] Although less appreciated, it is clear that multiple brain regions degenerate in PD,[3] and this pathology is likely responsible for the motor problems that respond poorly to L-dopa, as well as the numerous nonmotor symptoms of PD that include autonomic dysfunction, cognitive decline, sleep disturbances, constipation, anosmia, and depression. Finding novel therapeutic strategies for PD has been problematic due to the absence of appropriate models that mimic the temporal pattern of

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Interplay between oxidative stress and inflammation in Parkinson's Disease

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Experimental models of dopaminergic neurodegeneration play a critical role in our quest to elucidate the pathogenesis of Parkinson's disease (PD). Despite the recent development of "genetic models" which have emanated from the discovery of mutations causing rare forms of familial PD, toxic models remain at the forefront of this type of endeavor. Among these, the model produced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has a competitive advantage over all other toxic models because, upon intoxication, it induces in humans a syndrome virtually identical to PD. For the past two decades, the key steps in the MPTP neurotoxic process have been unraveled. These molecular events can be classified into three groups. First, those implicated in the initiation of toxicity, which include an oxidative stress originating from either mitochondrial defect, vesicular dopamine leakage, or both. While these alterations are crucial in triggering the toxic process, it seems that only a small fraction of dopaminergic neurons actually succumb to these early alterations. Instead, mounting evidence indicates that these early perturbations activate death-related molecular pathways which include apoptosis, JNK, and COX-2. The third component of the pathogenic cascade involves cellular and molecular changes which amplify the neurodegenerative insult and includes various pro-inflammatory factors. Among the latter, oxidative stress mediated by glial cells has emerged as a pivotal element in modulating the neurodegenerative process. During the present talk, these different contributing factors will be discussed. The sequence in which it is believed that various factors intervene within the pathogenic cascade, responsible for the death of dopaminergic neurons in the MPTP model and in PD, will also be discussed. In light of these results, it may thus be concluded that PD pathogenesis likely derives from a complex interplay between oxidative stress and inflammation. If correct, this multifactorial scenario may help in improving the design of neuroprotective strategies of this common disabling age-related disease.

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Toxic animal models

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Introduction

As discussed elsewhere (Przedborski *et al.*, 2003), the term 'neurodegenerative disease' refers to a group of neurological disorders with heterogeneous clinical and pathological expressions. These diseases are all characterized by a loss of specific subpopulations of neurons confined to functional anatomic systems, arising in most cases for unknown reasons and progressing in a relentless manner. Among the variety of neurodegenerative disorders, the lion's share of attention has been given only to a handful, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). So far, the most consistent risk factor for developing a neurodegenerative disorder, especially AD and PD, is increasing age. Over the past century the growth rate of the population age 65 and beyond in the industrialized countries has far exceeded that of the population as a whole. Thus it can be anticipated that, over the next generations, the proportion of elderly citizens will double and, with this, the number of individuals suffering from a neurodegenerative disorder. This prediction is at the centre of the growing concerns from the medical community and from legislators, as one can easily foresee a dramatic increase in the emotional, physical, and financial burden on patients, caregivers, and society related to these disabling illnesses. The problem is made worse by the fact that, although to date several approved drugs do, to some extent, alleviate symptoms of several neurodegenerative diseases, their chronic use is often associated with debilitating side effects, and none seems to stop the progression of the degenerative processes. It is now clear that the development of effective preventive or protective therapies has been thus far impeded by our limited knowledge of the causes and mechanisms by which neurons die in neurodegenerative diseases. Thanks to the development of experimental models, several neurobiological breakthroughs have brought closer than ever the day that the secrets of several neurodegenerative disorders will be unlocked and effective therapeutic strategies will become available. So far, among the various accepted experimental models, neurotoxins have been the most popular tools employed to produce selective neuronal death in both in vitro and in vivo systems. In this chapter, we will thus review the key neurotoxic models of neurodegeneration, which will be categorized based on their main molecular mechanisms as we currently know them. For each of the selected neurotoxins, attention will be paid to providing the reader with information regarding their respective mechanisms of action and the kinds of diseases that they model. Although in vitro data will be mentioned whenever necessary, this chapter will focus on in vivo studies.

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a byproduct of the chemical synthesis of a meperidine analog with potent heroin-like effects. MPTP can induce a parkinsonian syndrome in humans almost indistinguishable from PD (Langston & Irwin, 1986). Recognition of MPTP as a neurotoxin occurred early in 1982, when several young drug addicts mysteriously developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogs which, unknown to anyone, were contaminated with MPTP (Langston *et al.*, 1983). In humans and non-human primates, depending on the regimen used, MPTP can produce an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD; in non-human primates, a resting tremor

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characteristic of PD has only been demonstrated convincingly in the African green monkey (Tetrud *et al.*, 1986). It is believed that, in PD, the neurodegenerative process occurs over several years, while the most active phase of neurodegeneration is completed within a few days following MPTP administration (Langston, 1987; Jackson-Lewis *et al.*, 1995). However, recent data suggest that, following the main phase of neuronal death, MPTP-induced neurodegeneration may continue to progress 'silently' over several decades, at least in humans intoxicated with MPTP (Vingerhoets *et al.*, 1994; Langston *et al.*, 1999). Except for four cases (Davis *et al.*, 1979; Langston *et al.*, 1999), no human pathological material has been available for study and thus the comparison between PD and the MPTP model is mainly based on non-humam primates (Forno *et al.*, 1993). Neuropathological data show that MPTP administration causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD (Dauer & Przedborski, 2003), yet there is a resemblance that goes beyond the loss of SNpc dopaminergic neurons. Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area (Seniuk *et al.*, 1990; Muthane *et al.*, 1994) and, in monkeys treated with low doses of MPTP (but not in humans), a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus (Moratalla *et al.*, 1992; Snow *et al.*, 2000). However, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, pigmented nuclei such as the locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions, called 'Lewy bodies,' so characteristic of PD, thus far, have not been convincingly observed in MPTP-induced parkinsonism (Forno *et al.*, 1993); although, in MPTP-injected monkeys, intraneuronal inclusions reminiscent of Lewy bodies have been described (Forno *et al.*, 1986). Despite these imperfections, MPTP continues to be regarded as the best animal model of PD, and the belief is that studying MPTP toxic mechanisms will shed light on the molecular basis of PD.

Over the years, MPTP has been used in a large variety of animal species, ranging from worms to mammals. To date, the most frequently used animals for MPTP studies are monkeys, mice and rats. The administration of MPTP through a number of different routes using different dosing regimens has led to the development of several distinct models, each characterized by some unique behavioral and biochemical features. The manner in which these models were developed is based on the concept of delivering MPTP in a fashion that creates the most severe and stable form of nigrostriatal damage with the least number of undesirable side effects such as acute death, dehydration and malnutrition. Although MPTP can be given by a number of different routes, including gavage and stereotaxic injection into the brain, the most common, reliable, and reproducible lesion is provided by its systemic administration (i.e. subcutaneous, intravenous, intraperitoneal or intramuscular). The most commonly used regimens in monkeys are the multiple intraperitoneal or intramuscular injections and the intracarotid infusion of MPTP (Petzinger & Langston, 1998). The former is easy to perform and produces a bilateral parkinsonian syndrome. However, often the monkey exhibits a generalized parkinsonian syndrome so severe that chronic administration of drugs such as L-DOPA is required to enable the animal to eat and drink adequately (Petzinger & Langston, 1998). Although unilateral intracarotid infusion is technically more difficult, this regimen causes symptoms mainly on one side (Bankiewicz *et al.*, 1986; Przedborski *et al.*, 1991), so it has the advantage of allowing the monkey to maintain normal nutrition and hydration without the use of L-DOPA,. For many years monkeys were mainly, if not exclusively, treated with harsh regimens of MPTP to produce an acute and severe dopaminergic neurodegeneration (Petzinger and Langston, 1998). More recently, several investigators have treated monkeys with low doses of MPTP (e.g. 0.05 mg/kg 2–3 times per week) for a prolonged period of time (i.e. weeks to months) in an attempt to better model the slow neurodegenerative process of PD (Schneider & Roeltgen, 1993; Bezard *et al.*, 1997; Schneider *et al.*, 1999). While both the acute and the chronic MPTP-monkey models are appropriate for the testing of experimental therapies aimed at alleviating PD symptoms, the chronic model is presumably the most suitable for testing neuroprotective strategies. Among the numerous mammalian species susceptible to MPTP (Kopin & Markey, 1988; Heikkila *et al.*, 1989; Przedborski *et al.*, 2000), in addition to monkeys, mice have become the animals most commonly used. However, several problems need to be emphasized. First, mice are much less sensitive to MPTP than monkeys; thus, much higher doses are required to produce significant SNpc damage in this animal species. Higher doses present a far greater hazard, requiring tighter safety precautions (Przedborski *et al.*, 2001b). Second, unlike monkeys, mice treated with MPTP do not develop parkinsonism. Third, the magnitude of striatal dopamine nigrostriatal damage depends on the dose and dosing schedule (Sonsalla & Heikkila, 1986). The use of MPTP in rats presents an interesting situation (Kopin & Markey, 1988). For instance, rats injected with mg/kg doses of MPTP comparable to those used in mice do not exhibit any significant dopaminergic neurodegeneration (Giovanni *et al.*, 1994a,b). Conversely, rats

injected with much higher doses of MPTP do exhibit significant dopaminergic neurodegeneration (Giovanni *et al.*, 1994a,b) although, at these high doses, rats have to be pretreated with guanethidine to prevent dramatic peripheral catecholamine release and extensive mortality (Giovanni *et al.*, 1994a). These findings indicate that rats are relatively insensitive to MPTP, but regardless of this drawback, rats continue to be used quite often in MPTP studies (Storey *et al.*, 1992; Giovanni *et al.*, 1994a,b; Staal & Sonsalla, 2000; Staal *et al.*, 2000). In rats, the systemic administration of MPTP is rarely used and the vast majority of studies involve the stereotaxic infusion of 1-methyl-4-phenylpyridinium (MPP^+), a toxic metabolite of MPTP (Storey *et al.*, 1992; Giovanni *et al.*, 1994a,b; Staal & Sonsalla, 2000; Staal *et al.*, 2000).

Prior to using the MPTP model it is crucial to remember that several factors influence the reproducibility of the lesion in monkeys, rats and mice. However, to our knowledge, an extensive and systematic assessment of these factors has only been done in mice (Heikkila *et al.*, 1989; Giovanni *et al.*, 1991, 1994a,b; Miller *et al.*, 1998; Hamre *et al.*, 1999; Staal & Sonsalla, 2000) and the results can be summarized as follows: different strains of mice (and even within a given strain, mice from different vendors) can exhibit strikingly distinct sensitivity to MPTP. This differential seems to be inherited as an autosomal dominant trait (Hamre *et al.*, 1999). As discussed in greater detail in (Przedborski & Vila, 2001), gender, age and body weight are also factors that modulate MPTP sensitivity and reproducibility of the lesion.

MPTP mode of action

As illustrated in Fig. 16.1, the metabolism of MPTP is a complex, multistep process (Przedborski & Jackson-Lewis, 1998; Dauer & Przedborski, 2003). After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood–brain barrier (BBB). Once in the brain, the pro-toxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) within non-dopaminergic cells such as glial cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. Thereafter, MPP^+ is released (by an unknown mechanism) into the extracellular space. Since MPP^+ is a polar molecule, unlike its precursor MPTP, it cannot freely exit cells within which it has been produced or enter cells within which it will cause toxicity. It is thus likely that the translocation of MPP^+ from its site of production to its site of cytotoxicity depends on the plasma membrane carriers. Consistent with this view is the fact that MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT) (Mayer *et al.*, 1986), as well as for norepinephrine and serotonin transporters, enabling it to gain access to monoaminergic neurons and more particularly dopaminergic neurons. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol (Javitch *et al.*, 1985) or ablation of the DAT gene in

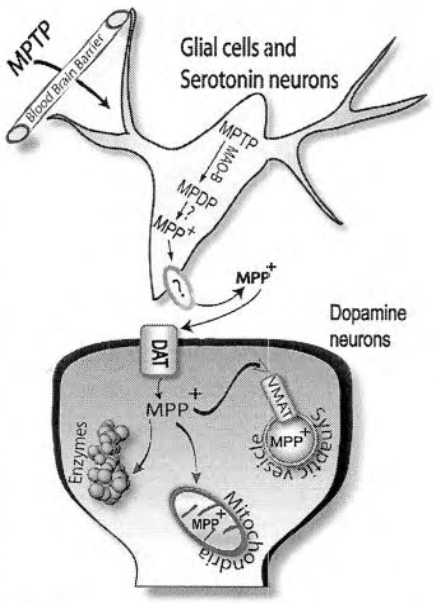

Fig. 16.1 Schematic representation of MPTP metabolism. After its systemic administration, MPTP crosses the blood-brain barrier. Once in the brain, MPTP is converted to $MPDP^+$ by MAO-B within non-dopaminergic cells, and then to MPP^+ by an unknown mechanism (?). Thereafter, MPP^+ is released, again by an unknown mechanism (?), into the extracellular space. From there, MPP^+ is taken up by the DAT and thus enter dopaminergic neurons. Inside dopaminergic neurons, MPP^+ can (i) bind to the vesicular monoamine transporters (VMAT) and translocate into synaptosomal vesicles, (ii) accumulate into the mitochondria by an active process, and (iii) remain in the cytosol and interact with different cytosolic enzymes.

mutant mice (Bezard *et al.*, 1999) completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP (Donovan *et al.*, 1999).

Once inside dopaminergic neurons, MPP^+ can follow at least three routes (Fig. 16.1): (i) it can bind to the vesicular monoamine transporters (VMAT) which will translocate MPP^+ into synaptosomal vesicles (Liu *et al.*, 1992), (ii) it can be concentrated within the mitochondria (Ramsay & Singer, 1986), and (iii) it can remain in the cytosol and interact with different cytosolic enzymes (Klaidman *et al.*, 1993). The fraction of MPP^+ destined to each of these routes is probably a function of MPP^+ intracellular concentration and affinity for VMAT, mitochondria carriers, and cytosolic enzymes. The importance of the vesicular sequestration of MPP^+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells (Liu *et al.*, 1992). Conversely, we demonstrated that mutant mice with 50% lower VMAT expression are significantly more sensitive to MPTP-induced dopaminergic neurotoxicity compared to their wild-type littermates (Takahashi *et al.*, 1997). These findings indicate that there is a clear inverse relationship between the capacity of MPP^+ sequestration (i.e. VMAT density) and the magnitude of MPTP neurotoxicity. Inside dopaminergic neurons, MPP^+ can also be concentrated within the mitochondria (Fig. 16.1) (Ramsay & Singer, 1986), where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain (Nicklas *et al.*, 1985; Mizuno *et al.*, 1987) through its binding at or near the site of the mitochondrial poison rotenone (Ramsay *et al.*, 1991; Higgins, Jr. & Greenamyre, 1996; Schuler & Casida, 2001).

MPTP mechanism of action

Currently, it is believed that the neurotoxic process of MPTP is made up of a cascade of multiple deleterious events, which can be divided into early and late neuronal perturbations and secondary non-neuronal alterations. All of these, to a variable degree and at different stages of the degenerative process, participate in the ultimate demise of dopaminergic neurons.

Early events

Soon after its entry into dopaminergic neurons, MPP^+ binds to complex I and, by interrupting the flow of electrons, leads to an acute deficit in ATP formation (Chan *et al.*, 1992). It appears, however, that complex I activity should be reduced >70% to cause severe ATP depletion in non-synaptic mitochondria (Davey & Clark, 1996) and that, in contrast to in vitro, in vivo MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (Chan *et al.*, 1991), raising the question as to whether MPP^+-related ATP deficit can be the sole factor underlying MPTP-induced dopaminergic neuronal death.

Another consequence of complex I inhibition by MPP^+ is an increased production of reactive oxygen species (ROS), especially of superoxide (Rossetti *et al.*, 1988; Hasegawa *et al.*, 1990; Cleeter *et al.*, 1992). Early ROS production can also occur in this model from the auto-oxidation of dopamine resulting from an MPP^+-induced massive release of vesicular dopamine to the cytosol (Lotharius & O'Malley, 2000). Dopamine can also be oxidized to the highly reactive dopamine-quinone by cyclooxygenase-2, an enzyme which is upregulated in SNpc dopaminergic neurons in MPTP-intoxicated mice and PD postmortem samples (Teismann *et al.*, 2003). The importance of MPP^+-related ROS production in the dopaminergic toxicity process in vivo is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1), a key ROS scavenging enzyme, are significantly more resistant to MPTP-induced dopaminergic toxicity than their non-transgenic littermates (Przedborski *et al.*, 1992). However, several lines of evidence support the concept that ROS exert many or most of their toxic effects in the MPTP model in conjunction with other reactive species such as nitric oxide (NO) (Schulz *et al.*, 1995b; Ara *et al.*, 1998; Pennathur *et al.*, 1999; Przedborski *et al.*, 2001a) produced in the brain by both the neuronal and the inducible isoforms of the enzyme NO synthase (Przedborski *et al.*, 1996; Liberatore *et al.*, 1999). A comprehensive review of the source and the role of NO in the MPTP model can be found in the following references (Przedborski & Vila, 2001; Przedborski & Dawson, 2001; Tieu *et al.*, 2003).

Late events

In response to the variety of functional perturbations caused by the depletion in ATP and the production of ROS, death signals, which can activate the molecular pathways of apoptosis, arise within intoxicated dopaminergic neurons. Although at this time we cannot exclude with certainty the possibility that apoptotic factors are not consistently recruited regardless of MPTP regimen, only prolonged administration of low to moderate doses of MPTP is associated with definite morphologically defined apoptotic neurons (Jackson-Lewis *et al.*, 1995; Tatton & Kish, 1997). Supporting the implication of apoptotic molecular factors in the demise of dopaminergic neurons after MPTP administration is the demonstration that the pro-apoptotic protein Bax is instrumental in this toxic model (Vila *et al.*, 2001a). Overexpression of the anti-apoptotic Bcl-2 also

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protects dopaminergic cells against MPTP-induced neurodegeneration (Offen *et al.*, 1998; Yang *et al.*, 1998). Similarly, adenovirus-mediated transgene expression of the X-chromosome-linked inhibitor of apoptosis protein (XIAP), an inhibitor of executioner caspases such as caspase-3, also blocks the death of dopaminergic neurons in the SNpc following the administration of MPTP (Xu *et al.*, 1999; Eberhardt *et al.*, 2000). Other caspases are also activated in MPTP-intoxicated mice such as caspase-8, which is a proximal effector of the tumor necrosis factor receptor family death pathway (Hartmann *et al.*, 2001). Other observations supporting a role of apoptosis in the MPTP neurotoxic process include the demonstration of the resistance to MPTP of mutant mice deficient in p53 (Trimmer *et al.*, 1996), a cell cycle control gene involved in programmed cell death, or the resistance of mice treated with inhibitors of c-Jun N-terminal kinases (Saporito *et al.*, 1999, 2000). Collectively, these data show that, during the degenerative process, the apoptotic pathways are activated and contribute to the actual death of intoxicated neurons in the MPTP model.

Secondary events

The loss of dopaminergic neurons in the MPTP mouse model is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes (Vila *et al.*, 2001b). From a neuropathological standpoint, microglial activation is indicative of an active, ongoing process of cell death. The presence of activated microglia in postmortem samples from MPTP-intoxicated individuals who came to autopsy several decades after being exposed to the toxin (Langston *et al.*, 1999) suggests an ongoing degenerative process and thus challenges the notion that MPTP produces a 'hit-and-run' kind of damage. Therefore, this important observation (Langston *et al.*, 1999) suggests that a single acute insult to the SNpc by MPTP could set in motion a self-sustained cascade of events with long-lasting deleterious effects. Looking at mice injected with MPTP and killed at different time points thereafter, it appears that the time course of reactive astrocyte formation parallels that of dopaminergic structure destruction in both the striatum and the SNpc, and that GFAP expression remains upregulated even after the main wave of neuronal death has passed (Czlonkowska *et al.*, 1996; Kohutnicka *et al.*, 1998; Liberatore *et al.*, 1999). These findings suggest that, in the MPTP mouse model (Przedborski *et al.*, 2000), the astrocytic reaction is secondary to the death of neurons and not the reverse. This conclusion is supported by the demonstration that blockade of MPP^+ uptake into dopaminergic neurons completely prevents not only SNpc dopaminergic neuronal death but also GFAP up-regulation (O'Callaghan *et al.*, 1990). Remarkably, activation of microglial cells, which is also quite strong in the MPTP mouse model (Czlonkowska *et al.*, 1996; Kohutnicka *et al.*, 1998; Liberatore *et al.*, 1999; Dehmer *et al.*, 2000), occurs much earlier than that of astrocytes and, more importantly, reaches a maximum before the peak of dopaminergic neurodegeneration (Liberatore *et al.*, 1999). In the light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc occurs within a timeframe allowing these glial cells to participate in the demise of dopaminergic neurons in the MPTP mouse model and possibly in PD. Activated microglial cells can produce a variety of noxious compounds including ROS and reactive nitrogen species (RNS) via NADPH-oxidase (Wu *et al.*, 2003) and inducible NOS (Liberatore *et al.*, 1999) as well as pro-inflammatory cytokines and prostaglandins. Observations showing that blockade of microglial activation mitigates nigrostriatal damage caused by MPTP supports the notion that microglia participate in MPTP-induced neurodegeneration (Wu *et al.*, 2002).

Reactive oxygen species-producing neurotoxins

Reactive oxygen species, such as superoxide radicals, are produced constantly during normal cellular metabolism, primarily as byproducts of the mitochondrial respiratory chain and, when it applies, catecholamine metabolism. Defense mechanisms, however, exist to limit the levels of ROS and the damage they inflict on cellular components such as lipids, proteins, and DNA. It has been hypothesized that the finely tuned balance between the production and destruction of ROS is skewed in a number of pathological conditions including neurodegenerative disorders such as Parkinson's disease, resulting in oxidative stress that leads to severe cellular dysfunction and, ultimately, to cell death. For this reason, ROS-generating neurotoxins such as 6-hydroxydopamine (6-OHDA) and paraquat have been extensively used to model neurodegeneration.

6-Hydroxydopamine and related compounds

Since the introduction of 6-OHDA more than 30 years ago, several related compounds have been synthesized and tested both in vitro and in vivo. So far, however, none of them have been found to be superior to 6-OHDA as a monoamine neurotoxin. Among the 6-OHDA analogues, only 6-OH-DOPA and 6-aminodopamine have been used to a certain extent, especially to damage noradrenergic pathways. Yet, the much greater body of information available on the use of 6-OHDA over any other of its derivatives

(Jonsson, 1980) may also explain why 6-OHDA is the preferred compound of its group for producing catecholaminergic lesions. With respect to the mode and mechanism of action, it is well established that 6-OHDA accumulates specifically in catecholaminergic neurons, causes depletion of norepinephrine and dopamine and, ultimately, inflicts structural damage to these neurons (Jonsson, 1983). The specificity of 6-OHDA's action on catecholaminergic neurons is explained by the high affinity of the toxin for the plasma membrane catecholamine transporter, allowing 6-OHDA to be rapidly and efficiently taken up by norepinephrine and dopamine neurons. Given this fact, it is not surprising that pre-treatment of animals with a norepinephrine (Herve *et al.*, 1986; Luthman *et al.*, 1989) or dopamine uptake blocker (Luthman *et al.*, 1987, 1989), by preventing the entry of the toxin in these neurons, mitigates 6-OHDA cytotoxicity. Once inside of the neurons, 6-OHDA accumulates in the cytosol where it produces quinones, which inactivate some biological macromolecules by binding to their nucleophilic groups (Saner & Thoenen, 1971), as well as a variety of ROS (Cohen & Werner, 1994). Although quinone-based and ROS-based mechanisms are probably both operative in 6-OHDA cytotoxicity, available evidence favors the notion that ROS play the dominant neurotoxic role (Graham, 1978; Cohen & Werner, 1994). Of note, since 6-OHDA forms the *para*-quinone it does not react as well with external nucleophiles as the dopamine *ortho*-quinone does. ROS generated by the metabolism of 6-OHDA emanate from its autooxidation and from its oxidative deamination mediated by the MAO. However, the finding that pretreatment with MAO inhibitor such as pargyline, rather than mitigating 6-OHDA toxicity, instead enhances it (Jonsson, 1980), suggests that auto-oxidation and not oxidative deamination is instrumental in 6-OHDA-induced neuronal death. Because auto-oxidation of 6-OHDA can occur both intra- and extraneuronally, this neurotoxin is prone to causing specific and nonspecific effects. Consistent with this view is the demonstration that, in mesencephalic cultures, 6-OHDA toxicity is not restricted to dopaminergic neurons (Lotharius *et al.*, 1999), and that several cell types devoid of catecholaminergic transporters including C6 glioma, NIH-3T3, and CHO cells can be damaged by the toxin (Blum *et al.*, 2001). It is thus important to use as low a dose of 6-OHDA as possible and to prevent auto-oxidation of 6-OHDA from occurring extraneuronally by, for example, administering a reducing agent such as ascorbic acid with 6-OHDA. Under experimental conditions devoid of non-specific toxicity, 6-OHDA affects primarily dopaminergic and noradrenergic neurons, much less peripheral adrenergic neurons, and, according to most reports, not at all central adrenergic and serotoninergic neurons (at least in rodents). Among the various central catecholaminergic pathways, the locus coeruleus noradrenergic system (nerve terminals) appears to be the one most sensitive to 6-OHDA, whereas the adrenergic neuron systems are most resistant (Jonsson *et al.*, 1976). The dopaminergic systems range from being relatively sensitive (e.g. the nigrostriatal pathway) to almost completely resistant (e.g. the tuberoinfundibular pathway) to 6-OHDA (Jonsson, 1980). In general, it has been observed that the nerve terminals appear to be the most sensitive, that the axons are less so, and that the cell body structures are least sensitive to the neurotoxic action of 6-OHDA (Jonsson, 1983). These differences can at least partly be related to differences in surface–volume relationships. Since the 6-OHDA dose, injection volume, time and route of administration, and the injection technique are the crucial factors, one may find it worthwhile to refer to Jonsson (1980) to optimize lesioning conditions.

In adult animals, 6-OHDA has been used most commonly in rats and to a lesser extent in mice, cats, dogs, and monkeys (Jonsson, 1983), but no one species has a real scientific advantage over the other. Since 6-OHDA does not easily cross the BBB it has to be injected intraventricularly, intracisternally, or intracerebrally to damage the central catecholaminergic systems (Jonsson, 1983). Intraventricular and intracisternal administration of 6-OHDA calls for high doses (500–1000 μg/kg in 25–50 μl) and produces a bilateral catecholaminergic lesion, observed within a few hours of the injection of the toxin, with generally limited regrowth of affected nerve fibers (Jonsson, 1983). Unexpectedly, these routes of administration produce a very heterogenous pattern of denervation with profound reductions in norepinephrine in the cerebral cortex and cerebellum and only mild reductions in the hypothalamus and brainstem (Jonsson, 1983). Similarly, reductions in dopamine are maximal at the level of the nigrostriatal pathway and minimal at the level of other dopaminergic pathways (Jonsson, 1983). Within the ventral midbrain dopaminergic neuronal groups, the highest loss is found in the substantia nigra, followed by the retrorubral field, and ventral tegmental area (Rodriguez *et al.*, 2001). When a bilateral 6-OHDA lesion is severe, rats will often die primarily due to the occurrence of marked aphagia, adipsia, and seizures (Ungerstedt, 1971a; Bourn *et al.*, 1972). However, the few rats which survive and recover normal ingestion and weight show interesting behavioral abnormalities including hypokinesia, purposeless chewing, and catalepsia which can, at least partially, be reversed by the administration of dopaminergic agonists (Rodriguez *et al.*, 2001). In contrast to this widespread mode of lesioning, local

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intracerebral injection of 6-OHDA is often used to target a specific catecholaminergic pathway of the brain (Ungerstedt, 1968, 1971c). This kind of lesion is achieved by stereotaxic administration usually of 1–10 μg of toxin infused in 1–10 μl at a rate of 1 to 5 μl/min. To avoid non-specific damage the solution injected should not contain more than 2.5 μg/μl of 6-OHDA (Przedborski *et al.*, 1995). Also it is important to keep in mind the fact that the size of the lesioned areas and the magnitude of the lesion (specific and non-specific) are dependent on the volume of 6-OHDA solution infused as well as its concentration and the rate at which it is infused (Agid *et al.*, 1973). To specifically target the nigrostriatal dopaminergic pathway, 6-OHDA must be injected stereotaxically into the substantia nigra, the nigrostriatal tract or the striatum (Javoy *et al.*, 1976; Jonsson, 1983). Following 6-OHDA injections into substantia nigra or the nigrostriatal tract, dopaminergic neurons start degenerating within 24 hours and die by non-apoptotic morphology (Jeon *et al.*, 1995). Maximal reduction of striatal dopamine is reached within 3 to 4 days post-lesion (Faull & Laverty, 1969) and, in most studies, residual striatal dopamine content is less than 20% of controls. When injected into the striatum, 6-OHDA produces a more protracted retrograde degeneration of the nigrostriatal system which can last from 1 to 3 weeks post-lesion (Sauer & Oertel, 1994; Przedborski *et al.*, 1995). Here, the death of nigral dopaminergic neurons occurs by retrograde degeneration and dying neurons exhibit a mixed morphology (Marti *et al.*, 1997), which is quite distinct from that seen following the injection of the toxin directly into the nigra (Jeon *et al.*, 1995). Regardless of the site of 6-OHDA injection along the nigrostriatal pathway, a number of pre- and postsynaptic changes occur, even with only moderate damage (Joyce *et al.*, 1996). These include increased dopaminergic turnover within the remaining dopaminergic terminals (Zigmond *et al.*, 1990) and upregulation of striatal dopamine D2 receptors (Ungerstedt, 1971b); the latter is only seen for a dopamine depletion greater than 90% and is maximal within 2–3 weeks postlesion (Marshall *et al.*, 1989). Usually, 6-OHDA is injected into one hemisphere while the other hemisphere serves as an internal control. Unilateral injections lead to asymmetric circling motor behavior whose magnitude depends on the degree of nigrostriatal lesion (Ungerstedt & Arbuthnott, 1970; Hefti *et al.*, 1980; Przedborski *et al.*, 1995) and is best observed after administration of direct (rotation away from the lesion) or indirect (rotation toward the lesion) dopamine agonists, due to physiological imbalance between the lesioned and the unlesioned striatum. Over the years, this turning behavior, which can be quantified, has been used extensively to assess the antiparkinsonian properties of new drugs, as illustrated in (Jiang *et al.*, 1993), or the success of transplantation and gene therapies in repairing the lesioned pathways (Kirik *et al.*, 2002; Bjorklund *et al.*, 2002). The 6-OHDA model does not mimic all the clinical and pathological features characteristic of PD nor does it result in formation of the cytoplasmic inclusions (Lewy bodies) so typical of PD. Furthermore, the acute nature of the nigrostriatal neurodegeneration seen after the injection of 6-OHDA differs from the more progressive time course of PD.

Studies in rodents have demonstrated that developing monoaminergic neurons respond differently to 6-OHDA than their mature counterparts (Jonsson, 1983). In neonatal rats 6-OHDA has been mainly administered systemically, which can only be done during the first postnatal week, due to the development of the BBB around 7–10 days of life, and intracisternally to produce widespread lesion of the nervous system. Both routes of administration induce profound alterations of the norepinephrine systems, particularly in the nucleus ceruleus and cerebral cortex as well as of the dopaminergic systems (Jonsson, 1983; Breese *et al.*, 1984). Upon reaching adulthood, in contrast to the noradrenergic denervation in the nucleus ceruleus and cerebral cortex, neonatally lesioned rats exhibit noradreneric and serotonergic hyperinnervation in other brain regions, such as the brainstem (Jonsson, 1983; Joyce *et al.*, 1996). Of note, some serotonergic sprouting in the striatum also occurs after 6-OHDA in adult animals, too. They also show increased serotonin receptor density and functional dopamine D1 receptor supersensitivity (Joyce *et al.*, 1996). The latter can be elicited by challenging neonatally lesioned rats with dopamine D1 receptor agonists, which results in behavioral abnormalities such as increased locomotion and self-mutilation (Breese *et al.*, 1990). The association of the aforementioned biochemical and behavioral alterations has led experts to propose neonatal 6-OHDA-lesioned rats as an experimental model of Lesch–Nyhan syndrome, a rare genetic disorder characterized by chorea, spasticity, and compulsive auto-mutilation (Moy *et al.*, 1997). Neonatal rats have also been lesioned with 6-OHDA by intra-striatal infusion to study the mode of cell death of injured developing nigrostriatal dopaminergic neurons (Marti *et al.*, 1997). In this study the authors demonstrate that the lesion results in an induction of apoptotic cell death in phenotypically defined dopaminergic neurons; this appears on the third postlesion day and persists until the tenth and is most marked when the lesion is performed before postnatal day 14 (Marti *et al.*, 1997).

Finally, 6-OHDA can be utilized to produce a chemical sympathectomy, which is preferentially achieved by intravenous injection of the toxin at a dose of 50 to

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500 mg/kg either once or twice at 16-hour intervals (Jonsson, 1983). Of note, while 50 mg/kg, which is well tolerated, reduces norepinephrine content by 90% in most peripheral organs such as the iris and heart, higher doses (e.g. 250–500 mg/kg), which cause high lethality, are needed to damage cell bodies in the sympathetic ganglia (Jonsson, 1983). Although 6-OHDA-induced sympathectomy is permanent in neonatal animals, it is transient in adults and, with time, there is regeneration of noradrenergic fibers (Jonsson, 1983). Over the years, 6-OHDA-induced sympathectomy has proved to be a useful model for studying the role of sympathetic innervation in a large variety of symptoms such as pain, and in physiologic functions such as immune, cardiovascular, bone, and glucose metabolisms (Khalil & Helme, 1989; Kaul, 1999; Sherman & Chole, 2000; Cao *et al.*, 2002).

Paraquat

The potent herbicide paraquat (*N,N'*-dimethyl-4-4'-bipiridinium) is another prototypic toxin known to exert deleterious effects through oxidative stress. It is now well established that paraquat toxicity is mediated by redox cycling with cellular diaphorase such as nitric oxide synthase (Day *et al.*, 1999), thereby elevating intracellular levels of the ROS superoxide. Since its introduction in agriculture, there have been several cases of lethal poisoning resulting from ingestion or even from dermal exposure (Smith, 1988). Until recently, most experimental studies of paraquat are related to its effects on lung, liver and kidney probably because the toxicity induced by this herbicide in these organs is responsible for death after acute exposure. However, the structural similarity of paraquat to the neurotoxin MPP^+ (Fig. 16.2) has sparked major interest in this herbicide as a potential environmental parkinsonian toxin. In support of this view are the published observations that significant damage to the brain is seen in individuals who died from paraquat intoxication (Grant *et al.*, 1980; Hughes, 1988) despite the fact that paraquat poorly crosses the BBB (Shimizu *et al.*, 2001). Furthermore, epidemiological studies have suggested an increased risk for PD due to paraquat exposure (Liou *et al.*, 1997). Yet, paraquat accumulation in the brain appears not to follow any known enzymatic or neurotransmitter distribution (Widdowson *et al.*, 1996a,b). While oral administration of paraquat to rats appears ineffective in lesioning the central nervous system (Widdowson *et al.*, 1996b), its intraventricular or intracerebral injection produces unequivocal neurodegeneration which, however, does not seem restricted to any specific neuronal groups (De Gori *et al.*, 1988; Liou *et al.*, 1996). When injected systemically into mice, conflicting results are obtained. Some

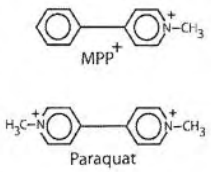

Fig. 16.2 Structural similarity between paraquate and MPP^+. The only difference between these two compounds is the second *N*-methyl-pyridium group that paraquat has instead of the phenyl group as seen in MPP^+.

authors have reported reduced motor activity and dose-dependent losses of striatal dopaminergic nerve fibers and substantia nigra neuronal cell bodies in paraquat-treated mice (Brooks *et al.*, 1999). Conversely, others have failed to see any behavioral abnormality or nigrostriatal dopaminergic pathway damage in similarly treated mice (Thiruchelvam *et al.*, 2000a,b). This discrepancy may be explained by the fact that the propencity of paraquat to damage the nigrostriatal pathway in mice is not only dose-, but also age-dependent (McCormack *et al.*, 2002; Thiruchelvam *et al.*, 2003). The use of Stereology to assess cell loss in some, but not all, studies may also be a reason for the previous inconsistent results. Aside from killing dopaminergic neurons, paraquat induces α-synuclein upregulation and aggregation (Manning-Bog *et al.*, 2002). This suggests that the paraquat model could be quite valuable to recapitulate some of the key neuropathological features of PD such as SNpc neuronal loss and α-synuclein-positive inclusions.

It is worth mentioning that manganese ethylenebisdithiocarbamate, or 'Maneb,' which is used in overlapping geographical areas with paraquat, has been shown to decrease locomotor activity and potentiate paraquat effects on the nigrostriatal pathway in mice (Thiruchelvam *et al.*, 2000a,b). In these studies, combined paraquat and Maneb exposures produce greater loss of dopaminergic neurons than either of the chemicals alone (Thiruchelvam *et al.*, 2000a,b). However, none of these studies has found meaningful reductions in striatal dopamine levels after paraquat injection, even when combined with Maneb. This paradoxical finding may be due to some kind of compensation mechanism that makes up for the modest loss of neurons caused by these toxins. In the light of this, the biochemical basis for the reported behavioural abnormalities found in these animals cannot be atrtributed to a deficit in brain dopamine as in PD; thus, based on our current knowledge of this model, those motoric perturbations cannot be used to mimic PD symptoms.

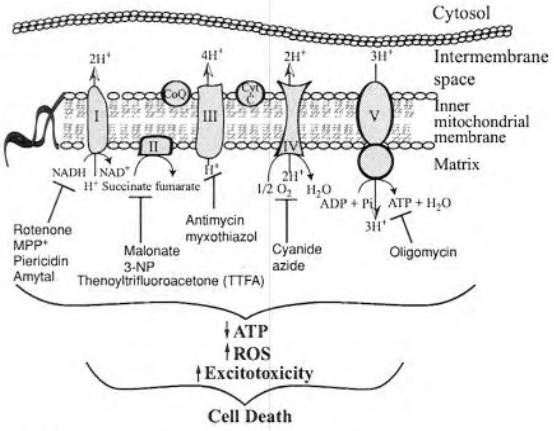

Fig. 16.3 Inhibitors of electron transport chain (ETC). Under normal physiological conditions, electrons donated from NADH to Complex I (NADH: ubiquinone oxidoreductase) or from succinate to Complex II (succinate: ubiquinone oxidoreductase) are passed to coenzyme Q (ubiquinone), ubisemiquinone, ubiquinol, Complex III (ubiquinol: cytochorme c oxidoreductase), cytochrome c, Complex IV (cytochrome c oxidase) and finally to O_2 to produce H_2O. The energy released from this ETC is used to pump the protons out of the inner mitochondrial membrane into intermembrane space creating a membrane potential gradient. This potential energy then drives the flow of protons back into the mitochondrial matrix via Complex V (ATP synthase) and thereby producing ATP. The specific sites of action of various inhibitors are illustrated here.

In contrast to SNpc dopaminergic neurons, GABAergic elements in the substantia nigra and the striatum are not affected by paraquat as evidenced by Western blot and immunohistochemical studies of glutamic acid decarboxylase (GAD) (McCormack *et al.*, 2002; Thiruchelvam *et al.*, 2003). Beyond these two neuronal populations, none of the studies cited above has systematically assessed the status of other neuronal types such as cholinergic, serotonergic or adrenergic in the brain. At this point, therefore, it remains unclear whether the observed paraquat/Maneb cytotoxicity is really specific to the dopaminergic systems and could thus be regarded as a reliable experimental model of PD.

Mitochondrial neurotoxins

Mitochondrion is a small organelle, which assures by itself a large number of vital cellular metabolic pathways such as the tricarboxylic acid cycle, the fatty acid beta-oxidation, and the electron transport chain (ETC), to cite only a few. Despite the multiplicity of potential mitochondrial metabolic targets, so far, investigators interested in producing toxic experimental models of neurodegeneration essentially have used natural or synthetic compounds capable of inhibiting various enzymatic components of the ETC to achieve their goals (Fig. 16.3). Presumably the main reason for this intense research effort is that defects in mitochondrial ETC function have been identified in several prominent neurodegenerative disorders in which they are believed to play a pathogenic role. Although a long list of ETC inhibitors has been tested, especially in rodents, only a handful, which include rotenone, malonate, and 3-nitropropionic acid (3-NP) have been used more than anecdotally and will be discussed here. Although these toxins will also be discussed by Timothy Greenamyre elsewhere is this book, here we will focus our review on the use of rotenone, malonate, and 3-NP in the production of

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experimental models of neurological diseases. Of note, MPTP and IQ, which are known to affect the ETC, but whose cytotoxic mechanisms may involve additional cellular targets, will not be discussed in this section.

Rotenone

Rotenone is the most potent member of the rotenoids, a family of natural cytotoxic compounds extracted from *Leguminosa* plants, used as insect and fish poison and more recently also regarded as a potential parkinsonian environmental toxin. Rotenone is highly lipophylic and can thus readily gain access to all organs with an apparent fast pharamacokinetic. For instance, after a single intravenous injection, rotenone reaches maximal concentration in the brain within 15 min and decays to about half of this level in approximately 100 min (Talpade *et al.*, 2000). Although it accumulates throughout the CNS, rotenone brain distribution is heterogeneous (Talpade *et al.*, 2000), apparently not because of regional variations in cerebral blood flow (Kilbourn *et al.*, 1997), but rather regional differences in oxidative metabolism (Talpade *et al.*, 2000). By virtue of its lipophilicity, rotenone freely enters all cells and, once inside, it probably diffuses into all organelles including mitochondria, where it blocks complex I of the ETC. The effect of rotenone on mitochondrial respiration is time and dose dependent and results from its insertion in a large binding pocket in the hydrophobic part of complex I (Okun *et al.*, 1999), probably made of the ND1 subunit. While rotenone has been used extensively as a prototypic mitochondrial poison in vitro, only a limited number of studies used rotenone to specifically kill dopaminergic neurons or to model PD. Treatment of embryonic ventral midbrain cultures with rotenone has been shown to cause major neurotoxicity (Marey-Semper *et al.*, 1995), especially in the presence of glial cells (Gao *et al.*, 2002). In both studies dopamine uptake was significantly more affected than GABA uptake, thus supporting the hypothesis of selective vulnerability of dopaminergic cells. In vivo, rotenone has been administered to animals by different routes. Oral administration of rotenone appears to cause little neurotoxicity (Betarbet *et al.*, 2000). By contrast, stereotaxic injection of rotenone into the median forebrain bundle causes substantial depletion of striatal dopamine and serotonin (Heikkila *et al.*, 1985), findings consistent with the notion that rotenone lesions the median forebrain bundle, which carries dopaminergic and serotoninergic fibers into the striatum. In rats treated for 7–9 days with high doses of rotenone (10–18 mg/kg/d) as a continuous intravenous infusion, there were major lesions in both the striatum and the globus pallidus, characterized by neuronal loss and gliosis (Ferrante *et al.*, 1997). The nigrostriatal pathway, however, appeared intact (Ferrante *et al.*, 1997). This latter finding is consistent with the demonstration that the systemic acute administration of high doses of rotenone (15 mg/kg), while causing high lethality, failed to affect striatal dopamine contents (Thiffault *et al.*, 2000). The possibility that nigrostriatal injury may be induced by rotenone under less severe experimental conditions and different paradigms of administration has also been evaluated. In one such study (Thiffault *et al.*, 2000), the effect of multiple subcutaneous injections of rotenone (1.5 mg/kg three times per week for 3 weeks) failed to cause any significant neurochemical signs of dopaminergic damage in rats. However, when rotenone was intravenously infused (2–3 mg/kg/day) for 1–3 weeks, treated rats developed no apparent striatal lesion, but exhibited the specific nigrostriatal pathway neurodegeneration associated with nigral intraneuronal proteinaceous inclusions (Betarbet *et al.*, 2000) reminiscent of Lewy bodies, a neuropathological hallmark of Parkinson's disease. However, in this study only 50% of the treated rats exhibited clear nigrostriatal damage and only Lewis rats showed a consistent lesion (Betarbet *et al.*, 2000), indicative of a significant inter-individual and inter-strain variability in the observed effect of rotenone. Although this toxic model of PD appears quite promising, it is also quite challenging. A review of the current problems and limitations that surround this model can be found in Perier *et al.* (2003).

Malonate and 3-nitropropionic acid

Malonate and 3-NP share similar mechanisms of action and both have been used to model the same type of neurological disorder; therefore, these two toxins will be discussed together. Although malonate and 3-NP can be readily synthesized, both are natural molecules in that the former is a product of animal fatty acid metabolism and the latter is a secondary metabolite of the fungus *Arthrinium*, considered to cause a form of acute food poisoning called 'moldy sugarcane poisoning.' It is now well established that malonate and 3-NP inhibit, in a competitive reversible and irreversible manner respectively, the binding of succinate to the enzyme succinate dehydrogenase (SDH) (Alston *et al.*, 1977; Cooper & Clark, 1994). Because SDH is a main enzymatic component of both the tricarboxylic acid cycle and the ETC complex II, its blockade impairs tricarboxylic acid metabolism and mitochondrial respiration. Not surprisingly, therefore, the administration of malonate and 3-NP is associated with impaired ATP synthesis (Beal *et al.*, 1993b; Zeevalk *et al.*, 1995; Kaal *et al.*, 2000) and increased local lactate production (Beal *et al.*, 1993a), metabolic alterations

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which, upon equimolar intrastrial injections, are less pronounced and short lasting with the reversible inhibitor malonate than with the irreversible inhibitor 3-NP. It is believed that the interference of electron flow in ETC by 3-NP and malonate leads to membrane depolarization which, in turn, activates the voltage-dependent NMDA receptors (Greene & Greenamyre, 1996). According to this scenario, once NMDA receptors are activated, a massive influx of calcium occurs, thus triggering numerous cell death pathways and stimulating ROS production which ultimately cause cell demise (Albin & Greenamyre, 1992; Dugan & Choi, 1994). Consistent with a role for NMDA receptors in this deleterious cascade are the autoradiographic and eletrophysiologic demonstrations that malonate- and 3-NP-induced SDH inhibition does activate NMDA receptors (Wullner *et al.*, 1994; Calabresi *et al.*, 2001) and that NMDA antagonists such as MK-801 can attenuate malonate- and 3-NP-induced neurotoxicity (Greene & Greenamyre, 1996; Massieu *et al.*, 2001). In addition, after exposure to these toxins, NMDA-dependent increased intracellular calcium (Calabresi *et al.*, 2001; Lee *et al.*, 2002), activation of proteases such as caspases (Schulz *et al.*, 1998; Lee *et al.*, 2002), and rise in ROS (Schulz *et al.*, 1996; Ferger *et al.*, 1999) were all documented. While most steps of the outlined sequence of events have been studied in great detail, there is a surprising lack of information about the process by which 3-NP and malonate actually enter the central nervous system and subsequently the neurons and mitochondria. Because these two toxins share structural similarity with succinate it may be surmised that malonate and 3-NP entry into the mitochondria may be facilitated by one of the succinate carriers (Kakhniashvili *et al.*, 1997). It should be mentioned that several variants of the mitochondrial succinate carriers are expressed at the level of the BBB and the plasma membrane of neurons (Pajor *et al.*, 2001). It is thus possible that these transporter variants participate in the translocation of 3-NP and malonate into the brain and neurons. If correct, it would appear that 3-NP benefits more from this mechanism than malonate since only 3-NP, and not malonate, can cause neurodegenerative changes after systemic administration. It would also suggest that the reported protective effects of large doses of succinate against malonate may be due, not to a rescue of SDH function as speculated by the authors (Greene *et al.*, 1993), but simply to the excess of succinate impeding malonate cellular and mitochondrial entry.

From the very beginning, the rat has emerged as the preferred species to study malonate and 3-NP neurotoxicity, although occasionally mice and monkeys have also been used (Brouillet *et al.*, 1999). Thus, most available data regarding the use of these toxins emanates primarily from investigations in rats and, as far as 3-NP is concerned, this toxin has been effectively administered systemically or intracerebrally to rats using very different regimens to cause very different behavioral and neuropathological alterations and death rates (Brouillet *et al.*, 1999). Higher doses of 3-NP have also been used in mice (Klivenyi *et al.*, 2000; Fernagut *et al.*, 2002) to induce striatal lesions; however, under these harsh regimens, death rate is as high as 50% in these animals. Surprisingly, while 3-NP is known to cause neurotoxicity following its ingestion, we did not come across any studies in which this toxin was administered orally. Among the different regimens used, it appears that conspicuous brain neuropathology with low lethality can be obtained in rats by intraperitoneal injections or subcutaneous infusion of 10 to 20 mg/kg/day of 3-NP for 5 to 30 days (Brouillet *et al.*, 1999). As depicted in (Brouillet *et al.*, 1999), after a prolonged low dose regimen, lesions are small and restricted consistently to the dorsolateral part of the anterior striatum, whereas after a brief high dose regimen, lesions are larger, invading the entire lateral striatum and involving, in several cases, the most caudal part of the striatum. In addition, while chronic 3-NP-treatment is not associated with extrastriatal lesions, acute and subacute 3-NP treatment is frequently associated with significant pallidal and hippocampal damage. These data indicate that the consistency and extent of the damage to the brain produced by 3-NP depends on the dose and rate of administration. In keeping with this, it should be emphasized that the chronic delivery of a low dose of 3-NP (e.g. 12 mg/kg/day) induces motor abnormalities and selective neuropathology in less than half of the treated animals (Beal *et al.*, 1993b). Other factors known to influence 3-NP neurotoxicity are the age and the genetic background of the rats (Brouillet *et al.*, 1993; Ouary *et al.*, 2000). As for malonate, because of its inability to cross the BBB, 2 to 4 μmol of the toxin is usually injected stereotaxically into the striatum. As with 3-NP, malonate neurotoxicity is age dependent as, after intrastriatal injections, 4- and 12-month-old rats show greater extent of striatal lesions than do 1-month-old animals (Beal *et al.*, 1993a). However, there is no difference in the extent of neuronal loss between 6- and 27-month-old rats (Meldrum *et al.*, 2000).

Microscopically, 3-NP injected systemically or intrastriatally produces tissue damage similar to that produced by stereotaxic injection of malonate in the striatum, consisting of a necrotic core at the centre of the lesion surrounded by an intermediate zone between the core and the normal striatal tissue in which there is gliosis and a loss of only certain subtypes of neurons. However, in the case of 3-NP, only with chronic low dosage are the size of the intermediate zone substantial and the pattern of neuronal loss

similar to that produced by malonate. For instance, harsh 3-NP treatment appears to affect all striatal subpopulations of neurons (Beal *et al.*, 1993b), whereas more gentle 3-NP treatment, like intrastriatal injection of 2 μmol of malonate, affects medium-sized GABAergic spiny neurons and spares the medium-sized aspiny NADPH-diaphorase in rodents and monkeys (Beal *et al.*, 1993a,b; Brouillet *et al.*, 1995). In addition, malonate, but apparently not 3-NP, also affects striatal dopaminergic structures and causes some degree of nigral dopaminergic loss through a retrograde degeneration process (Sonsalla *et al.*, 1997; Zeevalk *et al.*, 1997); why there should be no retrograde degeneration after 3-NP treatment is an enigma. Although not identical (Borlongan *et al.*, 1995; Sun *et al.*, 2002), this pattern of neuronal loss is reminiscent of that seen in Huntington's disease (HD), thus supporting the usefulness of both 3-NP and malonate in modeling HD. However, while malonate and 3-NP recapitulate rather faithfully HD's chemical neuropathology, neither in rats nor in monkeys do they reproduce so well HD's motor abnormalities. From the 3-NP perspective, both in rats and monkeys a harsh treatment produces mostly hypokinesia and limb weakness, and occasionally dystonia, uncoordination, and even somnolence (Brouillet *et al.*, 1995; Borlongan *et al.*, 1997; Sun *et al.*, 2002). In rats, the more chronic regimen was associated with hyperkinesia initially, followed by hypokinesia (Borlongan *et al.*, 1997) or subtle motor abnormalities only observable using sophisticated motor testing (Guyot *et al.*, 1997). In monkeys, the situation appears even more deceiving in that no spontaneous motor abnormalities were detected in three animals treated for 3–6 weeks with 8 mg/kg/day of 3-NP, and only foot dyskinesia and dystonia were seen in two others treated for an even longer period with a slightly higher dose (Brouillet *et al.*, 1995). Although the significance of this is unclear, these authors also show that a variety of abnormal movements, including chorea, could be induced in otherwise asymptomatic 3-NP-treated monkeys by the administration of a dopamine agonist such as apomorphine. Regardless of the type of abnormal movements observed in these animals, it was found that the degree of neurological impairment correlates with the severity of striatal lesions, consistent with the view that most of the observed motor symptoms directly result from striatal cell loss (Guyot *et al.*, 1997).

The reason that 3-NP and possibly malonate cause preferential damage to the striatum despite widespread brain SDH inhibition (Brouillet *et al.*, 1998) is unknown. To date, two hypotheses, which are not mutually exclusive, prevail. First, as discussed above, several in vitro and in vivo studies have demonstrated the importance of excitotoxicity in 3-NP's and malonate's deleterious effects (Brouillet *et al.*, 1999). Therefore, since the striatum receives prominent glutamatergic input from the cerebral cortex and the thalamus, it may be hypothesized that the rich striatal glutamatergic innervation is a key factor in 3-NP striatal selectivity. In favor of this view is the finding that the ablation of the cortical glutamatergic afferences to the striatum mitigates 3-NP striatal neurotoxicity (Beal *et al.*, 1993b). However, the fact that there is no evidence of increased striatal extracellular glutamate concentrations after systemic administration of 3-NP and that several brain areas not affected by 3-NP possess even greater glutamatergic innervation than the striatum represent strong arguments against this idea. Second, mounting evidence indicates that dopamine is instrumental in 3-NP and malonate's deleterious actions (Reynolds *et al.*, 1998; Maragos *et al.*, 1998; Moy *et al.*, 2000; Calabresi *et al.*, 2001; Xia *et al.*, 2001), which fits well with the fact that the striatum contains a conspicuous dopaminergic innervation and that virtually all striatal neurons are dopaminoceptive. It has been found consistently that striatal dopamine depletion, produced either by 6-OHDA lesion or by pharmacological inhibition of dopamine synthesis, attenuates the effects of malonate and 3-NP (Reynolds *et al.*, 1998; Maragos *et al.*, 1998; Moy *et al.*, 2000; Calabresi *et al.*, 2001; Xia *et al.*, 2001). So far, however, the molecular basis of the role of dopamine in the malonate and 3-NP models may be mediated by oxidative stress (mainly due to autooxidation of dopamine), dopamine receptor activation, or both. Relevant to both mechanisms are the demonstrations that antioxidant strategies protect from malonate- and 3-NP-induced toxicity (Schulz *et al.*, 1995a; Schulz *et al.*, 1996) and that dopamine D2 receptor agonists can restore the effect of malonate or of 3-NP in 6-OHDA-lesioned animals, whereas D2 receptor antagonists attenuate the effects of both neurotoxins (Calabresi *et al.*, 2001; Xia *et al.*, 2001). In conclusion, from all of the above evidence it appears quite obvious that, except for moldy sugarcane poisoning, both 3-NP and malonate do not represent perfect models of HD or any other neurodegenerative disorder of the basal ganglia. This fact should not undermine, however, the critical importance of these two neurotoxins as tools to further dissect the functional chemical neuroanatomy of the basal ganglia and the role of excitotoxicity and dopamine in the striatal neurodegenerative process.

Other ETC mitochondrial toxins

For each mitochondrial enzymatic complex of the ETC, numerous reasonably specific inhibitors are available (Cooper & Clark, 1994). Some of these, namely MPP^+, rotenone, malonate, and 3-NP, have already been discussed. Others such as piericidin (complex I), antimycin A (complex III), and cyanide and azide (complex IV), while

extensively used in vitro, have rarely been used in vivo to model neurological disorders. Because the rare surviving victims from cyanide suicide or homicidal attempts may develop parkinsonism and dementia or delayed onset dystonia combined with a severe parkinsonism (Sanchez-Ramos, 1993), animals have been treated with cyanide to elucidate the chemical neuroanatomy underlying the emergence of the movement disorders. In keeping with this, it has been reported that a third of the mice injected subcutaneously with potassium cyanide (6 mg/kg) twice daily for 7 days exhibited reduced locomotor activity, which could be reversed by L-DOPA, as well as catalepsy, which could not be reversed by L-DOPA (Kanthasamy *et al.*, 1994). Although behavioral abnormalities were only seen in a fraction of the treated mice, all had significant dopaminergic loss at the level of the basal ganglia (Kanthasamy *et al.*, 1994); since the authors only studied the dopaminergic system in these mice, it is unknown whether or not cyanide affects these neurons preferentially. While there is no in vivo evidence that any specific neurotransmitter pathway would be more sensitive to cyanide than any other, note that, following infusion of cyanide to cats, severe lesions were only seen in the corpus callosum, pallidus and substantia nigra (Funata *et al.*, 1984). More surprisingly and in contrast to neuropathological reports from human autopsy, the treated cats did not show significant lesions in the cerebral cortex and the hippocampus (Funata *et al.*, 1984). Except for the lack of the cortical and hippocampal damage, the topography of neuropathological changes resembles that reported in carbon monoxide intoxication, thus suggesting that cyanide may be a useful model of chemical brain hypoxia. Alternatively, given the prominent pallidal lesion, the administration of cyanide to animals may produce a unique means to explore the pathophysiology of akinetic-rigid syndromes.

Neurotoxic amphetamines

Amphetamines are potent, highly addictive and widely abused psychostimulants. Among the amphetamine derivatives, four have been shown to have neurotoxic effects on the nervous system, namely *p*-chloroamphetamine (PCA), methamphetamine (METH), 3,4-methylenedioxymethamphetamine (MDMA) and fenfluramine. These four compounds have been extensively used in various mammals to study the basis and the lasting consequences of amphetamine addiction on the brain. The neurotoxic profile of these four analogues, however, is not identical in that PCA, and fenfluramine, when used in moderate doses, tends to affect serotoninergic neurons specifically, while methamphetamine and MDMA are toxic to both dopaminergic and serotoninergic neurons.

While neurotoxic amphetamines can be administered effectively to animals through a variety of routes, systemic injection is by far the most popular. From these animal studies, we learned that, almost immediately after amphetamine administration, there is a massive release of monoamines from their storage sites in the nerve terminals from both the central and peripheral nervous system (Segal & Kuczenski, 1994); compared with the other amphetamines' analogues, methamphetamine causes more pronounced central effects and fewer peripheral ones. In the brain, the release of dopamine begins soon after the injection of amphetamines and peaks within 1 hour (Clausing & Bowyer, 1999; Kita *et al.*, 2000). This time course coincides with that of the brain levels of amphetamine following its systemic injection (Clausing & Bowyer, 1999). As in humans, during this early phase, animals such as rats and monkeys injected with low doses of amphetamines exhibit a range of acute behavioural changes such as increased locomotor activity (Segal & Kuczenski, 1994). However, as the doses increase the motor activity becomes more stereotyped (e.g. sniffing) (Segal & Kuczenski, 1994), and increased salivation and self-injurious behaviours occur (Kita *et al.*, 2000). In addition, animals develop a dose-dependent hyperthermia (potentially exceeding 41 °C) whose magnitude correlates with the levels of brain amphetamine and released dopamine and whose time course is delayed by about 30 minutes compared to that of brain amphetamine and released dopamine (Clausing & Bowyer, 1999). Although our knowledge of the pathophysiology underlying amphetamine-induced hyperthermia remains incomplete, it is well known that it correlates with lethality and neurotoxicity (Ali *et al.*, 1994; Albers & Sonsalla, 1995; Malberg & Seiden, 1998).

Following the acute phase, it is now well accepted that neurotoxic amphetamine analogs not only cause functional deficits, such as profound monoamine depletion, but also structural alterations. Amphetamine-induced neurotoxicity depends on the regimen used since only high dosage and repeated injections will produce significant and consistent neurodegeneration (Davidson *et al.*, 2001). As reviewed in Axt *et al.* (1994), neurotoxic regimens of amphetamine and analogs cause selective loss of dopaminergic and serotonergic nerve terminals with relatively little effects on the corresponding cell bodies. Furthermore, data obtained from rats and non-human primates show that the destruction of the serotonergic and dopaminergic nerve terminal is not even throughout the various regions of the brains. Amphetamines consistently destroy the fine serotonergic terminals that originate from the dorsal raphe nucleus but spare the beaded ones that

originate from the median raphe nucleus (Axt *et al.*, 1994). Similarly, amphetamines selectively destroy dopaminergic nerve terminals in the striatum, nucleus accumbens and frontal cortex but spare dopaminergic cell bodies in the ventral tegmental area and substantia nigra (Axt *et al.*, 1994). Although this represents the prevalent view, some studies have reported significant neuronal loss especially in the substantia nigra of rodents following very high levels of methamphetamine administration (Trulson *et al.*, 1985; Sonsalla *et al.*, 1996). Among the affected dopaminergic brain areas, the dorsal aspect of the striatum appears to be the most susceptible to the neurotoxic amphetamines, whereas the ventral aspect of the striatum and the shell of the nucleus accumbens are much less sensitive, and the olfactory tubercle and the septum are rather resistant (Axt *et al.*, 1994). Moreover, within the striatum it would appear that dopaminergic projections to the matrix are affected, while those to the patches are resistant (Axt *et al.*, 1994). Surprisingly, despite these dramatic biochemical and structural changes, there is still no consensus regarding the nature of their spontaneous behavioral correlates, neither in monkeys nor in rodents (Ricaurte *et al.*, 1994). However, several studies have reported strikingly abnormal responses to pharmacological challenges in amphetamine-treated animals (Ricaurte *et al.*, 1994), thus suggesting that such an approach may represent an effective and reliable means to reveal lasting behavioral alterations in these lesioned animals.

Several studies have shown that, after the administration of neurotoxic amphetamines to monkeys and rodents, monoaminergic innervation, especially in the forebrain, exhibit signs of recovery (Axt *et al.*, 1994). It appears that the milder the lesion the more extensive the re-innervation, but with the current regimens, recovery is moderate at best. Because this process is slow by nature, it is important to remember that it may take anywhere from several months to several years before re-innervation is completed and conclusions about the extent of the recovery can be drawn. For example, in non-human primates injected with MDMA (5mg/kg twice daily for 4 days, SC), the recovery of serotonin axon density in frontal cortex is only about 60% after 7 years (Hatzidimitriou *et al.*, 1999). Rostral neocortex and ventral forebrain are reinnervated sooner and more completely than the caudal neocortex and dorsal forebrain, which is a pattern of axonal re-growth reminiscent of that of axonal growth during development.

Major disagreements still persist about the mechanisms by which neurotoxic amphetamines destroy nerve terminals. All neurotoxic amphetamines cited above are quite lipophilic and can thus readily gain access to all organs including the brain. While amphetamine analogs may, to a certain extent, enter cells such as neurons by diffusion, their accumulation within dopaminergic neurons, for example, is facilitated by DAT (Zaczek *et al.*, 1991a,b). It is thus not surprising that mutant mice deficient in DAT are more resistant to methamphetamine (Fumagalli *et al.*, 1998). Once inside monoaminergic neurons, amphetamines inhibit MAO (Scorza *et al.*, 1997), promote flux reversal by plasma membrane monoamine transporters (Sulzer *et al.*, 1993), and induce monoamine efflux from vesicles (Sulzer *et al.*, 1995). The latter redistribution of monoamines from vesicles to cytosol is believed to be a pivotal event in amphetamine analog neurotoxicity as it gives rise to massive intracellular production of ROS (Cubells *et al.*, 1994; Fumagalli *et al.*, 1999). Although the evidence summarized above suggests that endogenous monoamines and monoamine-derived ROS play a critical role in amphetamine analog neurotoxicity, recent in vivo data indicate that at least some of the results implicating monoamines such as dopamine in the neurotoxic process may have been confounded by secondary drug effects on core temperature. The neuroprotective effects of several pharmacologic agents on MDMA and methamphetamine neurotoxicity appear to be related to their thermoregulatory actions, rather than to their primary pharmacologic actions (Miller & O'Callaghan, 1994; Albers & Sonsalla, 1995; Callahan & Ricaurte, 1998). One of the most damaging findings against the actual role of dopamine in the neurotoxic process is the demonstration that hypothermia rather than catecholamine depletion contributes to the neuroprotective effects of reserpine and α-methyl-*p*-tyrosine in MDMA and methamphetamine-treated animals, since prevention of drug-induced hypothermia abolishes the neuroprotection of reserpine and α-methyl-*p*-tyrosine, alone or in combination (Yuan *et al.*, 2001, 2002). In contrast to the above findings in animals, however, in vitro data obtained from cell cultures question whether change in body temperature is related to methamphetamine toxicity. Using postnatally derived ventral midbrain mouse cultures, it is shown that the specific loss of neurites in dopaminergic neurons induced by methamphetamine is a consequence of its upregulation of TH activity, leading to elevated levels of cytosolic dopamine and consequently increased production of toxic cytosolic ROS (Larsen *et al.*, 2002). This study also demonstrates that, in the absence of vesicular sequestration of dopamine as seen in cultures derived from VMAT-deficient mice, the toxicity of methamphetamine is exacerbated, further supporting the deleterious role of dopamine. Since all cultures are maintained at constant temperature, the authors argue that, in their system, temperature is not involved in the toxicity of methamphetamine. From all these studies, the issue of core temperature and toxicity

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of amphetamine and its analogs warrants further investigation. At the moment, at least in the animal models, hyperthermia is likely involved but itself *per se* may not be solely responsible for toxicity (Albers & Sonsalla, 1995). Caution should be exercised especially when these compounds are to be used in animals. The above discussion shows that administration of neurotoxic amphetamines to animals provides a powerful model to explore the molecular basis of acute and chronic amphetamine exposure on the brain. Aside from this, it also provides a unique model to elucidate the mechanisms governing regeneration in the adult brain, which is a topic of major importance in the field of neurorepair.

Isoquinolines derivatives

As reviewed in McNaught *et al.* (1998), isoquinoline derivatives refer to isoquinoline itself, substituted congeners such as 6-methoxyisoquinoline, and the various reduced species 1,2- or 3,4-dihydroisoquinolines, and 1,2,3,4-tetrahydroisoquinolines (TIQ), all of which may occur in their neutral or charged form, i.e. the isoquinolinium ion. These compounds are heterocycles in which a benzene ring and a pyridine ring are fused through carbon, and are formed from Pictet-Spengler non-enzymatic condensation of catecholamines (e.g. dopamine and L-DOPA) with aldehydes (Deitrich & Erwin, 1980). Among the isoquinoline derivatives, TIQ, 1-benzyl-TIQ and (R)-1,2-dimethyl-5,6-dihydroxy-TIQ ((R)-*N*-methyl-salsolinol) have the most potent toxicity (Nagatsu, 1997); of note, contrary to the other derivatives mentioned above, (R)salsolinol is formed enzymatically from dopamine and acetaldehyde by (R)salsolinol synthase (Naoi *et al.*, 1996). Many isoquinoline derivatives are found naturally in the environment and in foodstuffs as well as in human body fluids and brain tissues (McNaught *et al.*, 1998).

All available data support the notion that the mode and mechanism of action of isoquinolines is very close to that of MPTP and will thus not be discussed here. However, readers may find a comprehensive review on these topics in (Nagatsu, 1997; McNaught *et al.*, 1998). Isoquinolines have been used as neurotoxins both in vitro and in vivo, but, so far, they have produced much more consistent cytotoxicity in cell cultures than in animals (McNaught *et al.*, 1998). For instance, the administration of both TIQ and 1-benzyl-TIQ to monkeys produced behavioral and neuroanatomical abnormalities consistent with a lesion of the nigrostriatal dopaminergic pathway (Nagatsu & Yoshida, 1988; Yoshida *et al.*, 1990; Kotake *et al.*, 1996). In contrast, administration of *N*-methyl-TIQ, *N*-methylisoquinolinium, or *N*-methylnorsalsolinol to monkeys failed to produce any pathological change in the substantia nigra (Yoshida *et al.*, 1993). A similar confusing situation exist in rodents where both lesion (Yoshida *et al.*, 1993; Antkiewicz-Michaluk *et al.*, 2000; Lorenc-Koci *et al.*, 2000; Abe *et al.*, 2001) and no lesion of the nigrostriatal pathway has been reported following the systemic administration of various TIQ analogues (Perry *et al.*, 1988). More disturbing is the lack of dopaminergic neurotoxicity found following the stereotaxic injection of *N*-methyl-norsalsolinol into the medial forebrain bundle of rats (Yoshida *et al.*, 1993; Moser *et al.*, 1996). These discrepancies may relate to technical differences among the cited studies or to distinct physicochemical properties among the various analogues used, such as lipophilicity, making the particular isoquinoline derivatives differ in the degree to which they enter the brain. Regardless of the reason for this inconsistency, at this point the available data raise serious doubts about the ability of this group of toxins to produce a reliable experimental model of PD.

Glutamate and analogues

As discussed by Ikonomidou and Turski in this book, the amino acid glutamate is the main excitatory neurotransmitter in the brain implicated in a large array of physiological actions mediated through the activation of the glutamate receptors. These authors also remind us that, aside from its role in numerous normal functions of the brain, glutamate and its analogues have emerged as potent neurotoxins. The concept of glutamate and analogues as neurotoxins is quite interesting and emanates primarily from the demonstration that excitatory properties of various glutamate analogues correlate with their ability to cause neurotoxic damage (Olney *et al.*, 1971). Yet, while the list of identified neurotoxic excitatory amino acids, or 'excitotoxins,' is long, up to now only glutamate and a few of its analogues have been extensively utilized to lesion the nervous system, especially in vivo. By far the most popular excitotoxins include kainic acid (KA) and ibotenic acid (IA), which are isolated from the marine algae *Diginea simplex* off the coast of Japan and the mushroom *Amanita muscara*, respectively, and quinolinic acid (QA) which is formed endogenously from tryptophan through kynurenine pathways. Like other amino acids, neither glutamate nor KA, IA or QA efficiently cross the BBB, thus requiring their stereotaxic injection in the brain or CSF to produce significant excitotoxic lesions of the central nervous system. So far, excitotoxins have been regarded as unique chemical tools for killing intrinsic neurons while sparing passing axons, nerve terminals and glial cells in discrete regions

of the brain. Although this mode of lesioning may appear straightforward at first, accurate injury often requires laborious pilot studies to determine the appropriate coordinates, toxin concentrations, and injection volumes to produce a specific axon-sparing neuronal lesion with minimal necrosis confined within the boundary of the nucleus of interest. However, once those conditions are set, the lesion is quite reproducible, whether it is performed in rodents or in monkeys, in adult or immature animals, or to target large or small brain areas. Although all of the different excitotoxins mentioned above are effective in producing axon-sparing lesions, it may be important for certain investigations to remember that the actual morphology of the degenerating neurons (e.g. apoptosis or necrosis) is quite different depending on the excitotoxin used and the age of the animals (Portera-Cailliau *et al.*, 1997a,b). Beyond these technical considerations, it must be emphasized that, over the past decades excitotoxins, through their ability to produce axon-sparing lesions, have contributed tremendously in our understanding of the role of specific neuronal systems in both normal and pathological situations. This fact is particularly well illustrated in the context of the MPTP monkey, where the use of IA stereotaxic injection has led to the demonstration of the pivotal role of the subthalamic nucleus in the pathophysiology of parkinsonism (Bergman *et al.*, 1990).

In addition, excitotoxins are thought to be implicated in the pathogenesis of a variety of acute and chronic neurological diseases and have thus been tested with more or less success in various animal species in attempts to model these pathological conditions. For instance, it has been well established that the systemic, intracerebroventricullar, or intraparenchymal injection of kainic acid produces an excellent experimental model of epilepsy (Ben Ari & Cossart, 2000). KA, IA and QA have also been proposed as suitable neurotoxins to generate an animal model of HD given the fact that their intrastriatal injections replicate many of the biochemical, neuropathological and even behavioral hallmarks of this neurodegenerative disorder. Using choline acetyltransferase (CAT), GAD and TH as markers, intrastriatal injections of glutamate, KA and IA produce significant reduction in cholinergic and GABAergic markers with normal or even increased dopaminergic markers on the site of injection (McGeer & McGeer, 1976; Coyle & Schwarcz, 1976; Schwarcz *et al.*, 1978, 1979). These alterations occur as early as 6 h after injection, with maximal effect observed at 48 h and lasting up to 21 days after KA injection (Schwarcz & Coyle, 1977). Consistent with the known chemical neuroanatomy of the striatal projections, the excitotoxic lesion of the striatum is accompanied with a reduction of GAD activity (McGeer & McGeer, 1976) and substance P content (Hong *et al.*, 1977) in the ipsilateral substantia nigra. KA has also been demonstrated to induce other biochemical changes that parallel those found in HD such as reduced levels of the angiotensin converting enzyme as well as receptors for serotonin, dopamine, acetylcholine, and GABA (McGeer & McGeer, 1982). Despite these striking similarities to HD, the enthusiasm for the intrastriatal KA model, was dampened after it became clear that KA fails to spare striatal somatostatin-containing neurons (Araki *et al.*, 1985) as seen in HD, and, even more troublesome, that KA causes remote neurodegeneration in, for example, the hippocampus, following its intrastriatal injection (Zaczek *et al.*, 1980). Unfortunately, intrastriatal injection of IA has not been proven to be more selective than KA in killing neurons and has thus been usually regarded as not particularly suitable to model HD either (Kohler & Schwarcz, 1983; Beal *et al.*, 1986). In contrast to KA and IB, QA has been proposed as a more suitable agent to model HD, since intrastriatal injections of QA (240 nmol), unlike the other two, result in marked depletions of both GABA and substance P, with selective sparing of somatostatin and neuropeptide Y neurons (Beal *et al.*, 1986, 1991). Likewise, quantitative assessment of QA-induced striatal lesion in rats using radioligand binding assays has also shown the selectivity of the damage (Levivier *et al.*, 1994). However, other laboratories have failed to observe similar selective effects from QA (Davies & Roberts, 1987; Boegman *et al.*, 1987). Although technical differences may underlie the discrepancy, to date, the actual reason remains obscure.

From a behavioral point of view, rats receiving bilateral striatal injection of KA (1 μg) exhibit locomotor changes such as increased swing time and decreased stance time (Hruska & Silbergeld, 1979). These animals spend more time swinging their feet in the air than on the ground, movements that are somewhat reminiscent of chorea (Hruska & Silbergeld, 1979). Rats with this kind of lesion also show enhanced locomotor response to amphetamine but not to apomorphine (Mason *et al.*, 1978), a type of dissociate response that has been reported in HD patients. In addition to motor abnormalities, KA-lesioned rats also show impairments in learning (Dunnett & Iversen, 1981). Although, as with other animal models, the behavioral changes induced by KA are difficult to extrapolate to the abnormalities seen in humans, KA appears to mimic at least some behavioral features of HD. The behavioral pathological profiles of IA and QA have not been as well characterized as those of KA. In one study different doses of QA ranging from 75 to 300 nmol were injected to rats and behavioral testings were performed 2 and 4 weeks afterward (Sanberg *et al.*, 1989). The animals receiving 150 and 225 nmol were hyperactive

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and lost weight, whereas those that received 75 nmol were controls and those that received 300 nmol died. Abnormal movements closer to HD have been induced in monkeys with excitotoxic lesion of the striatum (Kanazawa *et al.*, 1986, 1990; Hantraye *et al.*, 1990; Burns *et al.*, 1995). However, as seen with 3-NP, typical chorea in these animals was only elicited after administration of dopaminergic agonists (Kanazawa *et al.*, 1986, 1990; Hantraye *et al.*, 1990; Burns *et al.*, 1995) and, more importantly, only in animals with lesions encompassing the posterior putamen (Kanazawa *et al.*, 1986; Burns *et al.*, 1995).

When using KA, IA and QA as a model for HD, there are several variables to consider (McGeer & McGeer, 1978; Coyle & Schwarcz, 1983). Dose-response studies (0.5–5 μg) of KA show the curve is steep and toxicity is not linear with increasing doses (Coyle & Schwarcz, 1976; Coyle *et al.*, 1978). For example, an injection of 0.5 μg reduces by 20% the activity of GAD but 2 μg causes a near maximal depletion (80%) (Coyle & Schwarcz, 1976). Similarly, a histological study from Nissl-stained sections shows that at a dose of 0.5 μg, KA produces a discrete spherical lesion with a radius of 0.3 mm, but at 1 μg, the lesion increases to 1.4 mm (Coyle *et al.*, 1978). Also, due to differing sensitivity to excitotoxins among cell types, the dose of glutamate or analogues selected is dependent on the type of targeted cells and the desired magnitude of lesion. However, as a rule of thumb, to avoid non-specific damage due to pressure, excitotoxins should be delivered in the smallest possible volume slowly over a prolonged period of time. Furthermore, variation in response to excitotoxins has been linked to differences in strains (Sanberg *et al.*, 1979) and age of animals (Gaddy *et al.*, 1979). When given unilateral intrastriatal injections of either 2.5 or 5 nmol of KA, Wistar are more sensitive than Sprague–Dawley rats (Sanberg *et al.*, 1979). Similar types of injections produce a greater magnitude of damage in older (69–172 days) than in younger (48–49 days) Sprague–Dawley rats (Gaddy *et al.*, 1979). Sex and hormones have also been shown to influence the effect of excitotoxins. Bilateral striatal injections of QA produces higher locomotor activity (Emerich *et al.*, 1991) but less weight loss (Zubrycki *et al.*, 1990) in female than in male or overiectomized female rats. Since KA induces seizures, if the animals were to be pretreated with anticonvulsants one should bear in mind that pharmacological agents such as diazepam and carbamazepine attenuate the excitotoxic effect of KA (Zaczek *et al.*, 1978). The type of anesthetics used has also been shown to influence the effect of excitotoxins. Anesthetics with a short half-life such as ether or hexobarbital potentiate the action of KA whereas those with a long half-life such as chloral hydrate or pentobarbital attenuate it (Zaczek *et al.*, 1978). When IA is used, one should be aware that the injected animals may sleep for a long time (up to 8h) even at a low dose (5 μg) (Coyle & Schwarcz, 1983). This might present a drawback for the behavioral assessment of the animals. Finally, care should also be taken to avoid non-enzymatic decomposition of IA since this chemical is thermolabile and photosensitive.

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13

Mitochondrial dysfunction and neurodegenerative disorders

Kim Tieu and Serge Przedborski

Introduction

Neurodegenerative disorders represent a large group of diseases with heterogeneous clinical and pathological expressions characterized by the demise of specific subsets of neurons within restricted anatomical areas of the nervous system.[1] As discussed in detail elsewhere,[1] neurodegenerative disorders typically arise for unknown reasons, progress in a relentless manner, and their incidence increases markedly with age. Currently, hundreds of different neurodegenerative disorders have been recognized, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), to cite some of the most prevalent and publicized ones.

Until now, both the causes and the mechanisms of neuronal death in many of these disorders have remained uncertain. Relevant to the subject of this book, however, is the speculation that a large number of neurodegenerative disorders might be linked to mitochondrial dysfunction. In some such diseases, impaired mitochondrial function has been proposed as the primary molecular cause of neuronal death, while in others, it probably represents a secondary pathogenic event which arises in compromised neurons and which ultimately contributes to their demise. At first glance, it may appear perplexing that mitochondrial dysfunction would lead to so many disparate neurological diseases. However, when one thinks about the pivotal roles of mitochondria in cellular homeostasis and survival,[2] it becomes more plausible that functional impairment of these organelles may have dramatic consequences and be implicated in a host of neurodegenerative situations. A quick survey of the literature on mitochondrial dysfunction in neurodegenerative diseases shows that these disorders can be divided into three categories: diseases linked to mitochondrial gene mutations; neurodegenerative diseases linked to mutations in nuclear genes encoding mitochondrial proteins; and neurodegenerative disorders linked to mutations in non-mitochondrial proteins or with unknown causes.

This chapter is organized into three parts. The first deals with specific biological aspects of the mitochondrion that are relevant to common features of neurodegenerative diseases. The second part is a systematic review of the three aforementioned categories of mitochondrial neurodegenerative diseases. The final part discusses the mechanisms by which neurons may die in these mitochondrial neurodegenerative diseases.

Mitochondrial biology and neurodegeneration

Besides the nucleus, the mitochondrion is the only other organelle in the cell that possesses its own DNA and genetic machinery. All mitochondrial DNAs (mtDNAs) of a zygote originate from the ovum. The latter point has led to the traditional view that diseases linked to mtDNA mutations are maternally inherited: mothers (not fathers) who carry mtDNA mutations pass them to the children, and then only the daughters may pass them to their children. Rarely, however, has a maternal inheritance been suspected in classical neurodegenerative diseases.[3] Instead, familial forms of neurodegenerative diseases are typically transmitted as simple Mendelian traits. Furthermore, most neurodegenerative diseases are sporadic, and if genetic factors do contribute to their development, these are more likely susceptibility genes or genetically deleterious environmental factors.[4,5]

Because mtDNA encodes only for polypeptide subunits of the respiratory chain, all pathogenic mtDNA mutations affect oxidative phosphorylation. The nervous system and its cellular constituent, neurons, are highly dependent on oxidative phosphorylation. Thus, CNS and neurons are especially vulnerable to the effect of mtDNA mutations. The retina, renal tubules, and endocrine glands, also have high oxidative metabolism demands and are also high-risk targets in typical mitochondrial cytopathy. It is thus not surprising that mitochondrial diseases are usually multisystemic.[2] This situation, again, is in striking contrast with most neurodegenerative diseases, which are typically pure neurological disorders.

There are thousands of mtDNAs in each cell, with about five mtDNAs per mitochondrion, and in patients, a pathogenic mitochondrial mutation may be present in some, but not in all, mtDNAs. This mixture of mutated and wild-type mtDNA (heteroplasmy) can vary greatly among patients, organs and even cells. In addition, replication of mtDNA occurs constantly in all cells, even in postmitotic, non-dividing cells such as neurons (relaxed replication), and upon cell division, the mitochondria are partitioned randomly among daughter cells (mitotic segregation). Over time, the proportion of mutated mtDNA in tissues may change, influencing the expression of the disease. Specific nuclear factors can also influence mitochondrial segregation,[6] supporting a role for nuclear–mitochondrial interactions in determining the degree of heteroplasmy. That said, it is important to remember that a minimal proportion of mutated mtDNA is required before an oxidative phosphorylation deficit occurs and symptoms arise (threshold effect), and that – as a general rule – the higher the proportion of mutated mtDNA, the more severe the phenotype of the diseases. Collectively, the mitochondrial characteristics mentioned above might provide an explanation for some of the peculiar features of neurodegenerative disorders, including the heterogeneous phenotypic expression, the consistent adult-onset, and the progressive nature of these neurological disorders.

Mitochondrial neurodegenerative diseases

Mitochondrial dysfunction in neurodegenerative diseases linked to mitochondrial gene mutations

The human mtDNA is a 16.6-kilobase circle of double-stranded DNA that comprises 37 genes. Of these, 13 encode for polypeptides of the respiratory chain whereas two are transcribed to ribosomal RNAs (rRNAs) and 22 are transcribed to transfer RNAs (tRNAs) which are required for the synthesis of the 13 respiratory subunit proteins. These include seven subunits of complex I (NADH dehydrogenase 1, 2, 3, 4, 4L, 5, 6), one subunit of complex III (cytochrome *b*), three subunits of complex IV (COX I, II, and III), and two subunits of

ATP synthase (A6 and A8). Any mtDNA mutation (including deletion) leading to a defect in any of these proteins may provoke a mitochondrial disease. A complete list of known mtDNA mutations is available through the MITOMAP database (www.mitomap.org). Among the diverse diseases due to mtDNA mutation, only a few – including Leber hereditary optic neuropathy (LHON), Leigh syndrome (LS), neuropathy, ataxia and retinitis pigmentosa (NARP), and 12SrRNA mutation-linked parkinsonism – are linked to selective neuronal degeneration. LHON, LS, and NARP will not, however, be discussed here because they are described in detail in Chapters 2 and 5.

12SrRNA mutation-linked parkinsonism

In the mid-1990s, Shoffner and collaborators found that a point mutation (A1555G) in the *12SrRNA* gene cosegregated with maternally inherited deafness and levodopa-responsive parkinsonism in several members of one kindred.[7] Subsequently, another heteroplasmic, maternally inherited 12SrRNA point mutation (T1095C) was found in another pedigree with sensorineural deafness, levodopa-responsive parkinsonism, and neuropathy.[8] Except for an early onset, parkinsonism in these patients appeared similar to that seen in PD. Serum lactate levels were normal, but muscle biopsy was not done. Spectrophotometric mitochondrial respiratory chain assays performed in transformed lymphoblasts from the proband showed a significant reduction in cytochrome *c* oxidase activity. The secondary structure predicts that this mutation disrupts a highly conserved loop in the small subunit ribosomal RNA, which is important in the initiation of mitochondrial protein synthesis. These findings led to the conclusion that this mutation was pathogenic and caused an oxidative phosphorylation defect by interfering with mitochondrial protein synthesis. This mutation was not found, however, in 20 cases of sporadic PD, suggesting that the 12SrRNA mutation is not likely to be a common cause of PD.

Mitochondrial dysfunction in neurodegenerative diseases linked to mutations in nuclear genes encoding for proteins targeted to mitochondria

The mitochondrion comprises 850 polypeptides encoded by nuclear DNA (nDNA) in addition to the 13 encoded by mtDNA. Of these, about 75 are structural components of the respiratory chain, and about 60 others are needed for its proper assembly and functioning. Thus, certain mutations, which affect genes that encode for mitochondrial proteins, can cause oxidative phosphorylation defects just as classical mtDNA mutation would do. LS, for instance, may arise not only from mtDNA mutations, but also from a variety of nDNA mutations affecting any one of the respiratory chain complexes.[9] Many other nDNA mutations do not affect the respiratory chain directly, but still produce mitochondrial dysfunction, thereby giving rise to an entirely distinct class of mitochondrial neurodegenerative diseases from that discussed above. Among this singular group of diseases, one finds Friedreich's ataxia, the hereditary spastic paraplegia, the deafness-dystonia syndrome, and Wilson's disease. In many of these genetic neurodegenerative diseases, as we will find below, the products of the mutated nuclear genes fail to carry out their normal function, which involve various aspects of the mitochondrial physiology.

Friedreich's ataxia

Friedreich's ataxia (FRDA) is an autosomal-recessive mitochondrial disorder with clinical manifestations of progressive limb and gait ataxia, peripheral neuropathy, and areflexia.[10] Pathologically, FRDA is characterized by degeneration of the spinocerebellar tracts and large sensory neurons, along with hypertrophic cardiomyopathy

and increased incidence of diabetes.[11] The disease gene has been mapped to 9q13 and encodes for a 210 amino-acid protein called frataxin. This protein is synthesized in the cytoplasm as a large precursor with a *N*-terminal presequence that targets frataxin to the mitochondrial matrix.[12] Frataxin precursor is then processed into a roughly 17-kDa mature protein by the mitochondrial processing peptidase.[13]

Most FRDA patients are homozygous for a GAA trinucleotide repeat expansion in the first intron of the *frataxin* gene,[14] which hampers its transcription.[15] Studies in a knock-in mouse with 230 GAA repeat expansion introduced into the *frataxin* gene exhibited merely 25% reduction in frataxin expression,[16] suggesting that very long repeats are probably needed to recapitulate the transcriptional defect observed in FRDA patients. This interpretation is consistent with the fact that FRDA is typically caused by large GAA repeat expansion (up to 1700 repeats).[17] Some patients carry compound heterozygote mutations, in that they have a GAA expansion on one allele and a truncating or missense mutation on the other allele.[18] In these patients, the clinical expression ranges from typical FRDA to a much milder phenotype, depending on the point mutation.[18] This observation suggests that not all point mutations affect *frataxin* transcription to the same extent, and consequently the defect in frataxin expression may be quite different among compound heterozygote patients.

Similar to other neurodegenerative disorders, FRDA overt pathology appears confined to specific regions of the brain and the heart, although frataxin is expressed in all tissues.[14] In addition, frataxin seems vital as its ablation, at least in rodents, is embryonically lethal.[19] It is thus not surprising to observe that patients with FRDA exhibit low (4–29% of normal levels) but not null expression of frataxin,[15] and that the level of residual frataxin expression correlates with the severity of the disease phenotype.[15] However, as stressed by Badhwar and collaborators,[20] the phenotype, even within the same family, cannot always be predicted from the repeat length, as factors such as somatic mosaicism and repeat interruptions may impact on the expression of the disease.

The exact function of frataxin remains uncertain. It is noteworthy that FRDA patients exhibit specific deficits in the enzymatic activities of iron–sulfur proteins such as aconitase and complexes I, II, and III.[21] In theory, the loss of iron–sulfur protein activity may be caused by either oxidative damage to labile iron–sulfur clusters (ISCs) or to alteration of the ISC biosynthesis, which occurs within mitochondria through a well-conserved ISC machinery.[22] Although some studies suggest a role for frataxin in the antioxidant signaling pathway,[23] others suggest that frataxin is involved in iron–sulfur protein biosynthesis. The latter hypothesis appears compelling in light of several observations. First, the synthesis of ISCs is decreased in isolated mitochondria and in mitochondrial extracts from frataxin-deficient or conditionally frataxin-depleted yeast.[24,25] Second, human frataxin binds iron ions and mediates the transfer of the bound iron to the iron–sulphur scaffold proteins Isu,[26] which are essential in the formation of ISC.[21] Third, yeast frataxin is part of a multiprotein complex comprising Isu1p and the Nfs1p cysteine desulfurase.[27]

Defects in the synthesis of iron–sulfur proteins are known to alter mitochondrial iron homeostasis and to cause an excess of intramitochondrial iron.[28] Likewise, mutations in the yeast homolog of frataxin, Yfh1p, lead to a massive increase in mitochondrial iron concentrations.[12] However, animal studies showed that impaired iron–sulfur protein activities arose prior to any detectable intramitochondrial iron accumulation,[29] suggesting that an alteration in mitochondrial iron metabolism in FRDA patients is likely the consequence and not the cause for the ISC defect. Nevertheless, we cannot exclude that, once it occurs, accumulation of intramitochondrial iron

Box 13.1 The biogenesis of iron-sulfur clusters

Iron–sulfur clusters are among the most ancient of proteins, because they evolved to perform a number of critical functions, most notably electron transfer (i.e. redox regulation). In particular, the electron transport chain contains a number of FeS-containing subunits that are required for the electron flow through the chain (see Box X, page X). What is particulary notable is that the biogenesis and maturation of FeS clusters requires the obligate participation of mitochondria. It is for this reason that the proteins associated with FeS cluster metabolism are found in mitochondria from every species examined. Also, in spite of the fact that most eukaryotic FeS proteins are mitochondrial (for example, aconitase, and some subunits of respiratory complexes I, II, and III), FeS synthesis requires extra-mitochondrial maturation proteins (Figure B.1).[1] Conversely, biosynthesis of extramitochondrial FeS proteins (e.g. iron regulatory protein 1 and glutamate synthase) requires a mitochondrial membrane potential (pmf) and involves a host of mitochondrial proteins (Figure B.1).

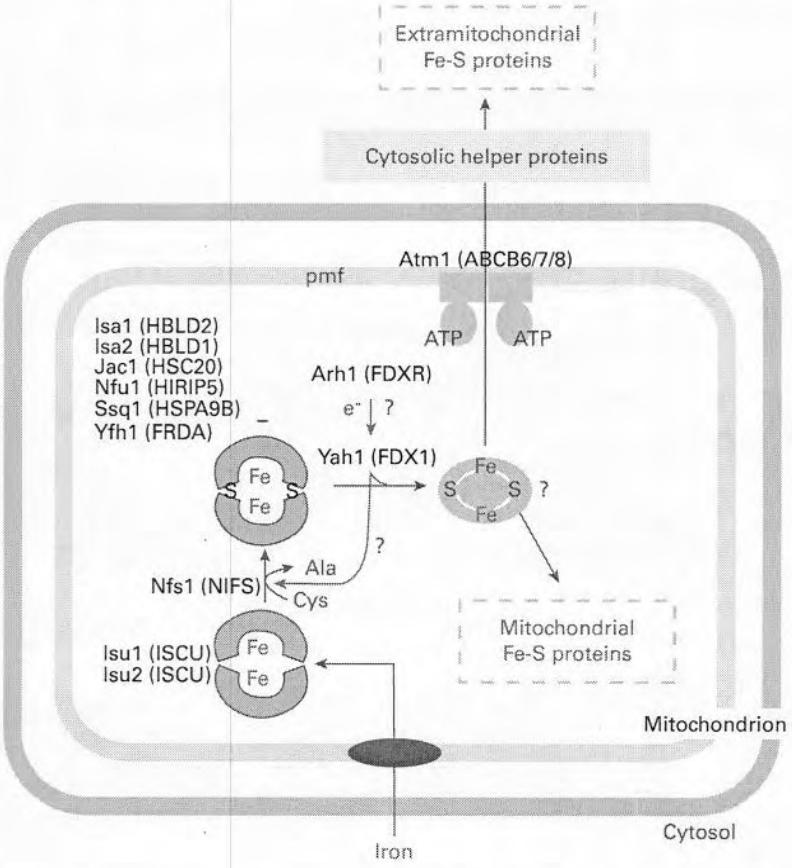

Figure B.1 Proteins associated with Fe–S cluster biogenesis. Shown are the yeast proteins and their human orthologs (in parentheses). Adapted from reference 1, with permission

(Continued)

Box 13.1 *(Continued)*

In bacteria, two operons are required for FeS biosynthesis, the *nif* (nitrogen fixation) and *isc* (iron–sulfur cluster) operons. The *nif* operon encodes proteins required for the formation of the FeS cluster present in nitrogenase (a metalloenzyme), while the *isc* operon encodes proteins necessary for the maturation of many bacterial FeS proteins. In eukaryotes, the mitochondrial ISC machinery is responsible for biogenesis iron–sulfur proteins both inside and outside the organelle.

Of importance clinically is the fact that mutations in two FeS proteins cause human disease. There are three human ABC (ATP Binding Cassette) orthologs of Atm1p (ABC transporter of mitochondria): ABCB6, ABCB7, and ABCB8. Mutations in ABCB7 cause X-linked sideroblastic anemia and ataxia, in which affected cells contain mitochondria harboring deposits of iron ('ring sideroblasts'). Since Atm1p/ABCB7 is required to transport mature FeS clusters from the mitochondria to the cytosol (see Figure B.1), defects in transport most likely cause the FeS clusters to accumulate within the organelle – hence the ring sideroblasts. Mutations in frataxin (FRDA), the human ortholog of Yfh1p (yeast frataxin homolog), cause Friedreich ataxia, a neurodegenerative disease associated with aberrant iron handling within mitochondria, although there is currently some debate regarding the actual pathogenetic mechanism.

Reference

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would promote local oxidative stress, and thereby exacerbate the mitochondrial defect found in FRDA patients. It is important however to stress the fact that while high intramitochondrial iron accumulation is well documented in FRDA cells, the nature of this iron, i.e. reactive (also called labile or chelatable iron) or unreactive, remains unclear. For instance, Sturm et al. have found no evidence of higher chelatable iron in fibroblasts from FRDA patients compared to controls, despite their elevated total mitochondrial iron content.[30] Should the latter observation be confirmed, it would then be unlikely that the excess of intramitochondrial iron in FRDA cells would contribute to a local oxidative stress.

If, as discussed above, the exact role played by intramitochondrial iron accumulation in FRDA is still believed to be instrumental in the pathogenesis of this illness, then, as such, it is not surprising to find that iron chelators and antioxidants have been intensively tested in these patients. Desferrioxamine and deferiprone have been tried to reduce intracellular iron, with questionable effects. Serum iron and ferritin concentrations are normal in FRDA patients and desferrioxamine is not lipophilic enough to permeate mitochondria.[31] Also, this compound cannot efficiently mobilize iron from iron-loaded mitochondria.[32] Additional concerns with deferiprone include its potential to cause liver fibrosis[33] and its lack of metal selectivity. Another group of iron chelators, the 2-pyridylcaroxaldehyde isonicotinoyl hydrazone analogs, has been designed to specifically target mitochondrial iron pools and is currently being assessed for use in therapies for FRDA.[34] Using the same strategy, the antioxidant mitoquinone (MitoQ) has been synthesized by linking ubiquinone to a lipophilic triphenylphosphonium cation.[35] In fibroblasts from FRDA patients, MitoQ conferred protection against endogenous oxidative stress induced by inhibition of glutathione synthesis.[36] Idebenone, an antioxidant short-chain analog of coenzyme Q10, inhibits lipid peroxidation in rat brain mitochondria[37] and reduces

urinary levels of a marker for oxidative DNA damage in FRDA patients.[38] In separate clinical trials, idebenone was found to reduced myocardial hypertrophy.[39–41] Other antioxidants, such as coenzyme Q10 and vitamin E, have also been evaluated for the treatment of FRDA. The results of their efficacy, however, have been conflicting.[42]

Hereditary spastic paraplegia

The term hereditary spastic paraplegia (HSP) describes a heterogeneous group of inherited neurodegenerative disorders in which the primary clinical feature is progressive bilateral spasticity and weakness in lower limbs.[43] Urinary urgency is also a common symptom. When these symptoms occur in isolation, HSP is called uncomplicated or pure, whereas when the symptoms occur in association with additional neurological abnormalities, such as mental retardation, extrapyramidal symptoms, deafness and optic neuropathy, the HSP is called complicated. Onset of symptoms usually is in childhood or early adulthood.[43]

Familial HSPs are most often transmitted as autosomal dominant traits and less commonly as autosomal or X-linked recessive traits.[43] Nine HSP genes and 27 HSP loci have been identified.[44,45] The few autopsy studies available show that the neuropathology of HSP is characterized by axonal degeneration in the corticospinal tract and the fasciculus gracilis without overt cell body loss. The pathogenesis of HSP is also poorly understood, but various molecular mechanisms have been proposed.[45] Patients with the recessive form of HSP are characterized by mutations in the *SPG7* gene, which encodes for the mitochondrial targeted protein paraplegin.[46] Paraplegin is a metalloprotease whose yeast homologs are Yta10p and Yta12p.[47] These proteins belong to a large family of ATPases that includes the ATPase associated with the diverse cellular activities (AAA) domain.[48,49] In yeast, multiple copies of Yta10p and Yta12p combine to form a high-molecular-weight complex located in the inner membrane of the mitochondria.[47] It is believed that this Yta10p/Yta12p complex mediates the ATP-dependent degradation of the mitochondrial inner membrane proteins,[47] and, by analogy, paraplegin is thought to play the same role in vertebrates. The pathogenic mutations in paraplegin are likely abrogating this function and thus may lead to accumulation of unwanted mitochondrial proteins in HSP patients, which, in turn, may cause mitochondrial dysfunction. Although highly speculative, this scenario is consistent with the demonstration that muscle biopsies obtained from some HSP patients show signs of mitochondrial dysfunction, such as cytochrome *c* oxidase-deficient fibers and ragged-red or ragged-blue (intensely SDH-positive) fibers.[46] In a paraplegin-deficient mouse model harboring a mutated *SPG7* gene, electron microscopy shows abnormal mitochondria in synaptic terminals and distal axons, a change that occurs prior to the appearance of axonal swelling and degeneration.[50] The massive accumulation of organelles and neurofilaments in the swollen axons suggest that the HSP-related mitochondrial dysfunction impairs the anterograde axonal transport. In addition to paraplegin, a mutation in another mitochondrial protein, the chaperone heat shock protein 60 (Hsp60 or chaperonin), has also been identified in some patients with autosomal dominant HSP (SPG13).[51] This study supports the notion that, in addition to the loss of mitochondrial protease activity, the loss of mitochondrial chaperones may also produce a HSP phenotype.[52] However the involvement of some sort of mitochondrial dysfunction in HSP pathogenesis is not always obvious. For instance, mutations in spastin, another AAA ATPase that are the most common cause of the autosomal dominant HSP,[53] seem to provoke the disease by impairing microtubule metabolism[54] via a mechanism whose link to a mitochondrial defect could not readily be established.

Treatments of HSP remain largely supportive at this moment. To reduce muscle spasticity, muscle

Box 13.2 Mitochondrial fusion, fission, and movement

Mitochondria are not the static entities that we see in textbooks. They are highly plastic, with shapes that vary from small spheres (~1 μm in diameter) to highly elongated spaghetti-like structures. They can exist as linear 'strings' or as highly branched, reticular structures, and like the bacteria from which they evolved, they can fuse and divide. Thus, mitochondrial morphology is influenced to a large degree by the balance between these two processes.[1] In yeast, three proteins are required for organellar fission: Dnm1p (dynamin-related protein; in the yeast nomenclature, the 'p' indicates the protein product (e.g. Dnm1p) encoded by the gene (e.g. DNM1)), Fis1p (fission-related protein), and Mdv1p (mitochondrial division protein) (Figure B.1). Two others are required for organelle fusion: Fzo1p (the yeast homolog of the *Drosophila* 'fuzzy onions' protein) and Ugo1P (ugo is Japanese for 'fusion').

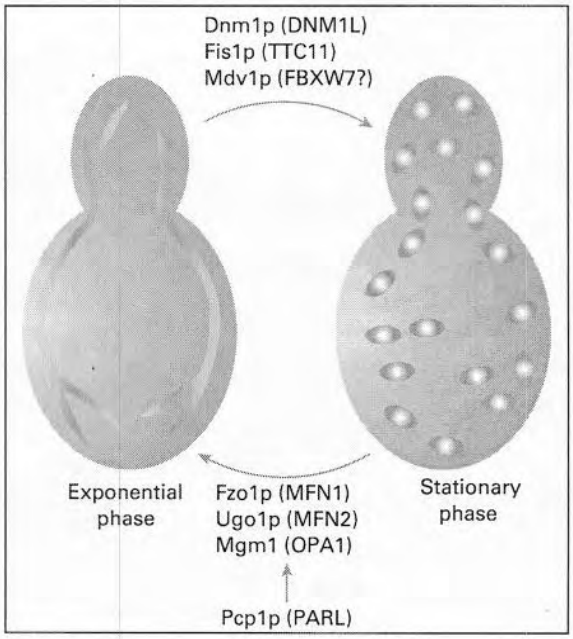

Figure B.1 Proteins associated with mitochondrial fusion and fission in yeast. Human orthologs of the yeast proteins are in parentheses. Adapted from reference 1, with permission

All five proteins are located in the mitochondrial outer membrane, but this raises a problem: the mitochondrion has two membranes (MOM and MIM), and presumably the inner membrane has to fuse/divide coordinately with the outer mambrane in order for the entire organelle to fuse/divide. Recently, inroads have been made into the solution of this problem. It turns out that Mgm1p (mitochondrial genome maintenance protein) is a fission-related protein that spans both the outer and inner membranes (Figure B.2), and the current idea is that, in fact, the MOM and MIM are 'pinched' together to form 'contact sites' at the point of division, presumably mediated by Mgm1p. A particularly noteworthy aspect of Mgm1p function is that it is a 'processed' protein: it is imported into the mitochondrion as a 'long' form (L-Mgm1p), which is anchored in the inner membrane, but is then processed into a

'short' form (S-Mgm1p) by a protease called Pcp1p (so-called because it also processes cytochrome c peroxidase), which releases the MIM-bound tail of L-Mgm1p and allows S-Mgm1p, now free in the intermembrane space, presumably to bind Ugo1p.[2] However, recent data also implicate Mgm1p, together with Tim11p (human ATP5I), a non-catalytic subunit of ATP synthase, in the formation of cristae (the invaginated folds of the inner membrane).[3]

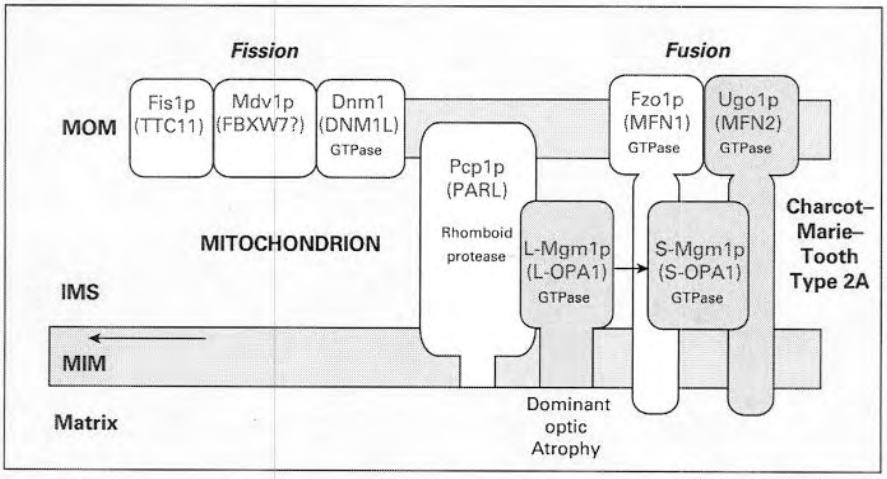

Figure B.2 Location of mitochondrial fission and fusion proteins. Note that mutations in two of these proteins – OPA1 and MFN2 – cause neurodegenerative diseases in humans

Remarkably, mutations in the human orthologs of both Mgm1p (OPA1) and Ugo1p (MFN2) cause human neurodegenerative diseases. Mutations in OPA1 cause dominant optic atrophy or DOA (hence the name OPA1),[4] and mutations in MFN2 (mitofusin 2) cause Charcot–Marie–Tooth disease, type 2A.[5] The reason why an error in organellar fusion should cause these disorders is the subject of much research, but it is already known that mitochondria in DOA cells have aberrant subcellular localization, implying that organellar fusion and fission is somehow connected to organellar mobility.

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relaxants such as baclofen, tizanidine, dantrolene, and botulinum toxin have been prescribed.[45] Oxybutynin may be used to alleviate urinary urgency.

Deafness-dystonia syndrome

Deafness-dystonia syndrome or Mohr–Tranebjaerg syndrome (MTS) is a rare X-linked recessive deafness syndrome associated with dystonia and other neurological abnormalities.[55] As with many other diseases discussed in this chapter, MTS also shows a high degree of phenotypic variability. Early-onset progressive hearing loss and dystonia are the only obligatory clinical symptoms.[55] In some but not all patients, cortical blindness can also be found. In addition, psychiatric symptoms, cognitive impairment, and behavioral problems can be seen late in the course of MTS.[55] The mitochondrial respiratory chain activity is normal in muscle, and there are no mitochondrial structural abnormalities. Functional and structural brain imaging studies revealed multiple loci of brain hypometabolism in the basal ganglia and parietal cortex and marked atrophy of the occipital lobes.[55]

MTS is due to loss-of-function mutations in *TIMM8A*, a small gene on Xq22,[56] which encodes for a small polypeptide of 97 amino acids named deafness-dystonia protein-1 (DDP1). Homology searches have shown that the DDP1 protein belongs to a family of proteins located in the mitochondrial intermembrane space.[57,58] In human mitochondria, DDP1 forms a multiprotein complex with Tim13,[59] which presumably stabilizes the precursors of hydrophobic inner membrane proteins during their translocation across the aqueous environment of the intermembrane space.[55]

Wilson disease

Hepatolenticular degeneration or Wilson disease (WD) is a rare familial disease whose mode of inheritance is autosomal-recessive and which is characterized by copper accumulation in a number of tissues.[60] Although WD is discussed here in the context of neurodegenerative diseases, the involvement of the nervous system invariably follows liver disease. Clinically, WD begins in most patients between the ages of 11 and 25 years. Liver manifestations range from asymptomatic hepatic enzymatic alterations to acute hepatic failure. Because the initial liver symptoms may be subtle, neurological signs are the second most frequent presenting features of WD. Neurological signs and symptoms associated with WD include dysphagia, dysarthria, parkinsonism, and dystonia.[60] Early on, one-fifth of WD patients also exhibit behavioral and psychiatric manifestations, which become more frequent and prominent in advanced cases. Among the clinical features associated with WD, the Kayser–Fleischer ring (intracorneal ring-shaped pigmentation) is the most important for the diagnosis. Indeed, the absence of a Kayser–Fleischer ring in an untreated patient with neurological manifestations rules out WD. The main laboratory features of WD are decreased serum copper and ceruloplasmin levels together with increased urinary copper excretion.[60] Biopsies also shows prominent copper deposits in kidney and liver associated with foci of tissue necrosis.[60] MRI studies, though useful, show non-specific abnormalities, such as ventricular dilatation and diffuse atrophy of the cortex, cerebellum, and brainstem.[60] From a therapeutic point of view, both asymptomatic and symptomatic WD patients must be treated. The goal of treatment is to remove the toxic accumulation of copper in tissues and to prevent its re-accumulation. Accordingly, copper chelators such as penicillamine or triethylene tetramine dihydrochloride are used. Agents that reduce copper digestive absorption such as tetrathiomolybdate and zinc are also used. Thanks to these therapeutic strategies, the survival of WD patients who have completed the first few years of treatment is comparable to that of the healthy population.

The disease is caused by mutations in the *ATP7B* gene, which encodes for a copper-transporting P-type ATPase.[61] Loss of function of this gene results in an accumulation of copper primarily in the liver, kidney, and brain; in the latter, the basal ganglia is especially involved. Abnormal copper accumulation in tissues and liver changes resembling cirrhosis are seen in knockout mice for the *ATP7B* gene,[62] thus confirming the pathogenic role of the ATP7B mutations in WD. ATP7B exists in two isoforms, a 159-kDa form that localizes to the transgolgi network, and a 140-kDa form that localizes to mitochondria.[63] The mitochondrial localization of the 140-kDa isoform suggests that ATP7B may participate in the delivery of copper to copper-containing enzymes, such as cytochrome *c* oxidase. Presumably, mitochondria in affected tissues exhibit morphological abnormalities, as well as a deficiency of liver mitochondrial enzymes, especially complex I and aconitase.[64] However, ATP7B is present in multiple subcellular compartments, raising the possibility that mitochondria dysfunction in WD may be the consequence, rather than the cause, of the disease.

Mitochondrial dysfunction in neurodegenerative disorders linked to mutations in non-mitochondrial proteins and in neurodegenerative disorders with unknown causes

This section is by far the most controversial as an unequivocal mitochondrial etiology has not been documented for any of the diseases to be discussed here. Still, for all these diseases the possibility of a mitochondrial link has been raised and has received much attention within the scientific and clinical communities. It is our opinion, however, that most data supporting mitochondrial involvement are circumstantial or indirect. Thus, whether mitochondrial involvement in neurodegenerative diseases such as HD, AD, or PD is a myth or a reality remains to be elucidated.

Huntington's disease

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by chorea, psychiatric disturbances and a decline in cognitive function. Symptoms usually begin in the fourth or fifth decade of life and worsen rapidly thereafter. Typically, the time from onset to death is about 18 years. HD is an autosomal dominant disease caused by the expansion of a trinucleotide CAG repeat that encodes the non-essential amino acid glutamine in exon 1 of the *IT15* gene on chromosome 4.[65] The normal range of CAG repeats – and thus of the glutamine tail in the encoded protein called huntingtin – is between six and 35. The age at onset is inversely correlated to the length of the repeat, but apparently not the severity or the rate of progression.[66] Environmental factors, however, also seem to play a major role in the pathogenesis of HD, as demonstrated in animal models[67,68] and in HD patients.[69] Pathologically, there is a selective neurodegeneration of medium spiny neurons in the striatum. Nuclear inclusions of protein aggregates are also a common feature in the striatum and cortex.

Twelve years after the discovery of the HD mutation,[65] the normal function of huntingtin is still unknown and the pathogenic mechanism leading to neurodegeneration in HD is elusive. Nonetheless, a defect in bioenergics has been proposed as a major pathogenic factor. Positron emission tomography studies with [^{18}F]-fluorodeoxyglucose have demonstrated a marked reduction in glucose utilization in the caudate and putamen of HD patients.[70] This hypometabolism precedes the detection of striatal atrophy, indicating that cell death occurs after metabolic impairment.[70,71] These results also suggest a dysfunction in nerve terminals since they are the sites of the greatest energy consumption by ATP-dependent pumps to restore ionic gradients following synaptic transmission.[72] The early damage to the terminals in HD is consistent with findings that various types of

Box 13.3 Mitochondrial protein importation

Of the thousands of genes presumably present in the endosymbiotic 'proto-mitochondrion', only a handful remain today in human mtDNA, and these are all associated with oxidative energy production. Where did the rest go? They were either lost, because they were unnecessary for the survival of the organelle as an endosymbiont, or they were retained by the host and incorporated into its nuclear DNA. Thus, many of these 'ancestral' genes, plus other 'newer' nuclear genes, not of direct proto-mitochondrial origin (more than 1200 in number (see Appendix 1)), encode proteins which are now synthesized in the cytoplasm and are then imported into mitochondria. How do these 1200 proteins know that they are supposed to go into mitochondria?

Mitochondrial importation is a complex process, with different pathways for the targeting and sorting of polypeptides to the four compartments of the organelle (the outer and inner membranes, the intermembrane space, and the matrix). Interestingly, key components of the import machinery are members of the so-called 'heat shock' protein family. These are 'molecular chaperones' that help unfold, and then refold, the mitochondrially targeted polypeptides as they are inserted through the import receptors and are sorted to the appropriate compartments.

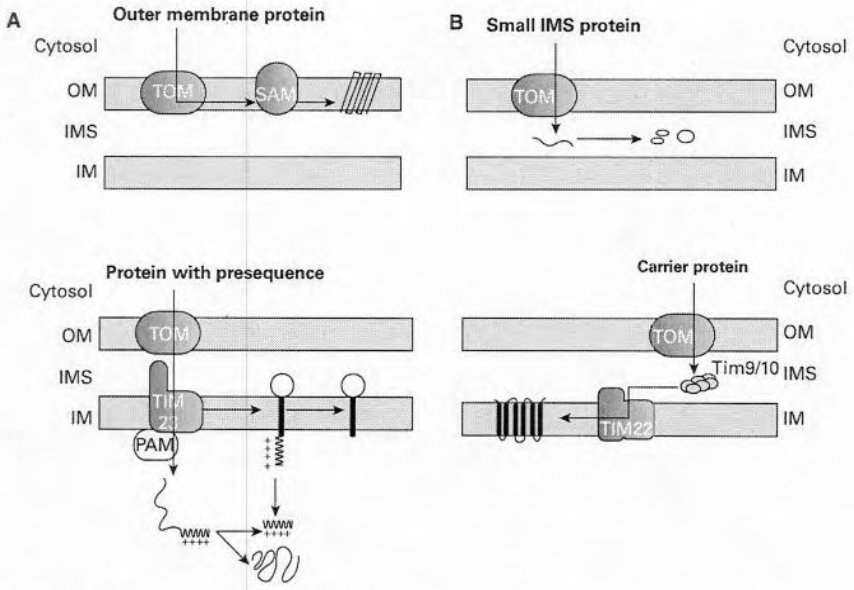

Figure B.1 Mitochondrial importation pathways. (**A**) Precursors of b-barrel proteins are translocated by the TOM complex. Their subsequent insertion and assembly into the outer mitochondrial membrane requires the SAM complex. (**B**) After their passage through the TOM channel, soluble proteins destined for the intermembrane space are recognized by specific factors that assist in folding and assembly. (**C**) Mitochondrial proteins carrying cleavable presequences utilize the TOM and TIM23 complexes to be translocated into the mitochondrial matrix or to be sorted into the inner membrane. (**D**) After translocation through the TOM complex, carrier proteins are guided by the small Tim proteins across the intermembrane space to the TIM22 complex, which then mediates their insertion into the inner membrane. From reference 1, with permission

For most of them, this 'addressing' capability is accomplished by the presence of a mitochondrial 'targeting signal' located at the N-terminus of the polypeptide (although some have C-terminal and even 'internal' targeting sequences).[1,2] Once inside the organelle, the N-terminal 'mitochondrial targeting signal' (MTS), also called a 'leader peptide' is cleaved to release the mature polypeptide. The leader sequence is usually highly basic (i.e. it contains many arginine and lysine residues, and few or no aspartate or glutamate residues), and it often contains peptide 'motifs' that determine the precise point of cleavage of the presequence inside the organelle. Almost all polypeptides destined for the matrix, and most destined for the inner membrane, have this type of MTS, to the point that there are a number of computer programs (IPSORT, Mitoprot, Predotar, Psort II, TargetP) that can predict with much success the probability that a polypeptide has such an MTS. A notable exception, however, is the 100 or so carriers and transporters, most located in the inner membrane. Some intermembrane space proteins have MTSs, but many do not; almost all outer membrane proteins have no recognizable MTS, and we still do not know what the targeting signals are in suffcient detail to be able to predict whether a protein is destined for that compartment.

In overview, the importation machinery consists of multi-subunit translocases that work in concert to parse out incoming polypeptides to the proper compartment. The translocase of the outer membrane (TOM) and inner membrane (TIM), in collaboration with Sorting and Assembly Machinery (SAM) proteins, directs polypeptides to the appropriate compartment, as shown in Figure B.1.

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neurotransmitter receptors are decreased in these patients[73,74] and in a transgenic mouse model of HD.[75] Because mitochondria are highly concentrated in nerve terminals,[76] these abnormalities could result from a mitochondrial dysfunction, and several lines of evidence are consistent with this idea. Muscle biopsies from HD patients show a reduced ratio of phosphocreatine to inorganic phosphate,[77] suggesting a build-up of precursor products due to decreased ATP production. Nuclear magnetic resonance spectroscopy shows elevated lactate levels in the cortex and basal ganglia of symptomatic HD patients,[78] indicating impairment of oxidative phosphorylation. In well-coupled mitochondria, a higher rate of oxygen consumption correlates with a higher rate of ATP production.[79] Polarography of mitochondria isolated from postmortem cortex and caudate of HD patients showed significant reductions in respiration.[80] This alteration is in agreement with the reduction in enzymatic activity of complexes II–III in these brain regions reported by others.[81,82] Complex II (succinate dehydrogenase) is a component common to both the tricarboxylic acid cycle and the electron transport chain. It catalyzes the oxidation of succinate to fumarate and transfers electrons to coenzyme Q. In animal models, inhibition of this enzyme by malonate or 3-nitropropionic acid produces striatal lesions similar to those seen in HD patients.[83] In these models, administrations of mitochondrial substrates such as creatine[84] and coenzyme Q[85] were neuroprotective, further reinforcing the hypothesis of metabolic defect as a pathogenic mechanism of HD.

Despite a wealth of data showing mitochondrial abnormalities in HD, the link between mutated

huntingtin and mitochondria is still largely speculative. Huntingtin is expressed ubiquitously in all brain cells and localized mostly in the cytoplasm and to a smaller extent, in the nucleus.[86] Yet, immuno-electron microscopy shows that the protein is also localized in the neuronal mitochondrial membrane[87] where it seems to accumulate at the level of the outer membrane.[88] Functionally, brain mitochondria from the HD mice have a lower membrane potential and depolarize at lower calcium concentrations than mitochondria from wild-type mice.[87] It has therefore been proposed that mutant huntingtin either induces the opening of the mitochondrial permeability transition (MPT) pore or decreases the threshold for calcium to induce MPT pore opening.[88] Of note, the release of cytochrome *c* as a result of huntingtin-induced MPT pore opening is inhibited by cyclosporin A.[88] In addition, mutant huntingtin increases the vulnerability of a striatal cell line, created from Hdh Q111 mouse embryos, to the mitochondrial toxin 3-NP.[89] This latter study implies that individuals with mutated huntingtin would be more susceptible to environmental mitochondrial toxins. Lastly, mutated huntingtin can also affect mitochondrial function indirectly through transcription factors. Huntingtin binds to and modifies the activity of transcription factors such as TATA-binding protein, cAMP-responsive element-binding protein (CREB)-binding protein (CBP), specificity protein 1 (Sp1), and p53.[90,91] These factors regulate the transcription of some mitochondrial proteins involved in energy metabolism and apoptosis. For example, CREB activation has been shown to be involved in mitochondrial dysfunction.[92] Huntingtin can also alter the activity of transcription factors through histone acetylation, which is regulated by both histone acetyltransferase and histone deacetylases. Indeed, huntingtin binds to and reduces the activity of histone acetyltransferase. The reduction in acetylation of histones leads to alterations in chromatin structure, which, in turn, alters gene expression.[93] It is thus conceivable that mutated huntingtin can perturb mitochondrial function by interfering with the activities of transcription factors; however, a study of HD transgenic mouse has clearly shown that these transcription factors are not affected.[94] Alteration of mitochondrial function in HD can also be indirect in that mutant huntingtin appears to impair axonal transport and, in so doing, the trafficking of organelles such as mitochondria.[95] This defect may be of pathogenic significance in HD since proper mitochondrial mobility has been linked to Ca^{2+} buffering and energy delivery to strategic subcellular sites of cells.[96]

Current therapeutic options for HD patients remain largely supportive. Nevertheless, major efforts are underway to find therapies that might delay or stop disease progression. Compounds that have been assessed in clinical trials and that are relevant to the mitochondrial hypothesis of HD include creatine and coenzyme Q10. Creatine, a mitochondrial 'energy-improving' compound, has been shown to prevent body weight loss and brain atrophy, improve motor performance, and extend life expectancy in HD animal models.[84,97-99] Coenzyme Q10, an electron carrier and a cofactor of the electron transport chain, also had similar protective effects in these animals.[85,100,101] The benefit of these compounds in animals, however, has not been replicated in human HD clinical trials.[102] Formation of protein aggregates has also been attributed to mitochondrial dysfunction. In keeping with this, administrations of congo red intraperitoneally[103] and of trehalose orally[104] inhibited protein aggregation and improved motor performance and survival in HD transgenic mice, supporting a very contentious argument that proteinaceous intranuclear inclusions in HD are cytotoxic.

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form

of dementia in the elderly. AD is characterized by impairments in memory and cognition. Increasing age and genetic mutations are both risk factors for AD. About 5% of AD cases are inherited in an autosomal dominant manner. Mutations in genes encoding amyloid precursor protein (APP) or APP processing proteins presenilin-1 (PS1) or presenilin-2 (PS2) cause the familial forms of AD. The remaining cases of AD are sporadic and their etiology is unknown. In both familial and sporadic forms of AD, the neuropathology is characterized by loss of cholinergic neurons and depositions of extracellular amyloid plaques and intracellular neurofibrillary tangles in the hippocampus and cortex. It is still a matter of debate whether these aggregates are neurotoxic.

As with HD and PD, knowledge of the mutated genes has not clarified the pathogenesis of AD. Although the discoveries of mutated APP and presenilins have strengthened the theory that amyloid β peptide (Aβ) is the culprit, strong disagreement exists regarding the mechanism by which Aβ induces neurodegeneration. Although highly controversial, mitochondrial dysfunction has also been suggested as a pathogenic mechanism in AD. Several circumstantial facts support this concept. For instance, a number of positron emission tomography investigations have shown that energy metabolism in AD brains is reduced.[105,106] Functional magnetic resonance spectroscopy has also shown a decreased ratio of phosphocreatine to inorganic phosphate in the AD brain.[107,108] In agreement with these studies are the reports of decreased glucose utilization in skin fibroblasts[109] and of decreased activities of pyruvate dehydrogenase[110] and -ketoglutarate dehydrogenase[111] in postmortem cortical tissue. In addition to these mitochondrial enzymes, the activity[112–114] and immunoreactivity[115,116] of cytochrome *c* oxidase is also reduced in the AD brain.

The mechanism by which mitochondrial dysfunction occurs in AD is unclear. The links between mitochondria and Aβ, PS1, and PS2 are still missing. Currently available transgenic animal models for AD[117] will potentially shed light on these missing links. For example, a confocal microscopy and immunogold electron microscopy study of transgenic mice overexpressing mutant Aβ, has shown that Aβ is present in mitochondria.[118] This study has also shown that Aβ binds to another mitochondrial protein, Aβ-binding alcohol dehydrogenase (ABAD), leading to the generation of ROS, release of cytochrome *c* and DNA fragmentation in cultured neurons obtained from transgenic mice.[118] Prior to this study, Aβ had been shown to inhibit mitochondrial respiration in isolated mitochondria.[119] Collectively, these studies suggest that one mechanism by which the mutated proteins in AD induce cell death could well be mediated by mitochondria. Until this mechanism is better understood and replicated by other laboratories, marginally effective cholinesterase inhibitors remain the major therapeutic option for AD patients. No pharmacological agent targeting mitochondria is currently being developed.

Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, after Alzheimer's disease.[120] PD is characterized by the loss of dopaminergic neurons in substantia nigra pars compacta (SNpc), leading to a reduction of dopamine in the putamen.[120] When the depletion reaches about 60% of SNpc dopaminergic neurons and 80% of dopamine content in the caudate, symptoms of PD appear.[120] Abnormal movements such as resting tremor, rigidity, and postural instability are common in PD patients.[120]

The etiology of PD is currently unknown. Environmental toxins have been hypothesized to play a dominant role based upon the observations that parkinsonism can be caused by encephalitic infection and accidental 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection.[120] However, more recent discoveries of various familial forms

of early-onset PD have given impetus to a genetic-based theory. Perhaps both mechanisms are involved and mitochondrial dysfunction could represent a common denominator that unifies these two theories. The notion of mitochondrial dysfunction as a pathogenic mechanism in PD emanated from the initial discovery that MPTP induced a parkinsonian syndrome in humans and various animal species.[120] Subsequently, 1-methyl-4-phenylpyridinium ion (MPP^+), the active neurotoxic metabolite of MPTP, was identified as an inhibitor of complex I of the mitochondrial electron transport chain.[121] This latter finding prompted many investigators to search for mitochondrial defects in PD patients. Soon enough, a plethora of publications reported more or less convincing respiratory defects in PD tissues including the SNpc[122] and platelets.[123,124] In cytoplasmic hybrid cell lines (cybrid cells), which contain mitochondria derived from platelets of PD patients, complex I activity was also reduced,[125] and this finding led the authors to conclude that complex I deficiency in PD is mitochondrially (i.e., maternally) inherited. Consistent with this, the same authors subsequently reported that complex I activity was reduced in maternal descendants of families with PD.[126] Furthermore, mutations and polymorphisms in subunits of complex I have been proposed as susceptibility genes in subsets of PD patients.[127,128] It has to be emphasized, however, that complex I deficiency only occurs in a subset of PD patients. This may partly explain the lack of complex I deficiency in a more recent study of cybrids.[129] Nevertheless, in aggregate, all of these studies suggest that genetic mutations, environmental toxins, or both, inhibit complex I activity and may play a pathogenic role in some PD patients. The recent demonstration that rats exposed to rotenone, a lipophilic complex I inhibitor, reproduce some key features of PD,[130] has revived interest in the relationship between complex I defects and sporadic PD.

The discoveries of various genes in early-onset PD have also suggested ways by which PD-causing mutations may provoke neurodegeneration via a mitochondrial mechanism.[131] By sequencing candidate genes within the PD locus *PARK6* region, two homozygous mutations have been identified in the PTEN-induced putative kinase 1 (PINK1)/BRPK gene.[5] This ubiquitously expressed protein has a serine/threonine kinase as the sole known functional domain.[132] Presumably PINK1 has a mitochondrial targeting motif because it localizes to mitochondria in transfected cell lines.[5] The two mutations thus far identified are expected to impair PINK1 kinase activity or substrate recognition and to cause PD by loss of function. Impaired phosphorylation of PINK1 substrate in mitochondria is likely to explain the pathogenic mechanism of the two mutations. While awaiting the elucidation of the critical substrates of PINK1, interesting data have been obtained in neuroblastoma cells transiently transfected with either wild-type or mutant PINK1.[5] There was no baseline alteration in viability with either the wild-type or mutated gene, but when neuroblastoma cells were challenged with the proteasome inhibitor MG132, those that overexpressed wild-type PINK1 survived the toxic insult, whereas those that overexpressed mutant PINK1 died to the same extent as non-transfected neuroblastoma cells.[5] These results suggest that wild-type mitochondrial PINK1 confers greater resistance to the mitochondria, and thus to the cell, against cytotoxic insults.

Eleven different DJ-1 mutations – including missense, truncating, splice site mutations, and large deletions – have been linked to an autosomal recessive form of PD.[133–135] In the normal situation, wild-type DJ-1 is in the cytosol, but, upon oxidation of one of its cysteine residues (C106), DJ-1 appears to translocate to the mitochondria.[136] The mitochondrial localization of DJ-1 seems to be critical, as mutation of C106 prevents DJ-1 from accumulating in the mitochondria and renders cells more susceptible to the toxic effects of MPP^+.[136] Other PD-causing DJ-1 mutations appear to also impair DJ-1

mitochondrial translocation.[133] Although the exact pathogenic mechanism of DJ-1 mutations remains to be determined, these studies suggest that the lack of DJ-1 translocation into mitochondria may be pivotal in the neurodegenerative process of PD.

Current therapeutic options for PD patients are symptomatic.[137] Among the agents being evaluated in clinical trials are the two mitochondrially targeted compounds, creatine and coenzyme Q10. These compounds were neuroprotective in the MPTP mouse model.[138,139] Results from one of these clinical trials has shown that coenzyme Q10 provides some therapeutic benefit to PD patients.[140]

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by the loss of motor neurons in the anterior horn of the spinal cord and cerebral cortex.[141] Onset usually is in the fourth or fifth decade of life, with rapid progression to paralysis and death within 2–5 years.[141] About 10% of ALS cases are familial (FALS), and 20% of these are caused by dominant mutations in Cu,Zn-superoxide dismutase (SOD1).[142,143] Thus far, >100 point mutations have been identified: they are scattered throughout the enzyme, and they are predominantly single amino acid replacements. Interestingly, these different mutations lead to the same clinical phenotype. The mechanism by which mutated SOD1 causes neurodegeneration in FALS is not clear. However, based on the observation that SOD1-deficient mice do not develop the hallmark features of the disease[144] whereas transgenic mice overexpressing mutated SOD1 do,[145,146] it has been suggested that mutated SOD1 provokes neurodegeneration by a toxic 'gain-of-function'.

Among the various pathogenic mechanisms proposed, mitochondrial dysfunction has also emerged. An early study of sporadic ALS showed abnormal mitochondrial morphology in muscle by electron microscopy.[147] Abnormal mitochondria were also reported in the anterior horn of the spinal cord,[148,149] corticospinal tract axonal swellings,[150] and muscle biopsies in a subset of sporadic ALS patients.[151,152] 'Bizarre giant mitochondria' were described in hepatocytes from 21 of 21 sporadic ALS patients.[153] In addition to abnormal morphology, reduction in mitochondrial number has also been reported in intramuscular nerves[154] and spinal cords[155] of sporadic ALS patients. In these same patients, mitochondrial respiratory chain activities were decreased in spinal cords[155,156] and muscles.[157] However, another group failed to confirm these biochemical defects in skeletal muscles from sporadic ALS patients.[158]

As for familial ALS linked to mutant SOD1, it should be mentioned that SOD1 had long been considered a cytosolic enzyme, but it has now also been identified in the mitochondria.[159–163] This finding strongly suggests a role for SOD1 in mitochondria, which may be relevant to the pathogenesis of FALS. Consistent with this idea, reduced respiratory chain functions and abnormally high release of apoptogenic mitochondrial molecules such as cytochrome *c* have been documented in transgenic mice expressing one pathogenic SOD1 mutation (G93A).[161,164–166] Furthermore, mutant SOD1 has been identified in several intramitochondrial compartments in the brain, and in the matrix, this mutant protein is misfolded and prone to aggregation.[167] Other investigators have described vacuoles in the spinal cord of these ALS mice, which originated from degenerating mitochondria and which preceded the death of motor neurons.[168,169] Supporting the pathogenic significance of these mitochondrial alterations in ALS-linked SOD1 mutations is the observation that creatine and coenzyme Q10 improve motor performance and survival in transgenic mice.[170] Together, these studies suggest that there is a relationship between mitochondrial dysfunction, death of motor neurons, and clinical symptoms in the transgenic mutant SOD1 mice. Consistent

with this is also the report that ALS transgenic mice are more vulnerable to mitochondrial toxins such as MPTP and 3-NP.[171]

Therapeutically, a few drugs with potential mitochondrial effects are being tested for ALS. For example, the antibiotic minocycline has been shown pre-clinically to improve motor performance and extend survival in ALS transgenic mice, presumably by preventing the release of apoptogenic molecules from the mitochondria.[172] Minocycline is currently in phase III clinical trials.[173] In contrast to the results obtained in ALS mice, a double-blind study of creatine failed to improve either clinical symptoms or survival in ALS patients.[174]

Mechanism of cell death in mitochondrial neurodegenerative diseases

One of the most intriguing and unresolved questions that pertain to mitochondrial diseases, including those associated with neurodegeneration, is how cells actually die in these pathological situations. As emphasized above, most of the definite mitochondrial neurodegenerative diseases are linked to some sort of defect in the respiratory chain. In keeping with this, it can be predicted that complete loss of mitochondrial respiration would be lethal, as suggested by the embryonic death of mutant mice deficient in mitochondrial transcription factor A.[175] In contrast, the late onset and chronic nature of the neurodegenerative diseases discussed in this chapter suggests that the mitochondrial defects are more subtle, causing a progressive worsening of mitochondrial and cellular function which culminates in cell demise.

Mitochondrial dysfunction, ROS overproduction, and ATP deficit

The extremely high energetic demand of the brain is illustrated by the fact that, while the brain represents only about 1/50 of the body weight, it consumes about 1/5 of the total oxygen inhaled. Of this amount of oxygen, about 90% is utilized by mitochondria to produce ATP. Although, through the course of evolution, mitochondria have provided eukaryotic cells with a very efficient aerobic metabolism, an estimated 1–2% of consumed oxygen is converted to ROS rather than water.[176] The inability of the respiratory chain to completely reduce oxygen to water, coupled with its high rate of oxygen consumption, contributes to the high ROS production of the mitochondrial respiratory chain. In situations of mitochondrial dysfunction this rate may increase dramatically. There are two main sites in the electron transport chain where ROS are generated: complex I and complex III (see Figure 13.1). A dysfunction of complex I, resulting either from a mutation or from toxicity by MPTP or rotenone, can dramatically increases ROS production. This elevation is a result of 'leakage' of electrons from complex I and the subsequent reduction of oxygen to superoxide. Increased production of ROS is also seen when complex III is inhibited by antimycin, leading to the accumulation of ubisemiquinone. Intrinsically, mitochondria are equipped with a defense mechanism against ROS, including antioxidant enzymes such as manganese superoxide dismutase and glutathion peroxidase. Any imbalance between the production of ROS and the antioxidant capacity of these enzymes leads to a local oxidative stress, potentially causing serious functional and structural damage to the mitochondria. How much of this mitochondrial oxidative stress can also damage cytoplasmic molecules and other organelles is still an unresolved question. The view that mitochondrial ROS leak out and inflict extramitochondrial oxidative damage is common in the literature, but the majority of ROS produced inside the mitochondria are not membrane permeant, and it is unclear how they get to the cytosol. This uncertainty does not undermine the potential significance of mitochondrial-derived oxidative

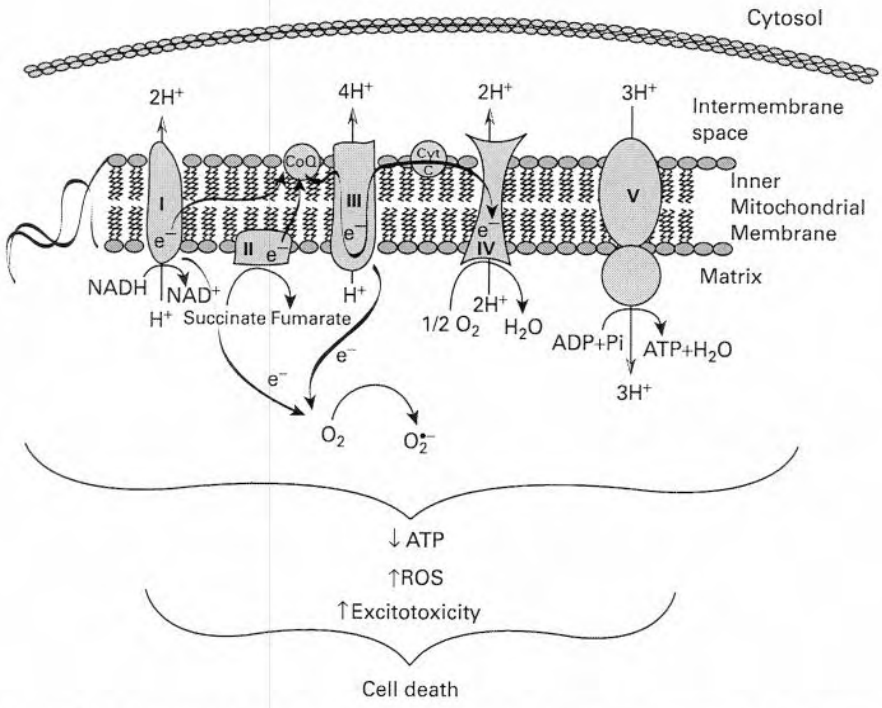

Figure 13.1 Schematic representation of electron transport chain (ETC). Complex I and complex III are the two major sites where ROS are produced. Dysfunction in the ETC, in general, as a result of mutations or blockade by specific inhibitors of the complexes, would lead to an increase in ROS production, reduction in ATP production, or increase in excitotoxicity. These abnormalities potentially result in cell death

stress in the cell death process underlying mitochondrial neurodegenerative diseases.

Defective mitochondria not only produce more ROS, but also less ATP, which is necessary for many critical cellular processes. However, it is unclear how low ATP synthesis must get to provoke cell damage. Studies in cultured cells expressing mitochondrial mutations known to impair ATP synthesis show that cellular stores of ATP drop to pathological levels only when the cells are placed under conditions of high ATP demand.[177] This suggests that cells such as neurons, which have variable ATP needs related to changes in ion fluxes in response to external stimuli, would experience energy crises only when ATP demands rise above basal levels. Thus, any prolonged energy crisis due to a sustained increase in ATP demand may not only impair basic ATP-dependent neuronal functions such as neurotransmission, but also increase the probability for that neuron to die. For instance, ATP deficiency may lead to Na^+/K^+-ATPase failure and subsequent neuronal membrane depolarization, which, via activation of NMDA receptors, may subject neurons to an excitotoxic injury.

Mitochondria and apoptosis

As indicated above, mitochondria also act as a major supplier of apoptogenic molecules (see Figure 13.2). Apoptosis is an active and programmed form of cell death regulated by multiple

molecular pathways.[178,179] In addition to representing the 'intrinsic' apoptotic pathway, mitochondria also serve as a central hub that interacts with other pathways of apoptosis. The surge of interest in the role of mitochondria in apoptosis was sparked by the discovery that apoptosis can be induced by cytochrome *c* released from mitochondria.[180] After being released into the cytosol, cytochrome *c* interacts with apoptosis proteases-activating factor-1 (Apaf1) and procaspase 9 to form a complex known as apoptosome, which then activates caspase 3.[181] Caspases are a group of proteolytic enzymes which cleave their substrates at specific aspartic acid residues. Activation of caspases potentially leads to apoptotic death. A second mitochondrial apoptogenic protein released into the cytosol is Smac/Diablo. In the cytosol, this protein inhibits the 'inhibitors of apoptosis proteins' (IAPs), leading again to activation of caspases. Two other mitochondrial proteins, apoptosis-inducing factor (AIF) and endonuclease G, are released and translocated from mitochondria to nuclei where they induce DNA fragmentation. In addition to these apoptogenic molecules, mitochondria also contain anti-apoptotic proteins, such as Bcl-2 and Bcl-XL. The balance between pro- and anti-apoptotic molecules must be tightly controlled to ensure proper execution of apoptosis.

Sequential, multifactorial scenario of neuronal death

The use of inhibitors of mitochondrial respiration shows that ATP depletion and ROS overproduction occur soon after the block of oxidative phosphorylation, subjecting the cells to energy crisis and oxidative stress. However, the time-course of these perturbations[182] appears to correlate poorly with the time-course of neuronal death,[120,181] suggesting that only a few neurons probably succumb to the early combined effects of ATP depletion and ROS overproduction. Instead, there is mounting evidence[183] that rather than killing the cells, alterations in ATP synthesis and ROS production trigger cell death-related molecular pathways, which, once activated, rapidly lead to the demise of neurons. Among these molecular pathways, the mitochondrial-dependent apoptotic machinery may play a critical role.[9] In the context of most mitochondrial neurodegenerative disorders, where defective mitochondrial respiration is unequivocally present, this cascade of deleterious events may be operative. In other diseases discussed above, in which mitochondrial protein degradation or ISC biosynthesis are deranged, apoptosis may be recruited by alternative mechanisms, including direct interactions between disease-causing mutated proteins and a key factor of the apoptotic cascade. This latter view is quite provocative and warrants, in our opinion, further investigation.

Conclusion

In the past decade, there have been some major discoveries on the role of mitochondria in neurodegenerative diseases. We now understand that through depletion of ATP, generation of ROS, and release of apoptogenic proteins, mitochondria may hold a key role in neurodegenerative processes. Initiation of these events, either individually or more likely in combination, would potentially lead to neuronal death. We also understand that mitochondrial abnormalities can arise from a variety of causes: mutations in mtDNA, mutations in nDNA encoding for mitochondrial proteins, and from mutations in genes apparently unrelated to mitochondria. What is clear is that a pathogenic scenario, inspired by definite or suspected mitochondrial alterations, can be envisioned to support a mitochondrial role in almost all neurodegenerative diseases. If this mitochondrially based pathogenic scenario is fairly acceptable in situations where genetic mutations impact on the mitochondrial function in a meaningful manner, the jury is still out as to whether such a scenario applies to sporadic neurodegenerative diseases. It

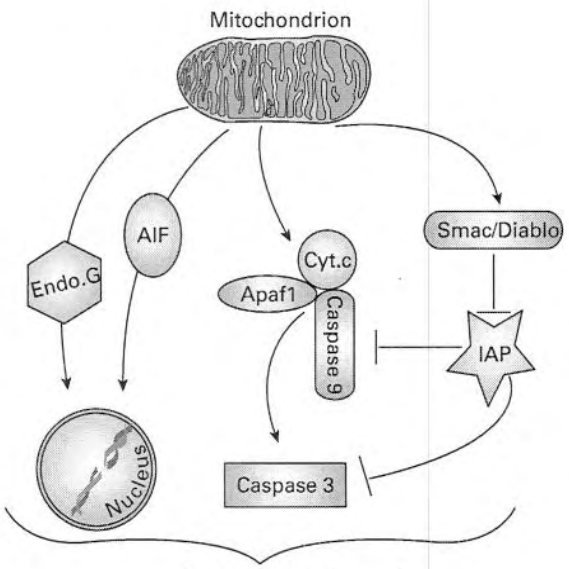

Figure 13.2 Mitochondria and apoptosis. As discussed in the text, mitochondria play a major role in the cascades of apoptosis. Shown here are the various apoptogenic molecules released from mitochondria, leading to caspase 3 activation and DNA fragmentation and ultimately cell death

is disturbing that pathogenic mutations in mtDNA do not cause the typical clinical manifestations of neurodegenerative diseases such as HD, AD, PD, and ALS. As emphasized by Schon and Manfredi, of the large number of pathogenic mtDNA mutations, only a handful cause chorea and dementia, parkinsonism, dystonia, or motor neuron disease and – almost without exception – these phenotypes are associated with atypical neurological features. Conversely, movement disorders are rare in 'primary' mitochondrial diseases. Some aspects of mitochondrial genetics, such as heteroplasmy, mitotic segregation, and the threshold effect, may contribute to our difficulty in linking common neurodegenerative diseases to mitochondrial defects. It is also important to remember that most studies on the mitochondrial link to neurodegeneration have been mainly performed in autopsy material, which often originates from terminally ill patients and is devoid of almost all neurons of interest, those that are proposed to die from mitochondrial dysfunction. Thus, many, if not all of these studies reflect analyses performed on a population of surviving cells (e.g., glia) not necessarily representative of the actual neuronal death mechanism. Finally, it is also crucial to remember that mitochondrial defects reported in postmortem tissues may simply reflect non-specific alterations that occur in dying neurons. The development of better in vivo experimental models of neurodegenerative diseases may provide us with the necessary tools to appropriately examine the mechanistic relationship between neurodegeneration and mitochondrial dysfunction and to address once and for all many of the pending issues that cloud the field of sporadic mitochondrial neurodegenerative diseases.

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7 Etiology and Pathogenesis of Parkinson's Disease

Serge Przedborski

Parkinson's disease (PD) is a common adult-onset neurodegenerative disorder whose disabling cardinal motor signs are mainly engendered by the loss of dopaminergic neurons in the substantia nigra. To date, researchers still have a limited understanding of the key molecular events that provoke neurodegeneration in this disease. A prevalent etiologic hypothesis is that PD may result from a complex interaction between environmental toxic factors, genetic susceptibility traits, and aging. As for its pathogenesis, the discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway may be pivotal in the cascade of deleterious events underling the demise of dopaminergic neurons. Previously implicated culprits in PD neurodegeneration, such as mitochondrial dysfunction, oxidative stress, and inflammation, also may produce deleterious effects on dopaminergic neurons. This chapter discusses the evidence and the ongoing lines of research relevant to the quest of unraveling the cause of and the mechanism of neuronal death in PD.

DEFINITION AND DIAGNOSTIC CONSIDERATIONS

Over the past decade, PD has garnered widespread interest, in part because several public figures and celebrities were identified as having this illness. PD is considered to be the second most common degenerative disorder of the aging brain after the dementia of Alzheimer's. In keeping with this, it is estimated that currently more than a million individuals are affected with PD in the United States alone, and, with the aging character of the society, this figure is forecasted to rise dramatically over the coming years. The overall incidence of PD in the United States (which surges upward after the age of 60) is roughly 13.4 per 100,000 people/year (1), and it is approximately twice as frequent in men than in age-matched women (1). Unfortunately, whether PD frequency varies among ethnic groups or geographic location remains an unsettled issue (1).

PD is progressive with a mean age at onset of 55, and, while the introduction of effective symptomatic treatment such as with levodopa has prolonged survival, PD patients, especially with severe parkinsonism or dementia (2), have a higher mortality risk compared to healthy controls (3). The mean disease duration of PD, as defined by the period between onset of the clinical manifestations to death, has been estimated to be 10.1 to 12.8 years (4,5). It remains uncertain whether the observed shortened life expectancy of PD patients results from the disease per se or rather from the ensuing motor and cognitive impairments, which increase the odds of fatal accidental falling, aspiration pneumonia, pressure skin ulcers, malnutrition, and dehydration. Relevant to this point is the finding that twice as many PD patients die from pneumonia as in the age-matched control population (4). This is more than a parochial discussion of semantics, as it goes to the heart of how best to care for PD patients.

As illustrated in figure 7.1, the main neuropathological feature of PD is the loss of neuromelanin-containing dopaminergic neurons of the nigrostriatal pathway (6),

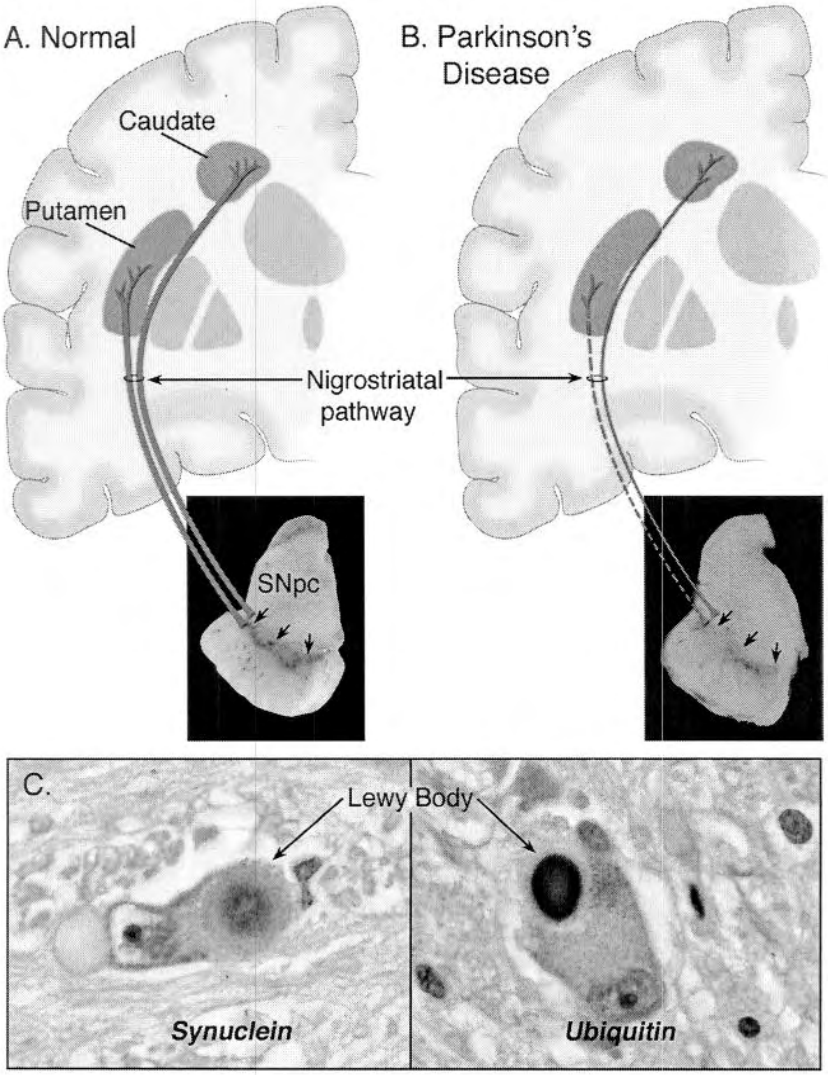

Figure 7.1 Neuropathology of Parkinson disease. **(A)** Schematic representation of the normal nigrostriatal pathway (*in red*). It is composed of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc; *see arrows*). These neurons project (*thick solid red lines*) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). The photograph demonstrates the normal pigmentation of the SNpc produced by neuromelanin within the dopaminergic neurons. **(B)** Schematic representation of the diseased nigrostriatal pathway (*in red*). In Parkinson disease, the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons that project to the putamen (*dashed line*) and a much more modest loss of those that project to the caudate (*thin red solid line*). The photograph demonstrates depigmentation (i.e., loss of dark-brown pigment neuromelanin; arrows) of the SNpc due to the marked loss of dopaminergic neurons. **(C)** Immunohistochemical labeling of intraneuronal inclusions (Lewy bodies) in an SNpc dopaminergic neuron. Immunostaining with an antibody against alpha-synuclein reveals a Lewy body (*black arrow*) with an intensely immunoreactive central zone surrounded by a faintly immunoreactive peripheral zone (*left photograph*). Conversely, immunostaining with an antibody against ubiquitin yields more diffuse immunoreactivity within the Lewy body *(right photograph)*. (Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron* 2003; 39:889–909, with permission.)

which, in depleting the brain of dopamine (7), leads to the emergence of motor abnormalities such as tremor at rest, rigidity, slowness of voluntary movement, and postural instability (8). By the time patients become symptomatic, however, ~60% of nigral dopaminergic neurons have been lost and striatal content in dopamine has been reduced by ~80%. It can thus be concluded that disease onset predates the expression of the motor manifestations of PD, which by 18F-fluorodeoxyglucose with positron emission tomography (PET) has been estimated to be about 4.5 years (9). In addition to demonstrating that the presymptomatic period of PD appears to be relatively short, this PET study also documented that the pattern of glucose metabolic alterations in PD is not consistent with the idea that neurodegeneration in this illness is a simple exacerbation of the normal age-related decay of the nigrostriatal dopaminergic system (9). Although the lion's share of attention is consistently paid to the nigrostriatal pathway, it must be remembered that in reality degenerative changes in PD are not restricted to the nigrostriatal pathway and that neuropathological findings can be found in many other dopaminergic and nondopaminergic cell groups, including the locus coeruleus, raphe nuclei, and nucleus basalis of Meynert (10). This is a particularly important notion with respect to the proper management of PD, as some quite disabling features, especially in advanced patients, such as postural instability and cognitive impairment, may not find their pathophysiology in the damage of the dopaminergic system and usually fail to improve with levodopa therapy.

Also important is the fact that more than 30 different neurological syndromes share PD clinical features (Table 7.1). Thus, a definite diagnosis of PD often is achieved only at autopsy and customarily relies not only on finding a loss of nigrostriatal dopaminergic neurons but also on the identification of intraneuronal inclusions, or Lewy bodies, that can be seen in many of the surviving cells of all affected brain regions (figure 7.1). Lewy bodies are spherical eosinophilic cytoplasmic aggregates of fibrillary nature that, as illustrated in Figure 7.1, are composed of a variety of proteins, including alpha-synuclein, parkin, ubiquitin, and neurofilaments (11,12). Many authorities, however, question whether identification of Lewy bodies should still be necessary for the diagnosis of PD, in light of the fact that cases of inherited PD linked to parkin mutations typically lack Lewy bodies and are still considered as cases of PD. These facts raise the question as to whether the current nosology of parkinsonian syndromes may not have to be revised to distance itself from the classification typically based on the disease's clinical and neuropathological hallmarks, to evolve toward a classification based on the disease's molecular characteristics. Conceivably, in this novel approach, parkinsonian syndromes that used to belong to distinct categories may become lumped together because of a common molecular defect. Although the usefulness of such a proposed recasting would have to be demonstrated, the idea deserves, at the very least, serious consideration as the issue of diagnostic heterogeneity is a well-recognized hurdle for clinical trials geared toward testing neuroprotective agents for PD.

TABLE 7.1
PARKINSONIAN SYNDROMES

Primary Parkinsonism
- Parkinson disease (sporadic, familial)

Secondary Parkinsonism
- Drug-induced: dopamine antagonists and depletors
- Hemiatrophy–hemiparkinsonism
- Hydrocephalus: normal-pressure hydrocephalus
- Hypoxia
- Infectious: postencephalitic
- Metabolic: parathyroid dysfunction
- Toxin: Mn, CO, MPTP, cyanide
- Trauma
- Tumor
- Vascular: multiinfarct state

Parkinson-Plus Syndromes
- Cortical-basal ganglionic degeneration
- Dementia syndromes: Alzheimer's disease, diffuse Lewy-body disease, frontotemporal dementia
- Lytico-Bodig (Guamanian Parkinsonism–dementia—ALS)
- Multiple system atrophy syndromes: striatonigral degeneration, Shy-Drager syndrome, sporadic OPCA, motor neuron disease–parkinsonism
- Progressive pallidal atrophy
- Progressive supranuclear palsy

Familial Neurodegenerative Diseases
- Hallervorden-Spatz disease
- Huntington's disease
- Lubag (X-linked dystonia–parkinsonism)
- Mitochondrial cytopathies with striatal necrosis
- Neuroacanthocytosis
- Wilson's disease

ALS, amyotrophic lateral sclerosis; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; olivopontocerebellar degeneration, OPCA.
Source: From Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron* 2003;39:889–909, with permission.

ETIOLOGIC THEORIES OF SPORADIC PARKINSON DISEASE

The etiology of almost all occurrences of PD remains unknown. In more than 90% of the cases, PD arises as a sporadic condition, i.e., in the absence of any apparent genetic linkage, but in the remaining instances the disease is unquestionably inherited (Table 7.2). Nonetheless, first-degree relatives of sporadic PD patients are two to three times more likely to have PD than relatives of controls (13). It is therefore not surprising that until now, all of the hypotheses regarding the etiology of sporadic PD have been focused primarily on environmental toxins and

genetic factors. As pointed out by Tanner et al. in the previous edition of this chapter (14), the dogma about the etiology of PD has for a long time been that a single or, at most, a few genes or environmental factors are sufficient to cause PD in most individuals and that both genetic and environmental risk would operate *independently*. The current school of thought, however, proposes a more multifactorial view of the problem by which diseases such as PD would result from a complex interplay of genetic and environmental factors. Based on this scenario, even in the presence of a known single-gene pathogenic mutation, PD would arise only when both the genetic variant and the deleterious environmental exposure coincide. In this context, a genetic variation would not necessarily cause disease but rather would influence a person's susceptibility to environmental factors. Hence, a person may not inherit the disease state per se but rather a set of susceptibility traits to certain environmental factors placing that person at higher risk of developing PD.

ENVIRONMENTAL TOXINS

According to the *environmental hypothesis*, PD-related neurodegeneration is provoked by exposure to a dopaminergic neurotoxin. The progressive nature of the neurodegeneration of PD would either result from a persistent toxic exposure that causes a sustained insult or from a circumscribed toxic exposure that initiates a self-perpetuating cascade of deleterious events. Relevant to the environmental hypothesis are numerous human epidemiological studies that have implicated consumption of well water, residence in a rural setting, farming and its associated exposure to herbicides and pesticides with an elevated risk for PD. These epidemiological notions have been reviewed comprehensively by Tanner et al. in the previous edition of this chapter (14), and will thus not be rehashed. Instead, only selected and recent epidemiology data will be discussed in the following pages.

From the outset it is stressed that none of the epidemiological studies to date have convincingly linked a specific environmental toxin to the cause of sporadic PD. Nevertheless, cigarette smoking and coffee drinking are inversely associated with PD (15), reinforcing the concept that environmental factors may indeed contribute to PD susceptibility or etiology. It is thus plausible that the lack of compelling evidence incriminating specific environmental factors simply reflects the limitations of our current analytic methodologies. Moreover, there are several known parkinsonian neurotoxins that potentially could be accumulating in our environment. For instance, exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of 1-methyl-4-phenyl-4-propionoxypiperidine synthesis (16), causes a clinical condition in human (17) and several other mammalian species almost identical to PD. Although autopsy studies of MPTP-intoxicated individuals with parkinsonism have consistently demonstrated a profound degeneration of the nigrostriatal dopaminergic pathway, no Lewy body has ever been found in these postmortem brain samples (18,19). Moreover, despite the impressive resemblance between PD and MPTP intoxication, MPTP has never been recovered from brain tissues or body fluids of PD patients.

Another potential environmental toxin is 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP, which has been developed as Cyperquat™, an herbicide (never commercialized), and paraquat, both of which are structurally similar (Fig. 7.2). Both MPP^+ and paraquat are polar molecules and consequently do not passively cross the blood–brain barrier (BBB). Of these two toxins, only for paraquat was an active transport system at the level of the BBB been identified (20), making it possible at least for the latter to accumulate in the brain and inflict damage. Consistent with this view is the demonstration that systemic administration of paraquat to rodents is associated with the degeneration of nigrostriatal neurons and the formation of Lewy-bodylike inclusions (21). Chronic exposure to paraquat has also been associated in a case-controlled study in Taiwan with an increased risk of developing PD (22). Interestingly, as noted by Thiruchelvam et al. (23), manganese ethylenebisthiocarbamate shows a striking geographic overlap with paraquat and has been implicated in cases of PD-like syndrome in agricultural workers, and it seems to exacerbate the dopaminergic neurotoxicity of paraquat. If paraquat were to be an environmental toxicant implicated in PD, the latter observation suggests that one should probably not think of the contribution of an environmental insult to the cause of PD in terms of a single toxin but rather in terms of

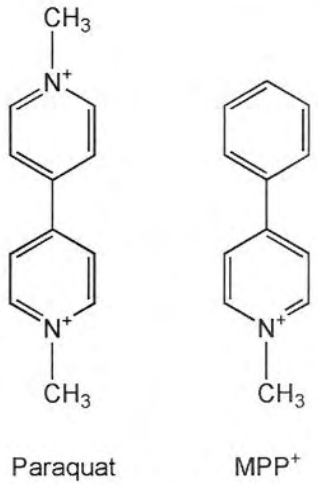

Figure 7.2 Structural similarity between paraquat (*left*) and MPP^+ (*right*). The only difference between these two compounds is the second *N*-methyl-pyridium group that paraquat has instead of the phenyl group seen in MPP^+.

a combination of toxic agents such as paraquat, manganese ethylenebisthiocarbamate, and possibly others.

What about rotenone as an environmental culprit? Rotenone is the most potent member of the rotenoids, a family of natural cytotoxic compounds extracted from various parts of *Leguminosa* plants. Like MPP^+, rotenone is a mitochondrial poison that is present in the environment and abundantly used around the word as insecticide and piscicide used to kill unwanted lake fish (24). Although rats chronically intoxicated with rotenone display nigrostriatal damage with Lewy-bodylike inclusions (25), it is unlikely to cause PD because rotenone is unstable, lasting only a few days in the environment (24). Indeed, rotenone breaks down readily by exposure to sunlight. Nearly all the toxicity of the compound is lost in 5 to 6 days of spring sunlight or 2 to 3 days of summer sunlight. Rotenone is also broken down rapidly in soil and in water. Also arguing against an etiologic role of rotenone in PD is the demonstration that chronic ingestion of rotenone for 24 months at doses 30 times greater that used to model PD by systemic infusion (25) failed to cause any behavioral or neuropathological features of the disease in rats (26). Nevertheless, there is one case of fatal rotenone poisoning following its acute ingestion (26). At autopsy, rotenone was found in the blood, liver, and kidney but not in the brain.

Another possibility, which does not, in the strict sense of the word, fall into the environmental category, is that an endogenous toxin may be responsible for PD neurodegeneration. As discussed elsewhere (27), distortions of normal metabolism might create toxic substances because of environmental exposures or inherited differences in metabolic pathways. One obvious source of endogenous toxins may be the metabolism of dopamine, which may generate harmful reactive oxygen species (ROS) (28). Consistent with the endogenous toxin hypothesis is the report that patients harboring specific polymorphisms in the gene encoding for the xenobiotic detoxifying enzyme cytochrome P450 may be at greater risk of developing young-onset PD (29). Further, isoquinoline derivatives and 6-hydroxydopamine, which are both toxic to dopamine neurons, have been recovered respectively from the brain (30) and urine of PD patients (31). Yet the link between polymorphisms in the xenobiotic-metabolizing enzymes and risk of PD remains equivocal, and a comprehensive discussion on this important topic can be found in Paolini et al. (32). From the preceding discussion, it can be concluded that, at this point, the environmental hypothesis of PD remains highly speculative but by no mean negligible.

MITOCHONDRIAL GENETICS AND PARKINSON DISEASE

For several decades, a mitochondrial defect has been proposed as an etiologic factor in PD, but when one scrutinizes the available evidence it appears that this appealing notion is nothing more than presumptive. For instance, all mitochondrial DNA of a zygote originate from the ovum, thus it is traditionally viewed that diseases linked to mitochondrial mutations are maternally inherited. To date, it is true that some epidemiological evidence supports a maternal inheritance pattern in a subset of PD patients (33,34). Furthermore, polymorphisms in subunits of complex I of the electron transport chain have been proposed as susceptibility genes in subgroups of PD patients (35,36). Although thus far true cases of PD linked to a mitochondrial mutation are still lacking, it should be mentioned that a point mutation (A1555G) in the 12SrRNA gene has been implicated in a maternally inherited deafness associated with levodopa-responsive parkinsonism (37). Subsequently, a distinct heteroplasmic, maternally inherited 12SrRNA point mutation (T1095C) was found in another pedigree with sensorineural deafness, levodopa-responsive parkinsonism, and neuropathy (38). These mutation were not found, however, in 20 cases of sporadic PD, suggesting that the 12SrRNA mutations are not likely to be a common cause of PD. It can thus be concluded that current data supporting the mitochondrial genetic hypothesis of PD etiology are only circumstantial and indirect. That said, mitochondrial alterations may still be instrumental in both the etiology and pathogenesis of PD (see the following) through molecular defects other than a mitochondrial mutation.

PARKINSON DISEASE MENDELIAN GENETICS

Since the discovery in 1997 that missense mutations in alpha-synuclein cause a rare form of PD, there has been a resurgence of interest in genetic factors that contribute to the etiology of the disease. Since mutations in parkin, ubiquitin C-terminal hydrolase L1 (UCH-L1), DJ-1, PINK1, and LRRK2/dardarin have also been identified as causes of familial forms of PD (see the following), and linkages for a number of other kindreds have been identified (Table 7.2). In addition to their detection in familial PD, mutations in parkin and LRRK2 were also found in a number of patients without apparent family history for PD (39,40). Moreover, large-scale genetic epidemiological studies have revealed a single-nucleotide polymorphism in tau to be associated with PD susceptibility (41), and both apolipoprotein E and probably more than one gene on chromosome 1p have been reported to influence age at onset for PD (42,43). Heterozygosity for a mutations in the glucocerebrosidase gene may also predispose to PD (44).

Worth noting is the fact that studies of monozygotic twins demonstrated a lack of concordance for PD, and thus this finding has been frequently cited as evidence arguing against a strong genetic contribution to sporadic PD (45). Yet, the interpretation of twin studies in PD is often complicated by the fact that twins may be clinically discordant for PD for up to 20 years (46). Moreover, a study utilizing

TABLE 7.2
GENES AND LOCI LINKED TO FAMILIAL PARKINSON DISEASE

Locus	Chromosomal Location	Gene/ Protein	Inheritance	Atypical PD Features	Lewy Bodies
PARK1	4q21	Alpha-synuclein*	AD	Early onset Lower prevalence of tremor	Yes
PARK2	6q25.2–q27	Parkin	AR	Early juvenile onset More frequent dystonia and levodopa-induced dyskinesias Slower disease progression	Mostly negative#
PARK3	2p13	Unknown	AD	Dementia in some patients Rapid progression	Yes
PARK4†	4p15	Unknown	AD	Early onset Rapid progression Dementia Autonomic dysfunction Postural tremor	Yes
PARK5	4p14	UCH-L1	AD	None	Unknown
PARK6	1p36	PINK1	AR	Early onset Slow progression	Unknown
PARK7	1p36	DJ-1	AR	Early onset Psychiatric symptoms Slow progression	Unknown
PARK8	12p11.2–q13.1	LRRK2/dardarin	AD	None	Some
PARK9	1p36	Unknown	AR	Juvenile onset Spasticity Supranuclear gaze paralysis Dementia	Unknown

AD, autosomal dominant; AR, autosomal recessive. * Including mutations and wild-type multiplications. # Lewy bodies reported in one patient with parkin mutations (64). †The initial PARK4 linkage to 4p15 could not be confirmed, and the PD phenotype in this family was subsequently linked to a PARK1 variant, i.e., alpha-synuclein triplication (56).
Source: Vila M, Przedborski S. Genetic clues to the pathogenesis of Parkinson's disease. *Nat Med* 2004;10(suppl):S58–S62, with permission.

18F-fluorodopa with PET to investigate the dopaminergic function in twin pairs clinically discordant for PD found significantly greater concordance for monozygotic but not dizygotic twins (47). These observations emphasize the need to utilize functional imaging to identify patients with subclinical disease in such studies and suggest that the actual contribution of genetic factors to the cause of PD may be more significant than thought initially.

Alpha-Synuclein Mutations and Overexpression

Three missense mutations (A53T, A30P, E46K) in the alpha-synuclein gene are linked to a dominantly inherited PD (48–50). Clinical and pathological features typical of PD have been found in brains from patients with any of the three mutations, although some atypical features have also been noted (49,51). Conversely, alpha-synuclein mutations have not been found in sporadic PD (52). Therefore, the idea that alpha-synuclein–mutant and sporadic PD share common pathogenic mechanisms relies predominantly on the observation that alpha-synuclein is a major component of Lewy bodies in sporadic PD (53). Transgenic overexpression of mutant alpha-synuclein in mice or flies has been generally associated with the development of some of the neuropathological features of PD, such as intraneuronal proteinaceous inclusions (27). These studies, together with the finding that alpha-synuclein ablation in mice does not lead to neurodegeneration (54,55), support the concept that alpha-synuclein mutations operate by a toxic gain-of-function mechanism.

Remarkably, multiplication of the gene encoding for wild-type alpha-synuclein can also cause an autosomal dominant PD phenotype (56–58). An important finding that has emerged from these studies is that relatively small alterations in alpha-synuclein expression can have marked impacts on the disease phenotype. In the study by Chartier-Harlin et al. (57), individuals with alpha-synuclein gene duplication had a mean age of onset of 48 years, with a disease duration of approximately 17 years. Similarly, in the

study by Ibáñez and collaborators (58), both patients with the duplication mutation had onsets of the disease at ages 46 and 50. Conversely, in the report by Singleton et al. (56), affected individuals carried four copies of the alpha-synuclein gene and developed a rapidly progressive (mean duration: 8 years) and young-onset form of PD (mean age: 38 years). It thus seems that the higher the expression level of alpha-synuclein, the more malignant the PD phenotype. The fact that increased amounts of wild-type alpha-synuclein can cause PD also advocates for the idea that the cytotoxic function gained by mutant alpha-synuclein proteins is not a newly acquired property but rather the enhancement of a native property. However, the nature of the gained function is still enigmatic, in part because the normal function of alpha-synuclein is just beginning to be elucidated. The fact that this prevalent presynaptic protein is abundant in Lewy bodies prompted many investigators to believe that its propensity to misfold and form alpha-synuclein fibrils may be responsible for its neurotoxicity. More can be read about the hypothesized toxic mechanisms of alpha-synuclein in the Dauer et al. (27) and Vila et al. (59).

Parkin Mutations

Loss-of-function mutations in the gene encoding parkin cause recessively inherited parkinsonism (60). Although this form of parkinsonism was originally termed *autosomal recessive juvenile parkinsonism*, the clinical phenotype includes a man with onset at age 64 and other older-onset patients. In general, however, parkin mutations are found in PD patients with onset before age 30, particularly those with a family history consistent with recessive inheritance (61). Clinically, parkin mutant patients display the classic signs of parkinsonism but with marked improvement of symptoms with sleep, abnormal dystonic movements, and a striking response to levodopa. Heterozygote mutations in parkin may also lead to dopaminergic dysfunction and later onset of parkinsonism, consistent with a mechanism of haploinsufficiency (62,63). Pathologically, parkin-related PD is characterized by loss of substantia nigra pars compacta (SNpc) dopaminergic neurons, but it is not typically associated with Lewy bodies (61), in that Lewy bodies were definitely identified in only a single patient harboring a parkin mutation (64). Unexpectedly, attempts to recapitulate parkin loss of function in mice and flies have not succeeded in producing a conclusive PD phenotype (65–67). Nevertheless, those engineered animals are not completely normal, as they appear to exhibit mitochondrial defects (66–68), suggestive of those described in sporadic PD (27).

To date, it is uncertain how parkin mutations lead to dopaminergic neuron degeneration, but one clue has emerged from the identification of its normal function, which is E3 ubiquitin ligase (69,70), a component of the ubiquitin-proteasome system. The way E3 ligases work is by conferring target specificity through their capacity of binding to specific molecules or classes of molecules, thereby facilitating the polyubiquitination necessary for targeting to the proteasome. Since many parkin mutations abolish its E3 ligase activity, it is suggested that the accumulation of misfolded parkin substrates could be responsible for the demise of SNpc dopaminergic neurons in PD. Several investigations highlight the multiplicity of parkin substrates and how these might play a key role in neuronal death (27). However, none of the identified parkin substrates appears specifically enriched in dopaminergic neurons. Also noteworthy is the lack of Lewy bodies in most PD patients carrying parkin null mutations (71), suggesting that parkin E3 ligase activity might be needed for Lewy-body formation.

Ubiquitin C-Terminal Hydrolase-L1 Mutation/Polymorphism

This enzyme is ubiquitously expressed in the brain and catalyzes the hydrolysis of C-terminal ubiquityl esters; it is thought to participate in recycling ubiquitin ligated to misfolded proteins after their degradation by the proteasome. A single dominant mutation (I93M) in ubiquitin C-terminal hydrolase-L1 (UCH-L1) has been linked with the development of an inherited form of PD (72). Although this I93M mutation presumably decreases UCH-L1 hydrolase activity, most experts still doubt that a loss of UCH-L1 function could generate a PD phenotype mainly because mice carrying a UCH-L1 null mutation do display dramatic foci of neurodegeneration in the nervous system, but without any evidence of dopaminergic pathology (73). Contrasting with the controversial deleterious role of UCH-L1 I93M, several studies have raised the prospect that a polymorphism (S18Y) in UCH-L1 could reduce the risk of developing PD, especially among young individuals (74). However, at this point none of the findings relative to UCH-L1 as either an etiologic or a risk factor in PD have received widespread acceptance, and the current consensus is that much work has to be done to clarify the possible involvement of UCH-L1 in PD.

DJ-1 Mutations

DJ-1 is a homodimeric, multifunctional protein ubiquitously expressed in human tissues, including the brain. Thus far, at least 11 different DJ-1 mutations, including missense, truncating, splice-site mutations and large deletions, have been linked to an autosomal recessive form of PD (75–77). Although DJ-1 mutations account for a small fraction of familial PD, it is now recommended to systematically screen for DJ-1 mutations in all cases of recessively inherited early-onset forms of the disease (78). These mutations are found throughout the four of the seven exons of the DJ-1 gene. Although little is known about the function of DJ-1, it is predicted that many, if not all of the identified mutations, would cause either a lack of DJ-1

expression or the transcription of an unstable and functionally deficient product.

Cells lacking DJ-1 have been reported to be more susceptible to a variety of stresses, including oxidative attack. These findings prompted researchers to propose that DJ-1 protects cells through some kind of antioxidant effect. However, whether this is indeed the case is still a matter of intense investigation. Structural studies also indicate that DJ-1 shares similarities with the bacterial heat shock protein Hsp31 a stress-inducible chaperone (79). In keeping with this observation is the demonstration that wild-type DJ-1 does exhibit chaperone activity (80). However, this Hsp31-like activity is not detectable when DJ-1 is in reducing conditions (which mimics the physiological intracellular environment) but is detectable when in oxidative conditions (which mimics an oxidative stress situation) (80). Furthermore, the PD-associated DJ-1 mutation L166M abolishes this redox-dependent chaperone activity (80). Collectively, these data are consistent with the emerging idea that wild-type DJ-1 functions as a redox-dependent chaperone that in response to intracellular oxidative stress becomes activated to assist the cell in coping with the rising amount of oxidatively damaged, misfolded proteins.

PINK-1 Mutations

Autozygosity mapping of a large consanguineous Sicilian family located the PARK6 locus linked to an autosomal recessive form of PD to chromosome 1p35–36 (81). PARK6 was then linked to an early-onset recessive PD in 8 additional families from 4 different European countries (82). By sequencing candidate genes within the PARK6 region in affected members from each family, two homozygous mutations were found in the PTEN-induced putative kinase 1 (PINK1)/BRPK gene (83). Several additional PINK1 mutations linked to PD have been reported subsequently (84,85). Although most data regarding PINK1 mutations argue for the mutant protein to exert its deleterious effects by a loss of function, reduced striatal [18F]dopa uptake found by PET in asymptomatic PARK6 heterozygote subjects raises the possibility that PINK1 mutations may in fact operate by haploinsufficiency or by a dominant negative effect (86).

To date, little is known about how mutant PINK1 could kill cells. The rare available data dealing with this question indicate that neuroblastoma cells transiently transfected with either wild-type or mutant PINK1 do not exhibit any impairment in viability (83). However, if neuroblastomas are challenged with a proteasome inhibitor such as MG132, neuroblastomas engineered to express high amounts of wild-type PINK1 resist the cytotoxicity of this drug, whereas neuroblastomas engineered to express high amounts of mutant PINK1 die (83). These results suggest that the loss of PINK1 function may render dopaminergic neurons more vulnerable to injury. In adult mice, PINK1 is ubiquitously expressed among tissues with an apparently high expression in the brain (87). Both human and mouse PINK1 possess a serine/threonine kinase domain as the sole known functional domain (87,88). Presumably PINK1 also has a mitochondrial targeting motif consistent with the observation that it localizes to the mitochondria in transfected cell lines (83). Of the two initially identified mutations, one is a missense mutation (G309D) in the putative kinase domain, while the second is a nonsense mutation (W437OPA) truncating the last 145 amino acids of the C-terminus of the kinase domain (83). Both of these mutations are expected to impair PINK1 kinase activity or substrate recognition. However, with the subsequently identified PINK1 mutations (84,85), it can now be concluded that most but not all known PD-causing PINK1 mutations are predicted to alter its kinase activity, a fact that casts doubts on a defect in PINK-1-mediated phosphorylation truly being the deleterious mechanism by which this mutant protein provokes neurodegeneration.

LRRK2/Dardarin Mutations

PARK8 locus linked to families with autosomal-dominant, late-onset parkinsonism has been located to chromosome 12p11.2–q13.1 (89). The subsequent sequencing of 29 genes within the PARK8 region revealed 5 missense mutations and 1 putative splice-site mutation in a gene encoding a large, multifunctional protein: leucine-rich repeat kinase 2 (LRRK2) (90) or dardarin (91). LRRK2/dardarin missense mutations were also found in 5 PD families of different origin (91). In both studies, the identified mutations segregated with disease and affected individuals were heterozygous for the mutation. Whether the Japanese Sagamihara kindred, in which the PARK8 linkage was initially identified (89), carries one of the identified LRRK2/dardarin mutations remains to be demonstrated. It has been estimated that LRRK2/dardarin mutations could account for more than 5% of all familial cases of PD (92), and even more remarkable is the finding that out of 482 sporadic PD patients, 8 were heterozygote for the G2019S LRRK2/dardarin mutation (40). As stated by the authors of this fascinating finding, the absence of family history in 5 of the PD patients harboring the LRRK2/dardarin mutation suggests either reduced penetrance or a de novo occurrence. As with the other PD-causing mutations, the function of LRRK2/dardarin in both normal and pathological situations remains unknown. At this point, it is believed that LRRK2/dardarin belongs to a family of multifunctional Ras/GTPase proteins, named *Roc* (for Ras of complex proteins). Moreover, specific motifs in LRRK2/dardarin sequence also suggest that protein–protein interactions play an important role in its function, a view that led to the idea that LRRK2/dardarin may function as a component of a multiprotein complex.

Postmortem examinations of several PD-linked PARK8 cases revealed a remarkable neuropathological heterogeneity. In all 6 patients carrying an LRRK2/dardarin mutation

who came to autopsy, there was neuronal loss and gliosis in the substantia nigra. In some cases, the loss of SNpc dopaminergic neurons was not associated with Lewy bodies as in parkin-linked PD, whereas in others, the loss of SNpc dopaminergic neurons was associated with Lewy bodies. In the latter cases, Lewy bodies were restricted to the brainstem as in typical PD, or widespread in brainstem and cortex as in diffuse Lewy-body disease. In one case, there were also tau-immunoreactive lesions in both neurons and glial cells, as in progressive supranuclear palsy. Finally, some patients also showed evidence of anterior horn motor neuron loss with spheroids, reminiscent of amyotrophic lateral sclerosis.

GENE DEFECTS AND MECHANISM OF CELL DEATH

Although genetic forms of PD are rare, the strong impetus for so avidly studying these uncommon occurrences of PD is fueled by the expectation that the phenotypic similarity between familial and sporadic PD indicates that both instances share key neurodegenerative mechanisms. As discussed previously (59), among various plausible mechanistic hypotheses (Fig 7.3), available data favor impaired protein degradation and accumulation of misfolded proteins as the unifying factor linking genetic alterations to dopaminergic neurodegeneration in familial PD. According to this reasoning, alpha-synuclein and DJ-1 mutations would cause abnormal protein conformations, overwhelming the main cellular protein degradation systems, namely the proteasomal and lysosomal pathways, whereas parkin and UCH-L1 mutations would undermine the cell's ability to detect and degrade misfolded proteins. The common end result of these different perturbations is thus expected to be a cellular buildup of altered proteins that should have been cleared. This scenario fails, however, to explain why an accumulation of "misfolded proteins," which is likely to occur in all cells, would inflict greater damage to dopaminergic neurons in familial PD. Perhaps nigrostriatal dopaminergic neurons are less amenable to coping with ìmisfolded protein stressî because of a higher basal load of damaged proteins due to dopamine-mediated oxidative events. Also poorly addressed by the preceding scenario is the link between previously identified factors in PD neurodegeneration, such as mitochondrial dysfunction or oxidative stress, which will be discussed in the following section, and the molecular events engendered by the PD-causing mutations. The hypothesized location in mitochondria of DJ-1 and PINK1 and the role in oxidative stress played by DJ-1 may emerge as critical in our effort to reconcile the different aspects of the unified pathogenic cascade of PD. Too little is thus far known about LRRK2/dardarin to speculate how and where this new mutant protein will fit into this proposed scenario (Fig 7.3).

Pathogenesis of Parkinson Disease

If etiology refers to the factor that initiates the disease process in PD, pathogenesis refers to the actual mechanisms by which the demise of dopaminergic neurons occur. Although there is no clear boundary between etiology and pathogenesis, this somewhat artificial dichotomy has proved useful from a didactic point of view. To date, there are two major theories regarding the pathogenesis of PD. One hypothesizes that misfolding and aggregation of proteins is key to the neurodegenerative process, whereas the other asserts that mitochondrial dysfunction and related oxidative stress, including toxic oxidized dopamine species, are responsible. Also, inflammatory events are a feature of PD neuropathology, and mounting evidence supports its contribution to neurodegeneration of PD (93). Until recently, most insights into disease pathogenesis have come from human autopsy specimens. Postmortem studies, although useful, also suffer major limitations. For instance, it is usually the case that the cells of main interest—that is, dopaminergic neurons—are greatly depleted in typical autopsy specimens, which usually consist primarily of glial cells and nondopaminergic neurons. Thus, in the absence of experimental models, it is often difficult to reach any reliable mechanistic conclusions, and one is usually left with the intractable dilemma of whether a reported alteration reflects the cause or is simply a consequence of the neurodegenerative process. It should also be mentioned that the pathogenic factors noted previously are not mutually exclusive, and one of the key aims of current PD research is to identify all of the various factors implicated in the death of dopaminergic neurons in PD and to determine in which sequence they intervene in this deleterious cascade. Of great importance for deciphering possible therapies for PD is to determine whether these pathogenic factors eventually converge to engage a common downstream pathway such as apoptosis, or whether they remain divergent until neuronal death.

Misfolding and Aggregation of Proteins

The abnormal deposition of proteinaceous material in the brain in the form of large aggregates detectable by light microscopy is a typical feature of many age-related neurodegenerative diseases, including PD. Even with differences in composition and location of these inclusions, their presence in many diseases of the brain might indicate that these protein aggregations or related events might be toxic to neurons. As previously proposed (27), the observed intraneuronal aggregates, such as Lewy bodies in PD, may cause damage directly, possibly by cell deformation or interference in intracellular trafficking. It also may be that Lewy bodies sequester proteins necessary for cell survival, so depriving the neuron. If so, should there not be a direct correlation between inclusion formation and neurodegeneration? The answer to this question is still ambiguous

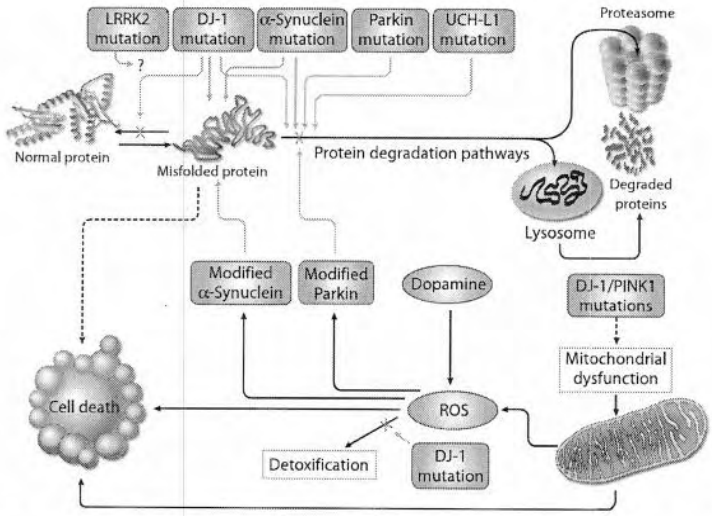

Figure 7.3 Genetic mutations and the pathogenesis of PD. Misfolded proteins may contribute to PD neurodegeneration. Mutant alpha-synuclein and DJ-1 may be misfolded (*blue arrows*), thus overwhelming the ubiquitin (proteasomal) and lysosomal degradation pathways. Other mutant proteins, such as parkin and UCH-L1, may lack their wild-type function. Both of these proteins, which belong to the ubiquitin-proteasome system, upon mutation may no longer exert their ubiquitin ligase activity, thus damaging the ability of the cellular machinery to detect and degrade misfolded proteins (*red arrows*). Mutations in DJ-1 may also alter its supposed chaperone activity, disrupting the refolding of damaged proteins or the targeting and delivery of damaged proteins for degradation (*red arrows*). These different alterations may lead to the accumulation of unwanted proteins, which, by unknown mechanisms (*dashed arrows*) may lead to neurodegeneration. Oxidative stress generated by mitochondrial dysfunction and dopamine metabolism may also promote protein misfolding as a result of post-translational modifications, especially of alpha-synuclein and parkin. Oxidative stress in PD may also originate from a defect in the reduced capacity of DJ-1 to detoxify reactive oxygen species, whereas the mitochondrial dysfunction may, at least in part, derive from defective activity and mislocation of DJ-1 and PINK1. Mitochondrial dysfunction, oxidative stress, and protein mishandling are thus tightly interconnected in this hypothesized pathogenic cascade. LRRK2 function is unknown. Additional possible interactions have been omitted for clarity. (Adapted from Vila M, Przedborski S. Genetic clues to the pathogenesis of Parkinson's disease. *Nat Med* 2004;10 Suppl:S58–62. With permission.)

as studies of Huntington's disease and other polyglutamine diseases (94,95) suggest that there is no straightforward link between the aggregate load and neurodegeneration, whereas studies of amyotrophic lateral sclerosis argue that there might be (96,97). Alternatively, cytoplasmic protein inclusions might not be passively derived from precipitated misfolded proteins but rather from an active process with intent to sequester misfolded proteins from the cellular environment. Accordingly, inclusion formation may be a protective process aimed at removing soluble misfolded proteins (94,98–100).

Aside from insoluble proteinaceous material, soluble misfolded proteins could also be toxic through a variety of mechanisms. For instance, soluble misfolded species seem capable of triggering apoptosis via a JNK-dependent mechanism (101). The identification of ubiquitin and chaperones in Lewy bodies (99,102) is further evidence of the presence of soluble misfolded proteins in PD, because chaperones bind specifically to the exposed hydrophobic surface of soluble misfolded proteins. The ability of chaperones such as Hsp70 to protect against neurodegeneration provoked by disease-related proteins also supports the view that soluble misfolded proteins are neurotoxic. Indeed, Auluck et al. (99) found that overexpression of Hsp70 prevented alpha-synuclein–mediated dopaminergic neuron loss in a *Drosophila* model of PD.

As described by Dauer and Przedborski (27), pathogenic mutations in patients with inherited PD are thought to cause disease directly by inducing abnormal and possibly toxic protein conformations or indirectly by interfering with the processes that normally recognize or process misfolded proteins. In sporadic PD, there is similar attention on both direct protein-damaging modifications and dysfunction of chaperones, proteasomes, or autophagosomes that may indirectly contribute to the accumulation of misfolded proteins. The triggers for dysfunctional protein metabolism in sporadic PD are now beginning to be revealed. One such initiator may be oxidative stress, long thought to play a key role in the pathogenesis of PD through damage caused by ROS. The tissue content of abnormally

oxidized proteins (which may misfold) increases with age, and neurons may be especially susceptible because they are postmitotic. In PD, Lewy bodies contain oxidatively modified alpha-synuclein, which in vitro exhibits a greater propensity toward aggregation than does unmodified alpha-synuclein (103). Environmental factors could also initiate protein aggregation in PD as several herbicides and pesticides induce misfolding or aggregation of alpha-synuclein (104–106).

There also appears to be an age-related decline in the ability of cells to handle misfolded proteins (107). Cells respond to misfolded proteins by inducing chaperones, but if not properly refolded they are targeted for proteasomal degradation by polyubiquitination. With aging, the ability of cells to induce a variety of chaperones is impaired. Similarly, there is loss of proteasomal function during aging, with one report of selective loss of the 20S subunit of the proteasome in the postmortem PD brain (108). Furthermore both in vitro (109–111) and in vivo studies (112) suggest that inhibition of proteasomal activity leads to accumulation of alpha-synuclein or the formation of ubiquitinated alpha-synuclein–containing aggregates. Conversely, alpha-synuclein interacts with and may be degraded by the proteasome (113,114) and autophagosome (115), and overexpression of alpha-synuclein leads to perturbations in the protein degradation systems (116). Proteasomal and autophagosomal dysfunctions and the accumulation of misfolded proteins may thus provoke a vicious cycle, with each insult reinforcing the other.

Mitochondrial Dysfunction and Oxidative Stress

The discovery that MPTP blocks the mitochondrial electron transport chain by inhibiting complex I allowed for the possibility that an oxidative phosphorylation defect could play a role in the pathogenesis of PD (117). Consistent with this view is that subsequent studies have identified abnormalities in complex I activity in PD, interestingly, not only in the brains of PD patients (118) but also in platelets (119). Low complex I activity in cybrid cells, which have been generated by the fusion of cells deficient in mitochondrial DNA with patient-derived platelets rich with mitochondrial DNA (120), led the conclusion either that the observed complex I deficit was inherited from the mitochondrial genome or that some systemic toxicity caused mutations in mitochondrial DNA. However, as mentioned previously, no mitochondrial DNA mutation has been reproducibly identified in PD patients.

Almost all molecular oxygen is consumed by mitochondrial respiration, and such oxidants as superoxide radicals and hydrogen peroxide are continuously produced as by-products. Inhibition of complex I increases the production of the ROS, which may form secondary oxidants, including hydroxyl radicals and peroxynitrite. All these oxidants may cause cellular damage by reacting with nucleic acids, proteins, and lipids, and one target of these reactive species may be the electron transport chain itself (121), leading to mitochondrial damage and further production of ROS. Supporting the occurrence of oxidative stress in PD are the demonstrations that several biological markers of oxidative damage are elevated in the SNpc of PD brains (122). Also, the content of the antioxidant glutathione is reduced in the SNpc of PD brains (123), consistent with increased ROS, although this could also indicate a primary reduction of protective mechanisms against ROS.

The presence of ROS would increase the quantity of misfolded proteins, increasing the demand on the ubiquitinproteasome system to remove them. Dopaminergic neurons may be a particularly fertile environment for the generation of ROS, as the normal metabolism of dopamine by monoamine oxidase (MAO) produces hydrogen peroxide and superoxide radicals, and autooxidation of dopamine produces dopamine quinone (124), a molecule that damages proteins by reacting with cysteine residues. Mitochondria-related energy failure may disrupt vesicular storage of dopamine, causing the free cytosolic concentration of dopamine to rise and allowing harmful dopamine-mediated reactions to damage cellular components. Dopaminergic neurons contain neuromelanin, which can bind ferric iron and reduce it to its reactive ferrous form (125). Midbrain dopaminergic groups differ markedly in the percentage of neuromelanin-pigmented neurons they contain, and PD-related neuron loss is more profound in cell groups that normally contain a greater percentage of neuromelanin-pigmented neurons (6). Further, there is a relative sparing of nonpigmented neurons in PD (6). Postnatal dopaminergic neurons exposed to low doses of levodopa accumulate a black pigment similar to neuromelanin (126), suggesting that the formation of neuromelanin is related to the presence of dopamine. Together, these results suggest that dopamine may be pivotal in rendering SNpc dopaminergic neurons particularly susceptible to oxidative attack.

Despite the large body of literature documenting mitochondrial dysfunction and indices of oxidative damage in autopsy tissue from PD patients, all these reported observations are correlative in nature. There are no data that convincingly link a *primary* abnormality of oxidative phosphorylation or ROS generation with PD; many of these abnormalities could be concomitant to dying cells. Indeed, of the many diseases known to result from mutations directly affecting the oxidative phosphorylation, i.e., mitochondrial cytopathies, parkinsonism is a rare feature and, when encountered, the parkinsonism is almost always accompanied by other symptoms that are not observed in PD.

Inflammation

The loss of dopaminergic neurons in postmortem PD brains is associated with marked microglial and, to a lesser extent, astrocyte activation (127–130). Activated microglial cells are found predominantly in proximity to free

neuromelanin in the neuropil, and they sometimes agglomerate onto remaining neurons, producing an image of neuronophagia (127). In PD, inflammation seems to be secondary to neuronal pathology and not a primary pathogenic event. It is thus hypothesized that signals arising from injured neurons stimulate the inflammatory response via subtle alterations in the CNS microenvironment (e.g., ionic imbalances) or rather gross spillage of intraneuronal contents. Also relevant to the initiation of neuroinflammatory response in PD is the demonstration that neurons treated with proteasome inhibitors accumulate ubiquitinated proteins and increase the production of prostaglandins, which can activate glial cells (131).

It is established that both microglia and astrocytes, once activated, acquire phagocytic properties and become capable of producing an array of inflammatory mediators and other deleterious molecules, including ROS and nitric oxide. In keeping with this, in the SNpc of PD patients (but not controls), numerous astrocytes are immunoreactive for inducible nitric oxide synthase (iNOS) (132) while microglia are positive for nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (133). Enhanced immunostaining for proinflammatory cytokines—such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and, for the macrophage cell surface, antigen FcεR11/CD23–is also observed in the SNpc from PD patients, in both astrocytes and microglial cells (134). These cytokines may act in PD on at least two levels. First, while cytokines are produced by glial cells, they can stimulate other astrocytes and microglia not yet activated, thereby amplifying the inflammatory response and consequently the glial-mediated assault on neighboring neurons. In vitro studies by Hunot et al. (134) found that IFN-γ together with TNF-α and IL-1β not only activates glial cells, but it also stimulates the glial expression of FcεR11/CD23, which leads to the induction of xxxx xxxx xxxx iNOS and the release of TNF-α. Second, glial-derived cytokines may also act directly on dopaminergic neurons by binding to specific cytokine receptors on the surface of these cells, such as those for TNF (135). Upon activation, these receptors trigger intracellular signaling pathways that may ultimately set off the programmed cell death (PCD) machinery, discussed in the following section. It is thus proposed that neuroinflammation does not initiate PD neurodegeneration but can promote its progression, whereby stimulating the worsening of PD symptoms.

Mode of Cell Death

Regardless of the mechanisms involved in the neurodegenerative process of PD, it may be asked whether a common downstream pathway mediates all PD-related neuronal loss, or whether there is significant heterogeneity in the pathways activated in different compromised neurons in a single patient, or among different patients with PD. The answers to these questions bear upon the development of therapeutic strategies for PD, and bear upon whether PD is a disease or rather a syndrome (i.e., multiple distinct pathological entities with similar phenotype). Over the past decade, PCD has emerged as an inescapable component in the mechanism of cell death in a wide variety of pathological situations, including diseases of the aging brain (136). Although physiological PCD is known to be crucial during normal development and as a homeostatic mechanism in some systems, inappropriate recruitment of this molecular machinery in the mature brain has been proposed as a main contributor to neurodegeneration. Until recently, investigators have explored the possibility that PCD occurs in PD autopsy specimens by searching for neurons that display features of apoptosis, a morphological correlate of PCD. As reviewed by Jellinger, these morphological studies have yielded conflicting results (137). Complicating matters, if apoptosis does occur in PD, it may be difficult to detect those apoptotic cells by morphological means because the rate of neuronal loss in PD may be low (138) and apoptotic cells reportedly disappear in a matter of hours (139). In addition, there may be nonapoptotic forms of PCD (140,141). Accordingly, most recent studies in PD have ascertained the content and status of molecular components of PCD instead of relying on morphological approaches. For example, studies of the PCD Bax molecule demonstrate an increased number of Bax-positive SNpc dopaminergic neurons in PD (142), and, compared to controls, there is an increased neuronal expression of the pro-PCD Bax protein in PD, suggesting that these cells are undergoing early PCD (143). SNpc dopaminergic neurons with increased expression and subcellular redistribution of the anti-PCD protein Bcl-xL and with activated PCD effector protease caspase-3 have also been found in greater proportion in PD (144,145). Remarkably, the Bcl-2 family member BAG5 has emerged as a potential promoter of neurodegeneration in PD by inhibiting both parkin E3 ligase activity and Hsp70-mediated refolding of misfolded proteins (146). Other molecular markers of PCD are altered in PD, including the activation of caspase-8 (147), caspase-9 (148), and the translocation of cytochrome c, an electron carrier and mediator of PCD (M. Vila, personal communication, xxx xxx). Studies of PCD in PD remain descriptive in nature, and it is uncertain whether the changes are a primary abnormality of PCD regulation or simply an appropriate "suicide" decision by cells seriously injured by any of the toxic processes reviewed in this section.

SUMMARY

Advances in epidemiologic, genetic, and basic science research are generating plausible hypotheses regarding the cause of PD. These advances, rather than narrowing the focus to a single genetic or environmental cause, have led to an understanding of PD as a complex disorder with

multiple etiologies and with a pathogenic cascade made of numerous deleterious factors. Thanks to rare genetic mutations causing familial forms of PD, a better understanding of mechanisms potentially important for the common sporadic form of PD has now been acquired. Alpha-synuclein is an important constituent in Lewy bodies, and environmental and genetic influences may lead to abnormal aggregation of this protein that, in turn, could cause neuronal dysfunction and death. The role of parkin and UCH-L1 in the ubiquitin-proteasomal system suggests that impaired regulation of protein degradation may play a role in PD, and promising insights into dopaminergic neurodegeneration are to be expected from studies on DJ-1, PINK1, and LRRK/dardarin. Exposure to environmental toxins may interrupt energy production in the mitochondria or cause increased levels of oxidative stress that, in turn, might lead directly to cell injury or death. Here, inflammation and PCD appear respectively as key modulators of the overall extent of the neurodegenerative process and as plausible downstream effectors of neuronal demise. An individual's ability to respond to environmental insults may be determined by genetic polymorphisms that code for metabolic enzymes with reduced or increased ability to metabolize exogenous or endogenous toxins.

Although the numerous potential causes of dopaminergic neuronal degeneration and clinical PD are complex and daunting, they also provide the opportunity for intervention at multiple steps. The development of successful future therapies and preventive measures depends on a sound understanding of the interaction of environmental, molecular, and genetic factors involved in PD.

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Chapter 26

Neuroinflammation and Parkinson's disease

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p0010 Parkinson's disease (PD) is the second most frequent neurodegenerative disorder of the aging brain after Alzheimer's dementia. Its clinical hallmarks include resting tremor, slowness of movement, rigidity and postural instability (Fahn and Przedborski, 2000), all of which have been attributed to a profound deficit in brain dopamine (Hornykiewicz and Kish, 1987). Given this fact, it is not surprising to notice that the lion's share of attention with respect to PD neuropathology has been paid to the dopaminergic systems of the central nervous system (CNS) and more particularly to the nigrostriatal pathway. Yet, it is now well established that degenerative changes in PD are not restricted to the nigrostriatal pathway or to other dopaminergic systems. Abnormal histological features can also be found in many non-dopaminergic cell groups, including the locus ceruleus, raphe nuclei and nucleus basalis of Meynert (Braak et al., 1995). Affected brain areas in PD are not only depleted of specific types of neurons, but also exhibit intraneuronal proteinaceous inclusions named Lewy bodies (Galvin et al., 1999) and inflammatory changes such as morphological and functional alterations in glial cells (McGeer et al., 1988b; Forno et al., 1992).

p0020 For decades, gliosis has been a well-recognized neuropathological feature of PD, thought of as secondary and insignificant as far as the pathogenesis of this illness is concerned. More recently, however, human epidemiological studies have suggested that inflammation increases the risk of developing PD (Chen et al., 2003) and investigations in experimental models of PD have shown that inflammatory response can modulate nigrostriatal dopaminergic neuronal death (Liberatore et al., 1999; Gao et al., 2002; Wu et al., 2002, 2003). These facts prompt many investigators to regard inflammation as a noxious factor in the neurodegenerative process in PD and related conditions. Nguyen and collaborators (2002), however, remind us that the inflammatory response is not always injurious and that it can provide beneficial effects in an otherwise compromised system, such as by stimulating the production of neurotrophic factors and some repair and remyelination mechanisms.

p0030 Based on the aforementioned premises, this chapter will review the topic of inflammation in PD. To achieve this goal, the notion of inflammation in neurodegenerative disorders will first be discussed to define the key players and to set the stage for the rest of the discussion. Then, the issue of how inflammation is triggered in PD and which place it occupies in the sequence of events that ultimately leads to the demise of dopaminergic neurons will be approached. A description of the composition of the inflammatory response in various parkinsonian syndromes including PD per se as well as in animal models of PD will follow. Minimal changes have occurred vis-à-vis the anatomical description of inflammation in all these different PD-related settings and thus they primarily represent a reiteration of previous publications I have written on this subject. And, finally, a discussion about the potential beneficial and deleterious role of inflammation in PD and how it can be targeted for therapeutic purposes will be offered.

26.1. Inflammation in neurodegenerative disorders

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p0040 Inflammation can involve any part of the body, including the brain. The inflammatory reaction of the CNS is a complex phenomenon which should not just be equated with the infiltration of the diseased brain

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parenchyma by blood-derived immune cells, since such infiltration only represents one particular type of inflammatory response, often called exudative inflammation. Although exudative inflammation can be observed in a host of acute insults of the CNS accompanied by a rupture of the blood–brain barrier, it is not the one expected to be found in chronic neurodegenerative diseases. Indeed, in these types of chronic brain disorders, rather than seeing infiltration of circulating macrophages and other bloodborne cells, the diseased brain tissue is populated with resident inflammatory cells such as microglia and astrocytes. In many neurodegenerative diseases, those innate immune cells are activated and produce a variety of inflammatory mediators (Eddleston and Mucke, 1993; Mennicken et al., 1999; Nguyen et al., 2002). In addition to the glial response, T-lymphocyte alterations have also been identified in several neurodegenerative diseases, including PD (Bas et al., 2001; Hisanaga et al., 2001), suggesting an involvement of the adaptive immune system in the inflammatory process seen in these illnesses.

p0050 In the context of this chapter, it is also important to discuss briefly the widespread misconception that necrosis, but not other forms of cell death such as apoptosis, elicits inflammation. This view likely finds its basis in the principle that, contrary to necrosis, non-necrotic forms of cell death (Clarke, 1990; Yaginuma et al., 1996) are not associated with a gross spillage of the intracellular content from degenerating cells, which is perceived by many as a determining factor in triggering inflammation. Although the inflammatory reaction is generally stronger in brain areas of necrosis than, for example, in areas of apoptosis, this may simply reflect the greater number of cells dying in necrotic areas. Moreover, authors who claim that non-necrotic forms of cell death such as apoptosis do not elicit inflammation are referring to exudative inflammation, as defined above (Wyllie et al., 1980). However, in the CNS, even in experimental situations associated with necrosis, the inflammatory response is largely local, i.e. mainly made of resident microglia and astrocytes, as seen in experimental situations associated with apoptosis. Therefore, whereas the intensity of the glial reaction may vary among the distinct forms of cell death detailed by Peter Clarke (1999), the occurrence of gliosis should not be regarded as a characteristic of necrosis only. The occurrence of inflammation in neurodegenerative diseases such as PD cannot and should not imply that dopaminergic neurons are dying by necrosis.

p0060 As previously stressed (Przedborski and Goldman, 2004), prior to embarking on the discussion of inflammation in PD, the meaning of *gliosis*, which is sometime also called *reactive astrocytosis*, must be addressed. Gliosis, in neurological diseases, normally refers to scarring produced by astrocytes; however, it is often loosely used to define simply increased immunoreactivity for the intermediate filament, glial fibrillary acid protein (GFAP). The extensive use of this term, especially in landmark studies, makes the interpretation of glial pathology difficult, as the limited range of techniques employed does not always allow an appropriate interpretation of the data. For instance, it is often unclear whether gliosis, evidenced by increased GFAP immunostaining, meant increased stainability of the tissue, increased numbers of astrocytes, increased size of astrocytes or a combination of all of these. It is also not always possible to comment on the status of other glial cells such as oligodendrocytes and microglia or T cells. Consequently, all efforts will be made to spell out what those studies actually show. Nonetheless, a more accurate and comprehensive analysis of the inflammatory reaction in PD may have to wait for this topic to be revisited using more modern techniques.

p0070 Even though the description of glial cells in normal and PD brain has already been discussed elsewhere (Przedborski and Goldman, 2004), it is essential that this aspect be revisited here. It must be remembered that astrocytes play an important role in the normal, undamaged adult brain for the homeostatic control of the neuronal extracellular environment. Contrasting with the large body of information about astrocytes, only scattered information is available regarding the role of microglia and T cells in the immature brain and even less in the normal adult brain. Following an injury to the brain, both astrocytes and microglial cells can undergo dramatic phenotypic changes that enable them to respond to and play a role in the pathological processes (Eddleston and Mucke, 1993; Gehrmann et al., 1995). For example, microglial activation is characterized by a series of alterations including proliferation; increased or de novo expression of marker molecules such as major histocompatibility complex antigens; migration; and eventually transformation into a macrophage-like appearance (Banati et al., 1993).

p0080 As indicated by Przedborski and Goldman (2004), in neurodegenerative diseases both microglia and astrocytes can become activated, producing an array of inflammatory factors and taking on phagocytic functions. Although some glial factors are specifically produced by reactive astrocytes or activated microglia, others, such as interleukin-1ß (IL-1ß), can apparently be produced by both (Rothwell, 1999). Also, it is believed that microglia are responsible for more generalized phagocytosis involving activation of the complement cascade, whereas astrocytes are implicated in

circumscribed phagocytic processes, such as the removal of individual synapses (Wyss-Coray and Mucke, 2002). The most efficient and aggressive phagocytes in the CNS are likely the round or ameboid microglia, which express high levels of macrophage markers, whereas ramified microglia have little phagocytic activity (Kreutzberg, 1996). In neurodegenerative diseases many microglia cells show a ramified morphology, although they express activation markers (Dickson et al., 1993), suggesting that they might be non-phagocytic (DeWitt et al., 1998). Astrocytes may also participate in phagocytosis, either directly (Shaffer et al., 1995) or by regulating microglial activities (DeWitt et al., 1998).

p0090 As indicted above, there is often T-cell infiltration in diseased brain areas in neurodegenerative diseases. Yet, the prevailing theory about the pathogenesis of neurodegenerative diseases, except for multiple sclerosis and related conditions, is that the observed inflammation results from a glial reaction and T-cell infiltration secondary to the loss of neurons; hence neurodegenerative diseases such as PD are not to be considered as autoimmune diseases in which a peripheral immune reaction directed against the CNS is the primary event. However, the identification of T cells in the diseased brain parenchyma raises intriguing questions, such as: How do T cells enter the brain? What subtypes are they? How are they recruited within diseased areas? What could be their role in the disease process? The CNS is an immune-privileged site due to the complexities of the blood–brain barrier and its lack of lymphatic drainage (Becher et al., 2000). The latter results in an ineffective dialog between the CNS and the immune system and is one mechanism preventing the exchange of CNS antigens with the peripheral immune system (Becher et al., 2000). It is proposed that such complications in this dialog result in the failure of the immune system to protect and to promote regeneration within the damaged CNS, in contrast to its role in such maintenance within the peripheral nervous system (Moalem et al., 1999). Despite these obstacles, compelling evidence indicates that activated, but not naive T cells, can enter the CNS regardless of whether the blood–brain barrier is inflamed (Hickey et al., 1991). It seems that activated T cells circulate peripherally until they are arrested by adhesive interactions with the endothelium (Hauzenberger et al., 1995). Adhesive molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which are instrumental in the extravasation of T cells, are minimally expressed in the normal brain but can be markedly upregulated in the diseased brain (McCluskey and Lampson, 2000). Once in the CNS, activated T cells must recognize their antigen in order to perform their effector functions (e.g. release of cytokines) and participate in the recruitment of additional T cells through the secretion of chemokines (McCluskey and Lampson, 2000). The basic requirement for antigen recognition by T cell is that the antigen be processed into a peptide, be complexed to major histocompatibility complex-encoded proteins and be translocated to the cell surface (McCluskey and Lampson, 2000). Whether infiltrating T cells correspond to specific antigen-activated T cells that can exert effector functions contributing to the disease process in neurodegenerative disorders, or merely to non-specific antigen-activated T cells which patrol the CNS, remains to be clarified.

26.2. How inflammation arises in Parkinson's disease and related conditions s0020

Most data available to date are consistent with the p0100
view that inflammation in PD results from the detection by defense mechanisms of ongoing neuronal perturbations. This implies that, as already alluded to above, microglial and astrocytic activation and T-cell infiltration within injured areas are not primary events but rather the consequence of the neuronal pathology. If inflammation in PD is a secondary event, i.e. rises after the demise of dopaminergic neurons has already started, does this fact preclude a role for inflammation in the neurodegenerative process? Prior to answering this question it is important to remember that neuronal death in diseases such as PD proceeds in an asynchronous fashion – not all dopaminergic neurons within the substantia nigra pars compacta die simultaneously. This implies that at any given time, only a small number of dopaminergic neurons are actually dying and among these many, if not all, are at various stages along the cell death process. Consequently, the very first neurons that succumb to the disease process are responsible for initiating inflammatory events. From then on, all compromised, but still living, neighboring neurons will now also be subjected to the effects of inflammation, which will intensify as more neurons die and the glial response grows. Assuming that inflammation exerts deleterious effects on dopaminergic neurons (see below), it can thus be easily understood how inflammation may amplify the neurodegenerative process and stimulate its progression.

Even if this scenario is correct, the nature of the p0110
signal that triggers inflammation and which emanates from the dysfunctional or dying neurons remains enigmatic. Because glial cells entertain intimate contacts with neurons, it is tempting to imagine that the initiation of the inflammatory response could derive from

a defect in the nature or quality of the neuronal contact with glia cells. Indeed, early on in the process of apoptosis, cells including neurons harbor plasma membrane alterations, which lead to the cell surface exposure of phosphatidylserine, as evidenced by the binding of annexin V (van den Eijnde et al., 1998). It can thus be hypothesized that such alterations of the neuronal membrane could be readily perceived by glial cells as a pathological signal, leading to their activation. Although not mutually exclusive, several cell culture studies show that inflammation can be triggered by soluble factors, either secreted by or leaking from 'sick' neurons. One such molecule is chromogranin A, a glycoprotein widely distributed in the CNS, which accumulates in areas with neuronal degeneration and in Lewy bodies (Nishimura et al., 1994). Upon its release or leakage from neurons, chromogranin A can activate microglia and promote neurodegeneration by a microglial-dependent mechanism (Ciesielski-Treska et al., 1998). In addition, stressed neurons in both PD and experimental models of PD expressed high amounts of the prostaglandin-synthesizing enzyme, cyclooxygenase-2 (Cox-2). Once produced by the neurons, prostaglandins can presumably reach the extracellular space and activated glial cells. Finally, the idea that misfolded neuronal proteins and protein aggregates could also trigger glial activation is particularly appealing in the context of PD, given the fact that mutations in the genes encoding for parkin and ubiquitin C-terminal hydrolase L1 (two enzymes of the ubiquitin/proteasome pathway) and for α-synuclein (a main component of the intraneuronal proteinaceous inclusions, Lewy bodies) lead to familial PD (Vila and Przedborski, 2004). There is no shortage of ideas about how glial cells may be activated in response to neuronal perturbations, but we still do not know with certainty how this occurs. Most data support the hypothesis that inflammation in neurodegenerative diseases such as PD is likely not a non-specific response to dysfunctional and dying neurons. Instead, it is more probable that the inflammatory response is related to the ligation of specific transmembrane receptors, such as toll-like receptors (TLRs), which are present on glial cells (Bowman, 2003; Zuani, 2003). A critical aspect of the TLR machinery is the fact that, among the 10 different TLRs, each is activated by a specific ligand (Iwasaki and Medzhitov, 2004). Although activation of TLRs has been studied mainly in the context of pathogens, one may hypothesize that the development of inflammation in PD could also arise from the activation of the TLRs by specific structural molecules originating from dysfunctional and dying neurons such as, for example, truncated or oligomeric species of α-synuclein.

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26.3. Description of the inflammatory response in Parkinson's disease s0030

In the normal adult brain, microglia, which constitute roughly 10% of all glial cells, appear not to be evenly distributed (Lawson et al., 1990) and for the most part they harbor a morphology of resting state: elongated, almost bipolar cell bodies with spine-like processes that often branch perpendicularly. With respect to the main areas of the brain affected in PD, the density of these cells seems much higher in the substantia nigra compared to any other brain regions (Kim et al., 2000). This observation, together with the demonstration that substantia nigra neurons are more susceptible to activated microglial-mediated injury (Kim et al., 2000), supports the idea that inflammation plays a meaningful role in the PD neurodegenerative process. In contrast with microglia, astrocytes in the normal adult brain display a rather homogeneous distribution except in midbrain, where the estimated density of GFAP-positive cells varies among the different catecholaminergic groups (Damier et al., 1993). For instance, the density of GFAP-positive cells is moderate in the midbrain areas known to be most severely affected in PD, such as substantia nigra pars compactam, and high in those least affected, such as the gray substance (Damier et al., 1993). Furthermore, as pointed out by Hirsch and collaborators (1999), within the substantia nigra pars compacta the density of GFAP-positive cells is lowest in the calbindin-D_{28K}-poor areas, where the loss of dopaminergic neurons is presumably the most severe (Damier et al., 1999). p0120

Although the neuropathology of PD goes well beyond the degeneration of dopaminergic systems (Braak et al., 1995), most attention has been paid to the nigrostriatal dopaminergic neurons, whose cell bodies are located in the substantia nigra pars compacta and their projecting nerve terminals in the striatum. It is thus not surprising that most data available about inflammation in PD pertain to the substantia nigra and the striatum. In keeping with this, several studies have reported that the loss of dopaminergic neurons in postmortem PD brains is associated with microglial and astrocytic alterations (McGeer et al., 1988b; Forno et al., 1992; Banati et al., 1998; Mirza et al., 2000). It also appears that the described changes in microglia and astrocytes are consistently more important in the substantia nigra pars compacta than in the striatum (McGeer et al., 1988b). This contrasts with the fact that the loss of dopaminergic elements is consistently more severe in the striatum than in the substantia nigra pars compacta. Although the explanation for this divergence is unknown, it may be due to the fact that the dopaminergic structures, which are p0130

degenerating, represent a larger fraction of the total pool of cellular elements in the substantia nigra pars compacta, but only a small one in the striatum; dopaminergic synapses represent less than 15% of the entire pool of synapses in the striatum (Pickel et al., 1981). It is thus possible that the pathological signal emanating from dysfunctional and degenerating dopaminergic elements is dampened to a greater extent in the striatum by the larger number of intact structures that surround them.

p0140 In addition to these topographical differences, the magnitudes of the microglial and astrocytic changes in PD brains also appear quite different. The substantia nigra pars compacta of postmortem PD brains exhibits, at best, slightly more astrocytes based on counts of GFAP- and metallothionein I/II-positive cells (Mirza et al., 2000). These data, however, do not indicate whether the finding results from glial hyperplasia (i.e. proliferation of astrocytes) or simply more stained astrocytes. Indeed, without having to invoke any change in number of astrocytes, it is well recognized that reactive astrocytes, by upregulating proteins such as GFAP, become readily visible by immunostaining. Remarkably, the majority of immunostained astrocytes in PD exhibit rather a resting-like morphology, with thin and elongated processes, and only few exhibit a true reactive morphology with hypertrophic cell body and short processes (Forno et al., 1992; Mirza et al., 2000). Thus, whether gliosis in PD results from astrocytic proliferation, increased stainability, or both, remains to be ascertained. Among other astrocytic pathologic features seen in PD is the intriguing finding of argyrophilic, tau-negative, α-synuclein-positive glial inclusions (Wakabayashi et al., 2000). These were only found in the brainstem: 40% of the PD samples studied showed these glial inclusions in the substantia nigra and 80% in other brainstem regions (Wakabayashi et al., 2000). Of note, in these tissue samples oligodendrocytes also contained these proteinaceous inclusions (Wakabayashi et al., 2000). More importantly, the number of such inclusions in astrocytes presumably did correlate with the severity of nigral neuronal loss (Wakabayashi et al., 2000).

p0150 Unlike astrocyte alterations, the microglial changes in PD are consistently more severe (McGeer et al., 1988b; Banati et al., 1998; Mirza et al., 2000; Imamura et al., 2003), but also less frequently addressed. Despite this caveat, the handful of studies cited above provides a rather similar picture about the status of microglial cells in PD tissues, at both the level of the midbrain and the striatum. Microglial cells can be readily evidenced in PD samples by immunostaining for various microglial markers such as, usually, human leukocyte antigen-DR (HLA-DR) and major histocompatibility complex class II (MHC-II) antigens and, less often, intercellular adhesion molecule-1 and leukocyte functional antigen-1 (McGeer et al., 1988b; Banati et al., 1998; Mirza et al., 2000; Imamura et al., 2003). Many of these markers, however, are not specific for activated microglia; hence positive cellular immunoreactivity due to the labeling of resting microglia is to be expected in normal control tissues. Thus, the assessment of the microglial response in pathological samples such as PD generally sits on the comparison of the morphology and the number of labeled cells between PD and controls. So, morphologically, microglial cells in PD tissues, unlike in control tissues, typically exhibit thick, elongated processes (McGeer et al., 1988b; Banati et al., 1998; Mirza et al., 2000); less often microglial cells in PD tissues also exhibit a round cell body devoid of processes, a morphology reminiscent of ameboid macrophages. Quantitative analysis shows that the number of activated microglia in the substantia nigra pars compacta, as evidenced by HLA-DR or ferritin immunostaining, is much higher in PD than in controls (Mirza et al., 2000; Imamura et al., 2003). Activated microglia in PD are predominantly found in close proximity to free neuromelanin in the neuropil and to remaining neurons in the substantia nigra, which they often surround or cover, realizing an image of neuronophagia (McGeer et al., 1988b). Similar microglial activation is also found in the putamen (Imamura et al., 2003).

26.4. Description of the neuroinflammatory response in Parkinsonian syndromes s0040

Although PD is the commonest cause of parkinsonism, more than 30 different neurological syndromes share PD clinical features (Dauer and Przedborski, 2003) and among these, PD does not have a monopoly on the association of nigrostriatal neurodegeneration and glial alterations (Oppenheimer and Esiri, 1997). Many of these non-PD parkinsonian syndromes exhibit both clinical features (e.g. ocular movement or upper motor neuron abnormalities) and loci of neurodegeneration in brain regions not typically seen in PD (striatum or corticospinal track pathology). Despite this, all of these syndromes show nigrostriatal dopaminergic neuronal loss, whose magnitude can vary greatly, and sometimes brainstem Lewy bodies. In addition, in most publications authors mention the presence of gliosis, usually to denote increased small non-neuronal cells, visualized by hematoxylin-and-eosin stain, or more GFAP-positive cells. When present these changes are found at the level of the nigrostriatal pathway, as well as at the level of the other affected regions of the brain not normally affected in PD. Even in the initial reports p0160

on progressive supranuclear palsy (Steel et al., 1964) and striatonigral degeneration (Adams and Salam-Adams, 1986), gliosis, as just defined, was already recognized as a prominent feature of the pathological changes seen in these syndromes. Over the last decade, several familial forms of parkinsonism have been identified (Vila and Przedborski, 2004). Like in sporadic PD, histological examination revealed similar glial alterations in most of the familial forms of parkinsonian syndromes, whether they are linked to unknown (Dwork et al., 1993) or known gene defects (Vila and Przedborski, 2004). In this vein, the situation reported for the autosomal-dominant form of PD linked to leucine-rich repeat kinase 2 (LRRK2)/dardarin mutations is particularly fascinating (Zimprich et al., 2004). Indeed, in the 6 patients carrying a LRRK2/dardarin mutation who came to autopsy there was neuronal loss and gliosis in the substantia nigra in all (Zimprich et al., 2004). Yet, although all 6 had parkinsonism, some cases only had dopaminergic neuronal loss and gliosis without Lewy bodies, whereas others had dopaminergic neuronal loss and gliosis associated with Lewy bodies (Zimprich et al., 2004). In the latter cases, Lewy bodies were restricted to the brainstem, or widespread in brainstem and cortex. In one case, there were also tau-immunoreactive lesions not only in neurons, but also in glial cells (Zimprich et al., 2004). These data support the view that inflammation is a generic phenomenon that arises from neuronal death irrespective of the type of parkinsonian syndrome or neuropathological picture. It also suggests that the presence of Lewy bodies is not a prerequisite for the occurrence of inflammation in PD and related conditions. This view is also consistent with *parkin* mutation-linked parkinsonism, which is a recessive form of PD with a loss of dopaminergic neurons, typically not associated with Lewy bodies, but with gliosis (Hayashi et al., 2000). To date, however, although the occurrence of gliosis in all of these conditions is clearly indicated, unlike in PD, no comprehensive qualitative or quantitative analysis of these alterations has been published and simple information such as whether the reported gliosis refers to astrocytes, microglia, or to both is in most instances lacking.

s0050 ## 26.5. Description of the neuroinflammatory response in experimental models of Parkinson's disease

p0170 Experimental models of PD are multiple and can be genetic or toxic (Dauer and Przedborski, 2003). The neuropathological picture found in these models is often very similar to that found in PD itself. In almost all of the PD models, some indications can be found about the fact that the demise of nigrostriatal dopaminergic neurons is associated with a glial response. However the amount of data regarding inflammation in these different models is quite disparate. For instance, if some data on inflammation are available for the herbicide paraquat (McCormack et al., 2002) and mitochondrial poison rotenone (Sherer et al., 2003) and even for transgenic mice expressing mutant α-synuclein (Gomez-Isla et al., 2003), it is incomparable to the wealth of information readily available for the 6-hydroxydopamine (6-OHDA) and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxic models of PD. Of note, the type and magnitude of glial alterations in rodents following the administration of 6-OHDA (Stromberg et al., 1986; Akiyama and McGeer, 1989; Sheng et al., 1993; Przedborski et al., 1995; He et al., 1999; Nomura et al., 2000; Rodrigues et al., 2001) appear very similar to those seen following the administration of MPTP (see below). Thus, to avoid unnecessary repetitions, the description of the glial response in experimental models of PD will be limited to those reported in the MPTP model. The beauty of the latter resides in the fact that data on the glial response are available from MPTP-intoxicated humans, to monkeys, to rodents.

p0180 In the few MPTP-intoxicated individuals who came to autopsy, postmortem examination revealed a paucity of nigrostriatal dopaminergic neurons accompanied by the presence of numerous small cells intensely immunoreactive for either the astrocytic marker GFAP or for the microglial marker HLA-DR (Langston et al., 1999). Almost all of the stained cells did show morphological characteristics of reactive astrocytes and activated microglia (Langston et al., 1999). Images of neuronophagia were also often seen in these nigrostriatal specimens (Langston et al., 1999). Although no formal quantification has been performed, it appears that the greater the abundance of GFAP- and HLA-DR-positive cells, the more profound the loss of dopaminergic neurons (Langston et al., 1999). Based on these neuropathological data (Langston et al., 1999), it also appears that more astrocytes adopted a reactive morphology in the postmortem samples from MPTP-intoxicated individuals than from PD. A comparable observation was made in 6 monkeys who survived 5–14 years after exposure to MPTP (McGeer et al., 2003) in that evidence of extracellular neuromelanin and activated microglia in the substantia nigra was documented (Table 26.1). From a neuropathological standpoint, microglial activation and neuronophagia are indicative of an active, ongoing inflammatory process. Although this assertion is consistent with the fact that PD is a progressive condition,

t0010 *Table 26.1*

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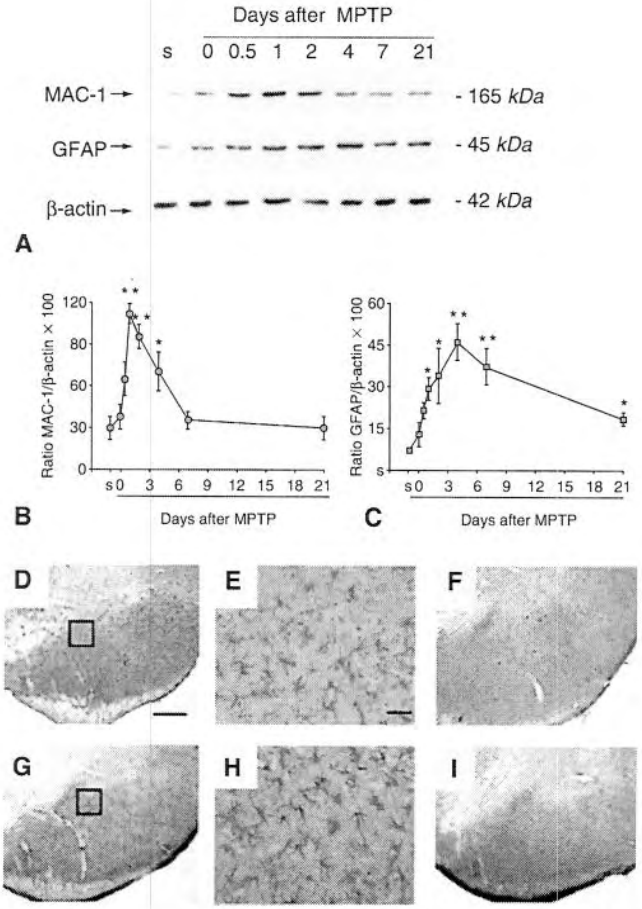
Monkey	Age (years)	Years after treatment	Type of treatment	Activated microglia[a]		SN cell loss[b]		Free melanin[c]	
				Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
M1	17	11	Right ICA, 0.95 mg/kg	3	2	4	1	3	3
M2	15.5	10	Bilateral, IV, 2 mg/kg	3	3	3	3	4	4
M3	11	5.5	Right ICA, 1.5 mg/kg	2	2	2	0	3	3
M4	18	14	Bilateral, IV, 2 mg/kg	4	4	3	3	3	3
M5	18	14	Right ICA, 0.7 mg/kg	3	1	2	0	3	2
M6	20	11	Right ICA, 0.51 mg/kg	1	0	2	1	4	4
M7	18	Control	No treatment	0	0	0	0	1	1
M8	16	Control	No treatment	0	0	0	0	1	1

[a]Microglial activation: 4, very strong; 3, strong; 2, moderate; 1, weak; 0, none.
[b]Degree of nigral TH-positive neuronal loss: 4, complete depletion; 3, very severe depletion; 2, severe depletion; 1, moderate depletion; 0, no depletion (normal).
[c]Amount of free melanin: 4, very large; 3, large; 2, moderate; 1, weak; 0, none.
SN, substantia nigra; ICA, intracarotid administration.
Reproduced from McGeer PL, Schwab C, Parent A, Doudet D (2003), with permission.

it challenges, as stated by McGeer and collaborators (2003), the tenet that MPTP would 'produce an acute loss of cells, followed by healing and long-term stabilization of surviving neurons'. Instead, both the human and monkey neuropathological data suggest that a single acute MPTP insult can set in motion a self-sustained cascade of cellular and molecular events with long-lasting detrimental effects on dopaminergic neurons. Supporting this interpretation is the positron emission tomography demonstration, performed twice, 7 years apart, on 10 individuals exposed acutely to MPTP, which revealed a worsening of striatal [^{18}F] fluorodopa uptake in these patients (Vingerhoets et al., 1994). Furthermore, among the MPTP-intoxicated individuals who participated in this study, 3 apparently developed parkinsonism between the first and the second scan (Vingerhoets et al., 1994).

p0190 Mice injected with MPTP and killed at different time points after the last injection show that the appearance of reactive astrocytes parallels the destruction of dopaminergic structure in both the striatum and the substantia nigra (Fig. 26.1) and that GFAP expression remains high even after the main wave of neuronal death has passed (Czlonkowska et al., 1996; Kohutnicka et al., 1998; Liberatore et al., 1999). Remarkably, the blockage of the uptake of the active metabolite of MPTP, 1-methyl-4-phenylperydinium (MPP^+), into dopaminergic neurons not only completely prevents substantia nigra dopaminergic neuronal death, but also GFAP upregulation (O'Callaghan et al., 1990). Collectively, these findings are consistent with the view that in the MPTP model, as in PD, the astrocytic reaction is consequent to the death of neurons and not the reverse. As for the activation of microglial cells (Fig. 26.1), which is well documented in the MPTP mouse model (Czlonkowska et al., 1996; Kohutnicka et al., 1998; Liberatore et al., 1999; Dehmer et al., 2000), it occurs earlier than that of astrocytes and, more importantly, it peaks before that of dopaminergic neurodegeneration (Liberatore et al., 1999). In light of the MPTP data presented above and illustrated in Fig. 26.1, it can thus be surmised that the response of both astrocytes and microglial cells to the demise of substantia nigra dopaminergic neurons clearly occurs within a timeframe allowing these glial cells to participate in the neurodegeneration of the nigrostriatal pathway in the MPTP mouse model.

p0200 There have also been some descriptive data from Czlonkowska and collaborators (Kurkowska-Jastrzebska



f0010 AU:9 **Fig. 26.1.** 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced glial reaction. (A–C) Ventral midbrain MAC-1 (A and B) and glial fibrillary acid protein (GFAP: A and C) expression is minimal in saline-injected mice (S), but increases in a time-dependent manner after MPTP injection. Data represent mean ± sem ($n = 4$–5). $^{**}P < 0.01$; $^{*}P < 0.05$, compared with saline, Newman–Keuls post-hoc test. (D–I) There is a robust MAC-1 (D) and GFAP (G) immunostaining in the substantia nigra pars compacta of MPTP-treated mice compared with that in saline-treated control mice (F and I) at 24 hours after injection. (E and H) Magnification of the boxed areas in D and G shows that the MAC-1- and GFAP-immunoreactive cells in the MPTP-treated mice seem to have a morphology typical of activated microglia cells (E) and of reactive astrocytes (H). Scale bars represent 200 μm (D, F, G, I; shown in D) and 15 μm (E, H; shown in E). Used with permission from: Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM, Przedborski S (1999). Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat Med 5: 1403–1409.

et al., 1999a,b) about the response of the adaptive immune system. For instance these authors have found in mice a marked increase of MHC-II antigen expression by microglia as well as a recruitment of T cells in both ventral midbrain and striatum after the administration of MPTP. Conversely, these researchers failed to identify any B cells in these tissue samples. Based on these studies, it appears that the infiltrating T cells were mainly of the CD8+ type, but some CD4+ were present too and more than 50% of the observed lymphocytes expressed the CD44 antigen.

26.6. What role does neuroinflammation play in parkinson's disease? s0060

In the context of neurodegeneration, the inflammatory and in particular the glial response has typically been regarded as triggered by the death of neurons and whose sole role was to eliminate the cellular debris. It is only recently that the idea has gained recognition that both innate and adaptive immune cells, such as microglia and T cells, could influence the fate of compromised neurons. One significant correlate of this assertion is that the death of specific neurons in a p0210

given neurodegenerative disease might not be as cell-autonomous as initially thought. Relevant to this concept is the study performed in chimeric mice that are mixtures of normal- and mutant superoxide dismutase-1 (SOD1)-expressing cells (Clement et al., 2003). In this work it appears that toxicity to motor neurons in this model of amyotrophic lateral sclerosis does require damage from mutant SOD1 acting within non-neuronal cells and non-neuronal cells that do not express mutant SOD1 delay degeneration of mutant-expressing motor neurons.

p0220 Regarding the role that glial cells could play within the neurodegenerative process, Streit (2002) points out that for the last decade there have been intense discussions on whether inflammation and especially activated microglia are beneficial or harmful to neurons. In PD, as in other pathological situations, the dispute emanates from the fact that studies in cell culture and to a lesser extent in animal models of PD have demonstrated both neuroprotective and neurotoxic effects of inflammation. One example that kindles the controversy is the demonstration that the blockade of the microglial activation by minocycline has been associated with either reduction or augmentation of dopaminergic neurodegeneration after MPTP administration (Du et al., 2001; Wu et al., 2002; Yang et al., 2003; Diguet et al., 2004). Perhaps the divergence of opinion regarding the role of inflammation in PD and related conditions could be resolved by accepting the idea that inflammation in general and microglia in particular are capable of performing both neuroprotective and neurodestructive functions and that, depending on local factors, extent of the degenerative process and even possibly the etiology of the disease in question, inflammation can give rise to quite distinct outcomes.

s0070 **26.6.1. Neuroinflammation is neuroprotective**

p0230 Although not performed in PD or in PD models per se, several in vivo observations strongly support a neuroprotective and neuroregenerative role of inflammation and microglia in the injured CNS. One of the most prototypical examples of such beneficial effects of inflammation is the facial nerve axotomy paradigm in newborn rats and rabbits in which axotomized motor neurons exhibit signs of recovery that coincide with the development of a glial response (Moran and Graeber, 2004). It has also been shown that 2–5 weeks after implantation of microglia cells into a small mechanically produced cavity in the rat spinal cord, prominent neuritic growth was observed in microglial grafts (Rabchevsky and Streit, 1997). These results agree with Streit's position (2002), which is that under both normal and pathological conditions, neuronal well-being and proper functioning are highly dependent on the presence of large numbers of glial cells that sustain an abundance of neuron-supporting functions.

p0240 In keeping with this latter statement, it should be remember that various types of glia and T cells in mature and, to a greater extent, in immature tissues can indeed provide a host of trophic factors that are essential for the survival of dopaminergic neurons. Among these, glial-derived neurotrophic factor (GDNF), which is released by reactive astrocytes (Schaar et al., 1993) and by activated microglia following a mechanical lesion of the striatum (Batchelor et al., 2000), seems to be the most potent factor supporting nigrostriatal dopaminergic neurons during their period of natural, developmental death in postnatal ventral midbrain cultures (Burke et al., 1998). It is also worth emphasizing that GDNF induces dopaminergic nerve fiber sprouting in the injured rodent striatum (Batchelor et al., 1999) and that this effect is markedly decreased when GDNF expression is inhibited by intrastriatal infusion of antisense oligonucleotides (Batchelor et al., 2000). Brain-derived neurotrophic factor (BDNF) is another trophic factor that can also be released by reactive astrocytes (Rubio, 1997; Stadelmann et al., 2002), by activated microglia (Batchelor et al., 1999; Stadelmann et al., 2002) and that can support the survival and outgrowth of dopaminergic structures in the striatum (Batchelor et al., 1999). It should also be emphasized that oligodendrocytes have emerged as a source of potent trophic factors (Du and Dreyfus, 2002). For instance, it was shown that striatal AU:2 oligodendrocytes greatly improve the survival and phenotype expression of mesencephalic dopaminergic neurons in culture, while simultaneously decreasing the apoptotic demise of these cells (Sortwell et al., 2000).

p0250 Aside from the fact that glial cells are pivotal to neuronal well-being, by maintaining ion and pH homeostasis and extracellular volume, they can also protect these cells against damage by scavenging toxic compounds released by the dysfunctional and dying neurons. With respect to the dopaminergic neurons, dopamine can produce reactive oxygen species (ROS) through different routes (Przedborski and Jackson-Lewis, 2000). Along this line, glial cells may protect remaining neurons against the resulting oxidative stress by metabolizing dopamine via monoamine oxidase-B and catechol-*O*-methyl transferase present in astrocytes and by detoxifying ROS through the enzyme glutathione peroxidase, which is detected almost exclusively in astrocytes (Hirsch et al., 1999). In contrast to glial cells, neurons lack the uptake system for cysteine (Pow, 2001) and thus rely heavily on astrocytes for

their synthesis of glutathione (Dringen, 2000), which is a tripeptide of great importance in the protection of the brain against ROS. Up to now, however, how astrocytes assist neurons in their production of glutathione remains incompletely understood. It is hypothesized (Dringen, 2000) that astrocytes achieve this goal by using various extracellular substrates as precursors for glutathione. The latter, once released from astroglial cells, would then become a substrate for the astroglial ectoenzyme gamma-glutamyltranspeptidase and the generated dipeptide CysGly would serve as a precursor of neuronal glutathione. It is also proposed (Dringen, 2000) that glutamine, which is released from astrocytes, would be used by neurons as a precursor for the glutamate necessary for glutathione synthesis. Finally, astrocytes, which can avidly take up extracellular glutamate via the glutamate transporters GLT1 and GLAST, may mitigate the presumed harmful effects of the subthalamic excitotoxic input to the substantia nigra (Benazzouz et al., 2000), which is hyperactive in PD (DeLong, 1990). Taken together, the data reviewed here support the contention that glial cells and especially astrocytes could play neuroprotective roles in PD. Whether any of those dampen the neurodegenerative process in parkinsonian patients remains to be demonstrated.

s0080 **26.6.2. Neuroinflammation and neurodegeneration**

p0260 If, as advocated by Streit (2002), the primary purpose of glial cells is to support neuronal function, it may be challenging to put forth a pathological scenario that transforms these cells into harmful effectors. Nevertheless, one cannot ignore the rapidly growing number of observations consistent with the point of view that activation of the innate immune system and especially of microglia exacerbates pre-existing or concomitant neuronal dysfunction, promoting neurodegeneration. The importance of activated microglial cells in the neurodegenerative process is underscored by the demonstration that the stereotaxic injection of bacterial endotoxin lipopolysaccharide (LPS) into the substantia nigra pars compacta of adult rats is associated with a local activation of microglia and an ensuing degeneration of dopaminergic neurons (Liu et al., 2000b), presumably mediated by IL-1β and caspase-11 (Arai et al., 2004). Similarly, LPS-induced microglial activation led to neurodegeneration of dopaminergic MES 23.5 cells or primary ventral midbrain neurons only when co-cultured with purified microglia (Le et al., 2001).

AU:3

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p0270 In an attempt to reconcile the inerrant supportive role of glial cells with their observed detrimental actions, Streit (2004) has pioneered the concept of glial cell senescence. According to this author, glial cells, such as microglia, would become progressively disabled during normal aging or in pathological situations, thus losing their functional capacity to support neurons and hence neurons would slowly degenerate. Although this idea is appealing, there is thus far no compelling data to support such a scenario in neurodegenerative diseases such as PD and investigations performed in animal models of PD rather suggest a more direct noxious role of inflammation in the demise of dopaminergic neurons. It has been proposed that perhaps the apparent detrimental actions of glial cells result from a phenomenon of facilitative neurotoxicity (Streit, 2002). Based on this model, glial cells will only eliminate neurons that have been compromised beyond viability and functionality by the primary pathological event. Here, glial cells would take on an active role in the demise of neurons that are destined to die and whose continued presence would not be beneficial in promoting neuronal recovery. Finally, as dubious as some experts believe this to be, one cannot exclude the fact that, upon activation, glial cells in brain parenchyma would exert indiscriminate neurotoxicity, which can significantly stimulate neurodegeneration and promote both the progression and propagation of a disease such as PD. This last scenario is not so unrealistic since it is well established that activated microglial cells can produce a variety of noxious compounds, including ROS, reactive nitrogen species, proinflammatory prostaglandins and cytokines.

p0280 Among the array of reactive species produced by glial cells, lately significant attention has been given to reactive nitrogen species due to the prevalent idea that nitric oxide (NO)-mediated nitrating stress could be pivotal in the pathogenesis of PD (Przedborski et al., 1996, 2001; Ara et al., 1998; Pennathur et al., 1999; Giasson et al., 2000). It is particularly relevant to mention that numerous astrocytes in the substantia nigra pars compacta of PD patients (Hunot et al., 1996) and microglia in the substantia nigra pars compacta of MPTP-intoxicated mice (Liberatore et al., 1999; Dehmer et al., 2000), but not of controls, are immunoreactive for inducible NO synthase (iNOS). Upon its induction, this NOS isoform produces high amounts of NO (Nathan and Xie, 1994) as well as superoxide radicals (Xia and Zweier, 1997) – two reactive species that can either directly or indirectly promote neuronal death by inflicting oxidative damage. It should also be mentioned that a main source of glial-derived ROS emanates from the microglial enzymatic complex NADPH-oxidase, which upon its induction and activation can produce large amounts of superoxide radicals (Colton et al., 1996). This parti-

cular multiunit enzymatic complex has been reported to be activated in the substantia nigra pars compacta of both PD patients and mice intoxicated with MPTP (Wu et al., 2002, 2003) and the genetic inactivation of NADPH-oxidase has been shown to mitigate MPTP-induced neurodegeneration in mice (Wu et al., 2003).

p0290 Prostaglandins and their synthesizing enzymes, such as Cox-2, constitute a second group of potential culprits. Indeed, Cox-2 has emerged as an important determinant of cytotoxicity associated with inflammation (O'Banion, 1999). In the normal basal ganglia, Cox-2 is minimally expressed (Teismann et al., 2003). However, in both PD and MPTP mouse tissues, Cox-2 expression in the brain can increase significantly, as do the levels of its products such as prostaglandin E_2 (Mattammal et al., 1995; Teismann et al., 2003). In the MPTP mouse model of PD, it was shown that Cox-2 was induced via a c-Jun *N*-terminal kinase-dependent mechanism (Teismann et al., 2003; Hunot et al., 2004), whose blockade, like that of Cox-2 itself, attenuates neurodegeneration (Teismann et al., 2003; Hunot et al., 2004). Of note, although Cox-2 does modulate MPTP-induced dopaminergic neurotoxicity, its isoenzyme Cox-1 was ineffective in doing so (Teismann et al., 2003).

p0300 A third group of glial-derived compounds that can inflict damage in PD is the proinflammatory cytokines. Several among these, including tumor necrosis factor-α (TNF-α) and IL-1β, are increased in both substantia nigra pars compacta tissues and cerebrospinal fluid of PD patients (Mogi et al., 1994, 1996, 2000), although some of the reported alterations may be related to the chronic use of the anti-PD therapy levodopa (Bessler et al., 1999). Nevertheless, at autopsy convincing immunostaining for TNF-α, IL-1β and interferon-gamma (IFN-γ) is observed in substantia nigra pars compacta astrocytes from PD patients (Hunot et al., 1999). These cytokines may act in PD on at least two levels. First, although they are produced by reactive astrocytes, they can stimulate other astrocytes and even microglia not yet activated, amplifying the inflammatory response and consequently the glial-related injury to neurons. Relevant to this view is the demonstration that astrocyte-derived TNF-α, IL-1β and IFN-γ stimulate the expression of the cell surface receptor Fc∈R11/CD23 by microglia (Hunot et al., 1999). Then, upon ligation of Fc∈R11/CD23, activated microglia induce iNOS expression, leading to NO production which, in turn, can amplify the production of cytokines by astrocytes and diffuse to neighboring neurons. Second, astrocytic and microglial-derived cytokines may also act directly on dopaminergic neurons by binding to specific cell surface cytokine receptors such as TNF-α receptor and FAS. In connection with these, it must be stressed that investigations on TNF-α have generated confusing results in the MPTP mouse model in that ablation of both TNF-α receptor-1 and -2 did not impact MPTP-induced dopaminergic neurodegeneration (Rousselet et al., 2002), whereas ablation of TNF-α and the pharmacological inhibition of its synthesis did attenuate MPTP toxicity in mice (Ferger et al., 2004). As for FAS, its expression appears increased in mouse ventral midbrain after MPTP injection and mice deficient in this plasma membrane receptor were found more resistant to MPTP injections compared to their wild-type littermates (Hayley et al., 2004). Once activated, these cytokine receptors trigger intracellular death-related signaling pathways, whose molecular correlates include translocation of the transcription nuclear factor-κ-B (NF-κ-B) from the cytoplasm to the nucleus and activation of the apoptotic machinery, whose implication in the overall mechanism of dopaminergic neuronal death seems quite significant (Vila and Przedborski, 2003). PD patients exhibit a 70-fold increase in the proportion of dopaminergic neurons with NF-κ-B immunoreactivity in their nuclei compared to control subjects (Hunot et al., 1997). Despite the robust recruitment of NF-κ-B, it is not clear whether this transcriptional factor is instrumental in PD pathogenesis, as mice deficient in one of NF-κ-B main polypeptides, P50, had their nigrostriatal pathway as severely damaged by MPTP as that in their wild-type counterparts (Hunot et al., 2004).

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26.7. Conclusion and therapeutic perspective s0090

p0310 It seem clear from the above discussion that several lines of evidence indicate that both morphological and functional indices of inflammation are encountered in the diseased areas of the PD brain, other prominent sporadic and familial parkinsonian syndromes, as well as experimental models of PD. Thus far, most attention has been given to the innate immune response, involving the resident inflammatory cells such as astrocytes and microglia. In striking contrast is the limited amount of information currently available about the adaptive immune response in PD and related conditions. This discrepancy may be explained by the fact that brain inflammation in neurodegenerative disorders, as stressed by McGeer and McGeer (2004), is thought to be primarily a local immune reaction that occurs without significant involvement of adaptive immune cells. However, there are some descriptive studies, performed in both PD autopsy material and in the MPTP mouse model, that have begun to examine the status of T cells in damaged

brain areas (McGeer et al., 1988a; Kurkowska-Jastrzebska et al., 1999a,b). As for, B cells and immunoglobulin, it has been reported by several groups that antibodies to dopaminergic neurons are found in the cerebrospinal fluid of PD patients (Emile et al., 1980; Defazio et al., 1994; Rowe et al., 1998) and that the stereotaxic injection of PD immunoglobulin G into the mouse substantia nigra provokes the degeneration of dopaminergic neurons (Chen et al., 1998). Collectively, these data support the idea that if the innate immune response is the chief cellular component of brain inflammation seen in PD and related conditions, there is unmistakable evidence supporting the involvement of the adaptive immune system as well. Given this fact, additional studies geared toward better characterizing the adaptive immune system in PD and related conditions are certainly warranted.

p0320 Popular experimental models of PD, such as that generated by the neurotoxin MPTP, produce a more severe and acute degenerative event accompanied with a more robust inflammatory reaction than that occurring in PD. Despite these striking departures from PD neuropathology, the experimental models of PD discussed in this chapter have been and continue to be critical in our ability to ascertain the role of inflammation in the degeneration of the nigrostriatal pathway. Based on the large body of literature discussed above, the current belief that emerged about inflammatory reaction in PD is that it appears more often endowed with deleterious properties, capable of exacerbating dopaminergic neuronal death, rather than with beneficial effects, capable of mitigating neurodegeneration or even promoting repair of the nigrostriatal pathway. Should this view be correct, targeting cellular and molecular aspects of brain inflammation may have far-reaching implications for the treatment of neurodegenerative disorders such as PD. Along this line, three very different strategies of treatment may be envisioned.

p0330 First, attempts to prevent the glial reaction and more specifically the microglial activation may be foreseen. Several preclinical studies have successfully prevented microglial activation, especially in the MPTP and 6-OHDA models of PD with a variety of agents, whose molecular basis of their anti-inflammatory actions often remains enigmatic. These agents include, for example, the antibiotic minocycline (Du et al., 2001; He et al., 2001; Wu et al., 2002), the peroxisome proliferator-activated receptor-gamma agonist pioglitazone (Breidert et al., 2002), the vasoactive intestinal peptide (Delgado and Ganea, 2003) and some opiate receptor antagonists (Liu et al., 2000a). Because we begin to acquire a deeper understanding of the microglial molecular pathways responsible for their activation (Bhat et al., 1998; Pyo et al., 1998; Taylor et al., 2005), more specific agents to prevent microglial activation are likely to emerge in the near future.

Second, therapeutic strategies can also be aimed at p0340 blocking the effects of specific proinflammatory mediators without searching to mitigate glial response per se. As discussed above, factors such as iNOS-derived NO (Liberatore et al., 1999), NADPH-oxidase-derived ROS (Gao et al., 2003; Wu et al., 2003) or caspase-11 (Furuya et al., 2004) could all be considered as suitable therapeutic targets. With no exception, preclinical studies, especially performed in the MPTP mouse model of PD, have shown that these kinds of proinflammatory factors are capable of causing neurotoxic phenotypes and that their ablation attenuates neurodegeneration. In all of these studies, however, the beneficial effects provided by the blockade of one of these proinflammatory factors provided only mild protection. This fact should not preclude the significance of these findings, but argues that multiple such factors may have to be inhibited simultaneously before any substantial neuroprotection is observed and that improvement in the quality of life of PD patients can be expected.

Third, immunization strategies with CNS antigens p0350 expressed at the lesion site have been shown to induce T cells to enter inflamed CNS tissue, attenuate innate glial immunity and increase local neurotrophic factor production. For instance, in the MPTP mouse model of PD, vaccination with glatiramer acetate, a random amino acid polymer that generates non-encephalitic T cells, which cross-react with myelin basic protein, did confer protection against dopaminergic neurodegeneration (Benner et al., 2004). In this work, glatiramer acetate-immune cells administered to MPTP-intoxicated mice by adoptive transfer entered inflamed brain regions, suppressed microglial responses and increased expression of GDNF (Benner et al., 2004). These preclinical data suggest that vaccination strategies with antigens derived from prominent proteins residing in the site of neurodegeneration deserve to be tested further for their potential in mitigating inflammation and ensuing demise of dopaminergic neurons in PD and related conditions.

Acknowledgments s0100

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Pathogenesis of nigral cell death in Parkinson's disease☆

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Abstract

Parkinson's disease (PD) is primarily a sporadic condition which results mainly from the death of dopaminergic neurons in the substantia nigra. Its etiology remains enigmatic while its pathogenesis begins to be understood as a multifactorial cascade of deleterious factors. As of yet, most insights into PD pathogenesis are derived from toxic models of PD and show that the earlier cellular perturbations arising in dopaminergic neurons include oxidative stress and energy crisis. These alterations, rather than killing neurons, trigger subsequent death-related molecular pathways including elements of apoptosis. The fate of dopaminergic neurons in PD may also be influenced by additional factors such as excitotoxicity, emanating from the increased glutamatergic input from the subthalamic nucleus to the substantia nigra, and the glial response that arises in the striatum and the substantia nigra. In rare instances, PD can be familial, and those genetic forms have also provided clues to the pathogenesis of nigrostriatal dopaminergic neuron death including abnormalities in the mechanisms of protein folding and degradation as well as mitochondrial function. Although more remains to be elucidated about the pathogenic cascade in PD, the compilation of all of the aforementioned alterations starts to shed light on why and how nigral dopaminergic neurons may degenerate in this prominent disease, that is PD.

Keywords: Parkinson's disease; MPTP; Neurodegeneration; Pathogenesis

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after the Alzheimer's dementia. It is estimated that more than one million individuals in the United States of America alone are affected with this disabling disease and that more than 50,000 new cases arise each year [1]. PD is progressive with a mean age at onset of 55, and with an incidence that increases markedly with age [2]. Clinically, PD is characterized by the cardinal features of tremor at rest, slowness of voluntary movements, rigidity, and postural instability [1]. Like many other neurodegenerative diseases, PD presents itself mainly as a sporadic condition, meaning in absence of any genetic linkage, but in rare instances, PD can also arise as a simple Mendelian trait, linked to defects in a variety of genes [3]. Although, clinically and pathologically, sporadic and familial PD may differ on several significant aspects, they all share the same biochemical brain abnormality, namely a dramatic depletion in brain dopamine [2].

The reason why PD patients exhibit low levels of brain dopamine stems from the degeneration of the nigrostriatal dopaminergic pathway, which is made of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compact and whose projecting axons and nerve terminals are found in the striatum [2]. Yet, it is important to emphasize the fact that the neuropathology of PD is far from being restricted to the nigrostriatal pathway, and histological abnormalities can be found in many other dopaminergic and even non-dopaminergic cell groups [2]. The second most prominent neuropathological feature of PD is the presence of intraneuronal inclusions called Lewy bodies (LBs) in the few remaining nigral dopaminergic neurons [2]. LBs are spherical eosinophilic cytoplasmic aggregates composed of a variety of proteins, such as α-synuclein, parkin, ubiquitin and neurofilaments, and they can be found in every affected brain region [2].

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Over the past few decades a large core of data originating from clinical studies, autopsy materials, and in vitro and in vivo experimental models of PD has been accumulated, which led us to begin to have some level of understanding of the pathogenesis of sporadic PD [2]. Available data would argue that the mechanism of neuronal death in PD starts with an otherwise healthy dopaminergic neuron being hit by an etiological factor, such as mutant α-synuclein. Subsequent to this initial event, it is proposed that a cascade of deleterious factors is set in motion within that neuron made not of one, but rather of multiple factors such as free radicals, mitochondrial dysfunction, excitotoxicity, neuroinflammation, and apoptosis to cite only some of the most salient. Still based on this proposed scenario, all of these noxious factors will interact with each other to ultimately provoke the demise of the injured neuron.

Despite unquestionable major advances made in the molecular and cellular biology of PD and other neurodegenerative diseases which brought us closer than ever to being capable of unraveling the pathogenesis of PD, several critical questions remain unanswered. In this paper, three pending questions pertinent to the mechanisms of neuronal death in PD are discussed and form the body of this review. To be discussed first will be the question of what do we know about the nature of the pivotal factors and the sequence in which they act within the proposed pathogenic cascade that leads to neuronal death in PD. Second is the question to know whether the overall neurodegenerative process in PD is truly a cell autonomous process will be briefly addressed. Finally, one cannot avoid discussing the contribution of rare, inherited forms of PD to our current understanding of the pathogenesis of sporadic of PD.

2. Nature and sequence of action of pathogenic factors in PD

The current model of pathogenesis that most investigators in the field utilize has been outlined above. To confirm the actual role of these different presumed factors and the sequence by which they, respectively, intervene in this multifactorial cascade has been primarily, if not exclusively, studied in toxic experimental models of PD, which are numerous. Findings from these models and especially from that produced by the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) indicate that the initial cellular perturbations include inhibition of mitochondrial respiration. Indeed, soon after the systemic administration of MPTP to mice, its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP^+), does concentrate in the mitochondrial matrix, where it binds to complex I of the electron transport chain [4]. MPP^+ binding interrupts the flow of electrons along the mitochondrial electron transport chain, thereby leading to an increased production of reactive oxygen species (ROS), especially of superoxide radicals [4]. Previous work has clearly demonstrated that the magnitude of MPP^+-related ROS production within the mitochondria is a dose-dependent phenomenon [5]. The pathogenic significance of such a local excess of ROS production is supported by the demonstration that mitochondrial aconitase activity is reduced in ventral midbrain of MPTP-treated mice [6]. MPP^+-related loss of electron flow is also associated with a drop in ATP production [4], which in vivo is found only in susceptible areas of the brain such as ventral midbrain and striatum [7]. Remarkably, this work shows that ATP deficit develops very rapidly after MPTP injection and lasts only for a few hours, as by one day post-MPTP tissue content in ATP seem to return to normal values [7].

In addition to provoking mitochondrial oxidative stress and energy crisis, MPP^+ also interacts with synaptic vesicles through its binding to vesicular monoamine transporter-2 [8]. In so doing, MPP^+ translocates into synaptic vesicles where it stimulates the extrusion of synaptic dopamine [9,10], reminiscent of the effect of methamphetamine. The resulting excess of cytosolic dopamine can readily undergo autooxidation, thus generating a huge burst of ROS, subjecting nigral neurons to an oxidative stress [11]. Alternatively, oxidation of cytosolic dopamine can also be catalyzed by enzymes such as cyclooxygenase-2 [12], which is upregulated in the remaining nigral dopaminergic neurons in both MPTP-treated mice and in human post-mortem samples [13]. Supporting this proposed event is our demonstration that cyclooxygenase-2 promotes dopamine–quinone formation following MPTP injection, and the production of protein-bound 5-cysteinyl-dopamine adducts in the brain of MPTP-injected mice [13]. In addition, excess of cytosolic dopamine can stimulate the formation of neuromelanin [14], a dark intraneuronal pigment implicated in the greater susceptibility of nigral neurons to PD neurodegeneration [15]. Although it remains uncertain how neuromelanin does actually contribute to the demise of dopaminergic neurons, it has been hypothesized that this pigment can do so by a *macromolecule crowding effect* or by playing the role of *intraneuronal toxic reservoir* by binding different transitional metals, such as iron, and various toxicants, such as MPP^+ [16].

All of the studies discussed above point toward the superoxide radical being pivotal in MPTP neurotoxicity. However, superoxide radical is known to not be highly reactive, and thus it is unlikely that it may be directly responsible for the damage inflicted by MPTP. Instead, it is much more likely that superoxide neurotoxicity results from superoxide reacting with other reactive molecules to generate what are called *secondary reactive species* of much greater tissue damaging potential, such as peroxynitrite. Consistent with this view are the demonstrations that the production of peroxynitrite, evidenced by quantifying tissue content of protein-bound 3-nitrotyrosine, is increased after MPTP injection [17] and that peroxynitrite is likely implicated in the nitrative post-translational modifications

of pathogenically meaningful proteins such as α-synuclein and parkin [18,19].

Collectively, the aforementioned findings indicate that early pathogenic events following MPTP administration include mitochondrial and cytosolic oxidative stress and ATP deficit. Yet, when one compares the time course of these cellular perturbations with the actual phase of neuronal degeneration found after MPTP injections, it clearly appears that oxidative stress and energy crisis precede the peak of dopaminergic neuronal death in the substantia nigra of mice which is situated around 24–48 h after the last injection of MPTP [20]. This finding suggests that these early events may kill some dopaminergic neurons, but that most of the neurons injured by this parkinsonian toxin fail to succumb to this early attack. Instead, it is believed that rather than killing a large number of neurons, early oxidative stress and energy crisis activate cell death-related molecular pathways which are the real executioner of the injured neurons. Among these are c-Jun *N*-kinases [21], cyclin-dependent kinases [22], and various components of the apoptotic machinery [23]. To illustrate the critical role of these molecular pathways in the MPTP-induced neurodegenerative process, two studies that pertain to apoptosis will be discussed here. First is the work done on the pro-cell death protein Bax, demonstrating that not only is Bax highly expressed in nigral dopaminergic neurons, but that ablation of Bax renders mice more resistant to the dopaminergic neurotoxicity of MPTP [24]. The second study deals with apoptotic protease activating factor 1 (APAF-1), one of the critical components of the apoptosome complex [25]. In the latter work, the authors have unilaterally delivered a viral vector expressing a dominant negative mutant of APAF-1 by stereotaxic injection in the substantia nigra [25]. Then, they have subjected these mice to a systemic administration of MPTP and found that the blockade of APAF-1 did mitigate the death of dopaminergic neurons [25]. These two studies clearly demonstrate the importance of downstream molecular pathways such as apoptosis in the death of nigral dopaminergic neurons and are consistent with the sequential pathogenic model proposed above.

3. Is the neurodegenerative process in PD cell autonomous?

This question is of critical importance both for pathogenic and therapeutic reasons. Indeed, it is quite important to determine whether the demise of nigral dopaminergic neurons strictly results from the cellular perturbations that arise within these neurons due to the disease's etiology, or from a complex interaction between what are called intrinsic and extrinsic perturbations. The answer to this question is complicated and far from straightforward. For instance, if one reviews the body of literature on cultured neurons exposed to MPP^+ or overexpressing PD-causing proteins such as α-synuclein [2], there is no doubt that these catecholaminergic neurons die in absence of any other intervening exogenous factors such as other cell types. However, when one looks at more complex systems, such as post-mortem tissues from PD patients or in vivo experimental models of PD, there is mounting evidence that indicates that the surroundings of the nigral dopaminergic neurons appear to play a critical role in influencing the fate of these dopaminergic cells. Among the potential culprits is the increased glutamatergic input to the nigra and which originates from the hyperactive subthalamic nucleus [26,27]. Moreover, the glial response that is found in both striatum and nigra of PD patients and MPTP-mice is also likely to exert deleterious effects on the remaining dopaminergic neurons [28]. This view has led many investigators, including those in my laboratory, to aggressively examine the potential role of neuroinflammation in the pathogenesis of PD. This important topic, however, will not be reviewed here as it is discussed in-depth in the accompanied paper written by Dr E. Hirsch. Based on these data and those presented by Dr Hirsch in this special issue, it is our opinion that several factors, exterior to dopaminergic neurons, contribute to creating a hostile environment, which increases the stress on already compromised dopaminergic neurons present in the vicinity. These factors, while likely not capable of initiating the disease, are nevertheless likely to amplify the neurodegenerative process and stimulate the progression of a chronic disease such as PD. If this view is correct, one then must take into account those exogenous factors if one wishes to completely and accurately comprehend the pathogenic cascade underlying the neurodegenerative process of PD and to develop effective neuroprotective therapies for this illness.

4. Insights from the rare inherited forms of PD

Until recently, all of the hypotheses regarding the cause and the mechanisms of PD neurodegeneration came from investigations performed in autopsy material from sporadic PD cases or in neurotoxic models [2]. However, less than a decade ago this situation changed with the identification of a mutation in α-synuclein associated with PD in an Italian kindred [29]. Since then, four additional PD-causing genes have been identified, and a linkage has been reported for at least five more. Although rare, these inherited cases have opened new directions of research which have already led to the integration within the proposed pathogenic cascade of new molecular components. In particular, familial cases of PD have brought to our attention the potential importance of protein aggregation and abnormalities in protein turnover in the overall process provoking neurodegeneration in PD. The contribution to our understanding of the pathogenesis of sporadic PD from the different PD-causing mutations has been discussed elsewhere [3], and thus readers

interested in this question are urged to consult this paper. Since the publication of this latter review, several important new findings have been published and, of these, three are worth discussing briefly.

As mentioned above, protein degradation has emerged as a potentially important theme in PD pathogenesis, especially in the context of alterations of the proteasome/ubiquitin pathway. Yet, protein degradation does not solely rely on the proteasome/ubiquitin pathway, but also on autophagy. With respect to autophagy, it has been reported that both wild-type and mutant α-synuclein can be degraded by lysosomal enzymes and that both bind to the autophagy chaperone [30]. Remarkably, however, mutant α-synuclein binds with much greater avidity to the autophagy chaperone than its wild-type counterpart [30]. Furthermore, contrary to wild-type α-synuclein which after binding to the chaperone is rapidly taken up by autophagic vacuoles for lysosomal degradation, mutant α-synuclein remains tightly attached to the chaperone and is never taken up or degraded [30]. These striking observations indicate that mutant α-synuclein fails to be properly degraded by the chaperone-mediated autophagy (CMA). It can, thus, be speculated that part of the neurotoxic mechanism of mutant α-synuclein may be related to the blockade of CMA and the consequent accumulation of unwanted proteins that are no longer eliminated by CMA.

The two other studies to be discussed here pertain to observations made on DJ1 and PINK1, the products of genes which upon mutation are linked to familial forms of PD, whose sub-cellular locations are mitochondrial [31,32]. In both cases, it was shown that either the abrogation of the mitochondrial localization or the loss of activity of these proteins renders cells subjected to mitochondrial poisons or proteasome inhibitor more prompt to degeneration [31,32]. Although the exact functions of DJ1 and PINK1 remain to be elucidated, these results already indicate that wild-type DJ1 and PINK1 assume some type of mitochondrial functions which confer resistance of the whole cell to a variety of stressors. Worth noting is the fact that, at this point there is no evidence to indicate that the putative mitochondrial action of DJ1 or PINK1 is linked to the electron transport chain machinery.

5. Conclusion

In this short review, an attempt has been made to stress the fact that the current consensus regarding the pathogenesis of sporadic PD is based primarily on information gathered from neurotoxic models of the disease. Based on these data, it appears that nigral dopaminergic neuron degeneration does not result from the action of a single deleterious factor, but rather from the convergence of multiple pathogenic factors. Many of these noxious factors emanate from within the dopaminergic neurons, whereas several others originate from outside the dopaminergic neurons such as glutamatergic input and glial cells.

We also know by now that while uncommon, there is much to learn from these rare familial cases of PD linked to gene mutations. In a matter of a few years, thanks to several elegant investigations performed in genetic cases of PD, we have become aware of the importance of excess protein aggregation with respect to mechanisms of neuronal death, perturbations in protein degradation systems such as proteasome and autophagy, and accumulation of unwanted proteins. These genetic cases have also shed light on new mitochondrial mechanisms other than those related to the electron transport chain which may have great pathogenic significance.

Despite enormous advances, it is fair to conclude by saying that much remains to be done to completely unravel the pathogenesis of PD. Among specific aspects that may deserve particular attention are the identification of where within the proposed pathogenic cascade do mutation-related deleterious mechanisms intersect with those mediated by the parkinsonian toxins. Why nigral neurons are more vulnerable than other dopaminergic neurons to the PD neurodegenerative process is also paramount to a comprehensive understanding of the neurobiology of this prominent neurodegenerative disease.

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THE MPTP MODEL OF PARKINSON'S DISEASE

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INTRODUCTION

In his monograph "The case of the frozen addicts," J. William Langston reports the story of six patients, who in 1982, in California, developed mysterious symptoms provoked by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that were reminiscent of Parkinson's disease. While since that time MPTP is recognized as a highly selective neurotoxin, this compound, in reality, has a much longer history (1). Apparently, the synthesis of MPTP was first reported by Ziering and colleagues in 1947 as part of a series of papers on meperidine derivatives (2), and using these methods it was shown that piperidols may be acylated with a propionic group to produce MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), a compound 25 times more potent than morphine in the rat (2,3). Since then, MPTP has been used extensively as a synthetic intermediate in meperidine chemistry and has become commercially available (Aldrich Chemical Company, Milwaukee, Wisconsin) for this purpose long before it had been identified as a potent neurotoxin.

Early studies involving MPTP received little attention and were probably not even published. Ironically, MPTP was first investigated in the early 1950's as an anti-parkinsonian agent (Dr. Sorter, personal communication, 1983). Rats treated with MPTP failed to develop any neurological problems while two monkeys given a single dose of MPTP (0.5 mg/kg subcutaneously) developed severe limbs rigidity less than two days after the injection. Two other monkeys given a higher dose of MPTP (2 mg/kg) became extremely rigid and totally immobile by the third day after the injection; both were dead by end of the second and third weeks. Despite these reported findings in monkeys, investigators did not come to the conclusion that MPTP administration caused behavioral changes resembling parkinsonism. As part of the same investigation, MPTP was

administered to six parkinsonian patients in doses ranging from 50 to 300 mg/kg daily for 3 weeks. Two of these patients died during or shortly after the trial from presumably uremia, pancreatitis, cholecystitis, and congestive heart failure. Following this observation, MPTP was abandoned because of its toxicity. No further details regarding these patients had been collected.

The first published case of MPTP-induced toxicity in humans involved a graduate student who succeeded in producing MPPP for his personal consumption using the Zeiring's method (4). After several months of MPPP abuse, he apparently decided to increase the reaction temperature and to abandon the purification step to reduce the preparation time of the compound. Unfortunately, the amount of MPPP produced by this method was markedly dependent upon experimental conditions and drastic conditions, such as those caused by using excess acid or higher reaction temperatures dramatically increase the amount of MPTP generated. Not surprisingly therefore, shortly after he altered the protocol, he became severely parkinsonian. His symptoms were well controlled by levodopa/carbidopa and large doses of bromocriptine. Less than two years after the onset of illness, the patient was found dead of an overdose. Neuropathological changes were characterized mainly by a loss of nigrostriatal dopaminergic neurons (see *MPTP-induced parkinsonism in humans* below for details).

Four years later, in Vancouver, another young drug addict attempted to synthesize MPPP using the Schimdle and Mansfield formula. After he snorted his home-synthesized meperidine analogue daily for 7 days, he was admitted to the hospital in an immobile, mute state (5). This addict was first diagnosed as having an acute psychotic episode with catatonia and was treated with neuroleptics for about a year without any significant improvement. At that time, the patient was admitted to another institution, where he was found to present severe bradykinesia, stooped posture, shuffling gait, excessive drooling and severe dysarthria. He was diagnosed as having parkinsonism and was started on

levodopa/carbidopa, to which he responded dramatically. About 20 months after the onset of the illness, he died in a drowning accident. The delay between the death and autopsy was too long and thus no appropriate examination of the basal ganglia or the substantia nigra could be done. Even though in these two first cases MPPP or an intermediate compound was the suspected damaging agent, a definite identification was not made.

The next case of possible MPTP toxicity in humans found in the literature is of particular interest since it may represent an example of cutaneous absorption or vapor inhalation (6). In 1964, an organic chemist working for a major pharmaceutical company began synthesizing many compounds which required repeated preparation of MPTP (he synthesized more than 3 kg MPTP). There were many opportunities for cutaneous contact or inhalation of MPTP, which was purified by vacuum distillation, since no evidence of intravenous exposure to MPTP could be found. In 1970, when the patient was 37 years old, he began experiencing coordination difficulty in his left hand and, within a year, he was diagnosed as having PD. There has been some progression of his symptoms since that time, with generalized cogwheel rigidity, bradykinesia, facial hypomimia, and stooped posture; no tremor was present. In 1991, he was reported to take levodopa/carbidopa, and had an excellent clinical response to this therapy (Dr. L. Côté, personal communication, 1991).

Recognition that MPTP, a by-product of MPPP synthesis, was toxic finally occurred after MPPP was again produced for illicit use in northern California during 1982 (Dr. Heagy, DEA Western Regional Laboratory, San Francisco, personal communication, 1982). This time, however, it was destined for mass distribution. In the early spring and summer of 1982, several young drug addicts mysteriously developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogues sold as heroin, synthetic heroin, Mexican brown heroin ("organic heroin"), and "china white" (high-grade heroin from south-east Asia). After tracking down

samples of the "heroin" for analyses and searching for additional cases, it became evident that MPTP was most likely the offending agent (7). In addition, this view was further supported by the fact that the "epidemic" started not long after contaminated batches of MPPP containing MPTP (one contained almost pure MPTP) began appearing on the street (Dr. Heagy, DEA Western Regional Laboratory, San Francisco, personal communication, 1982).

MPTP-INDUCED PARKINSONISM IN HUMANS

It is estimated that more than four hundred drug addicts might have been exposed to MPTP (Langston, personal communication, 1994). However, among them only a small number have been interviewed and examined. Therefore, it is important to emphasize that the epidemiologic data available to date on MPTP-induced parkinsonism in humans derived from studies performed on a limited number of affected individuals (8,9).

Index cases

Case histories of the seven individuals discovered in 1982 to have MPTP-induced parkinsonism have been reviewed by Ballard et al. (8). In 4 of the 7 cases, samples of the drug they had been using were analyzed and were found to contain a high proportion of MPTP. All of the 7 patients displayed a characteristic clinical syndrome involving two stages: an acute phase followed by a chronic phase.

During the acute stage, all patients noted a burning sensation when the drug was injected intravenously. The immediate subjective effect included a heroin-like euphoria, but more dream-like. Disorientation, blurred vision, visual delusions or hallucinations

were also noted by some patients. The initial motor symptoms included intermittent jerking movements of the limbs, increasing slowness, or both. Jerking movements generally disappeared within a few days, to be followed by slowness and stiffness, difficulty in speaking and swallowing, and in some cases, tremor. This progressed over several days to 3 weeks. By the time they reached the chronic stage of the disease, patients displayed most of the hallmarks of PD (Table 1). There was no autonomic impairment, cerebellar or pyramidal sign. Six of these patients were also studied to assess intellectual functions (10). The authors found a slight but significant difference between the MPTP group and healthy controls in the level of general intellectual function on the modified mini-mental status examination which was due to poor performance by the MPTP group on orientation, naming presidents and construction subtests. MPTP patients also exhibited a significantly poorer performance on tests of construction (Rosen Drawing Test), verbal fluency (Category Naming) and executive function (Stroop Word-Color Test). Although patients with MPTP-induced parkinsonism performed less effectively than controls on these tasks, none met criteria for dementia. Performance on attentional tests (reaction time and continuous performance tests), memory, attention, digit span, calculation and overall language test (other than verbal fluency), was comparable in the two groups. Interestingly, the authors observed that the tasks on which the MPTP group exhibited a poorer performance than controls were comparable to the pattern of neuropsychological deficits seen in PD (11). Spinal fluid analyses performed in six of the patients showed that homovanillic acid (HVA) levels, the major metabolite of dopamine, were markedly reduced, whereas the levels of the serotonin metabolite, 5-hydroxyindoleacetic acid and of 3-methoxy-4-hydroxyphenylethylene glycol, the major metabolite of norepinephrine, were normal and elevated respectively in comparison to the levels measured in six healthy subjects (12). In all seven patients, the administration of levodopa/carbidopa alone or with dopamine agonists resulted in

dramatic improvement. However, all developed early side effects to the treatment such as "wearing-off" effect, "peak-dose" dyskinesias or "on-off" fluctuations.

Other MPTP-exposure cases in humans

Ruttenber et al. (9) described the results of interviews and physical examinations of 83 individuals who allegedly were exposed to MPTP. Among them, 80% had at least one of the following clinical signs of parkinsonism and 60% had two or more: lack of facial expression, "en bloc" turning, action tremor, bradykinesia, impairment in rapid alternative movements, seborrhea or resting tremor. In addition, 49% had increased tone and 64% had cogwheel rigidity in at least one limb. Although the acute and chronic symptoms of these patients were similar to those found in the index cases, they were less severe. This finding supports the hypothesis that the index cases received a heavy exposure to MPTP (9).

Another group of MPTP affected individuals was described by Tetrud et al. (13). In their study, the authors compared 22 individuals with mild parkinsonism resulting from MPTP exposure to 130 patients with early, untreated PD patients and 51 intravenous narcotic users not exposed to MPTP. The MPTP-exposed group was highly comparable to the patients with early PD, except for a lower prevalence of resting tremor. Interestingly, intravenous narcotic users not exposed to MPTP had no signs of parkinsonism, suggesting that drug abuse alone does not cause these neurological manifestations.

The parkinsonian syndrome induced by MPTP in humans is clinically indistinguishable from PD except for a few features. First, after onset, parkinsonism worsens slowly in PD patients, but much faster in MPTP-intoxicated individuals. Second, whether an acute exposure to MPTP causes a progressive neurodegeneration like in PD remains a debatable issue. Indeed, Burns and collaborators reported the case of a young chemist who developed parkinsonism after substantial laboratory exposure to

MPTP and who failed to show any evidence of worsening of his neurological condition over several years (14). Yet, positron emission tomography (PET) performed twice, seven years apart, on 10 individuals exposed to MPTP, revealed worsening of striatal [^{18}F]fluorodopa uptake in these patients (15). Moreover, postmortem studies in three individuals who survived 3-16 years after exposure to MPTP (16) and in six monkeys who survived 5-14 years after exposure to MPTP (17) showed evidence of extracellular neuromelanin and activated microglia in the substantia nigra, two neuropathological features consistent with an ongoing degenerative process. Third, dementia has not been documented in individuals with MPTP-induced parkinsonism either.

Brain pathology is another aspect upon which PD and MPTP are somewhat divergent. At autopsy, brains from PD patients exhibit a severe loss of large neuromelanin-containing neurons of the substantia nigra. This cell loss is associated with characteristic eosinophilic intracytoplamic inclusions called Lewy bodies. Other areas showing degenerative changes include the locus coeruleus, the dorsal motor nucleus of the vagus and the basal nucleus of Meynert; these changes are also accompanied by the presence of Lewy bodies. In the four known brains from MPTP-intoxicated individuals which underwent neuropathological examination (4,16) there was a marked neuronal loss and gliosis limited to the pars compacta of the substantia nigra and minor changes in the locus coeruleus. The other pigmented nuclei did not show pathological changes nor could any compelling evidence of Lewy body in the diseased areas be found, and that in contrast to the situation encountered typically in PD. The fact that the dopaminergic system in MPTP-exposed individuals is affected has been further documented by using PET with [^{18}F]-fluorodopa to label the dopaminergic neurons *in vivo* (18). The study demonstrated that the striatal uptake of 6-fluorodopa was markedly reduced in all six MPTP-exposed individuals compared to normal controls (18). Subsequently, the pattern of striatal loss of [^{18}F]-fluorodopa uptake was compared

between nine MPTP-exposed individuals with parkinsonism and six PD patients (19). This study demonstrated that there was an equal degree of reduction of dopaminergic function in the caudate and putamen in the MPTP group, whereas there was the typical greater putaminal than caudate loss in the PD group (19). These findings suggest that while MPTP damages the nigrostriatal pathway, the injury may be too harsh or acute to elicit the PD-like putaminal/caudate gradient of loss. Consistent with this view is the work of Moratalla and collaborators (20) which demonstrates that the regional pattern of dopaminergic loss seen in the striatum of PD patients could be recapitulated in monkeys only if the intoxication was achieved with low doses of MPTP.

MPTP-induced parkinsonism in animals

Shortly after MPTP was discovered to provoke a parkinsonian syndrome in humans, researchers were prompted to test MPTP toxicity in a variety of animal species including monkeys, dogs, cats, and rodents (21,22). These investigations showed that most of the vertebrates and invertebrates tested were sensitive to the neurotoxic effects of MPTP (21,22), but with marked differences among species. Among mammals, monkeys, for example, were by far the most sensitive to MPTP whereas rats and guinea pigs were resistant and mice were of intermediate susceptibility.

Behavioral effects

All species of monkeys tested thus far (i.e. rhesus, squirrel, and cynomegalus monkeys, marmoset and baboons) exhibit the motor features of PD (23) with the exception of resting tremor. MPTP induces a resting tremor with a frequency of 4-5 hertz indistinguishable

from that of PD only in the African Green monkey (24), whereas mainly action and postural tremor were observed in other species. Levodopa and dopamine agonists alleviated the behavioral deficits induced by MPTP in all monkeys (25) and the chronic use of levodopa in these animals is, as in PD, accompanied with motor side effects such as dyskinesias (25). This fact has made the MPTP monkey the preclinical model *par excellence* to better understand the pathophysiology of levodopa-induced dyskinesia (26) and to develop effective pharmacological strategies to alleviate this disabling problem (27). In addition to monkeys, MPTP has been reported to also induce a state of paucity of spontaneous motor activity resembling bradykinesia in sheep (28), dog (29), and cat (30).

In mice the notion that MPTP administration induces behavioral manifestations reminiscent of PD is more controversial. Most studies claiming to see motor abnormalities in MPTP-intoxicated mice refer to reduced spontaneous motor activity and exploratory behavioral within the first days post-MPTP (31). From our experience, within the first 24-48 hours post-MPTP mice will often exhibit episodes of generalized shivering (or seizure) and unsteady gait, resembling cerebellar ataxia. During that time mice are usually markedly hypothermic and hypotensive, especially if they were injected with the typical high doses of MPTP. Again, in our hand, these arrays of motor abnormalities – abusively labeled PD-like symptoms – never responded to levodopa or apomorphine and disappear spontaneously (if the animal survives) in a few days even in the presence of more than 90% depletion in striatal dopamine. It is our experience that irrespectively of MPTP regimen, mouse strain, or degree of striatal dopamine depletion, MPTP-intoxicated mice appear normal, with respect to their spontaneous motor behavior, by 7 to 14 days after the last injection of the toxin. It has always been our assertion that the aforementioned acute behavioral abnormalities seen in mice injected with MPTP do not reflect dysfunction of the nigrostriatal dopaminergic system but rather a state of generalized toxicosis. Supporting this view are the observations of depressed cardiac contractility (32), damaged spleen

with defective immunoresponsiveness (33), and altered blood chemistry consistent with liver dysfunction (23) in animals soon after MPTP administration. It is thus advisable to remain highly circumspect about the PD relevance of such *acute* motor manifestations in MPTP animals. That said, some other studies, including some of our own, have evidenced motor abnormalities in MPTP mice by device such as rotarod more than seven days post-MPTP injections (34,35). Yet, these abnormalities, which can be reversed by levodopa administration, are often subtle and only observed in animals with extreme nigrostriatal damage. Perhaps behaviors that involve striatal function, such as habituation to a novel environment or the ability to learn a stimulus-response paradigm, may prove to be more fruitful and reliable for assessing the striatal dopaminergic function in MPTP mice.

Neurochemical effects of MPTP

In PD neurochemical alterations not only involve dopamine, but also other monoaminergic and non-monoaminergic systems including acetylcholine and several neuropeptides (36). Although following MPTP administration, changes in a numbers of neurotransmitter and neuromodulator systems have also been documented especially in monkeys, the most consistent and significant alterations occur in the dopaminergic systems (21). Overall, it is fair to say that among the different species sensitive to MPTP, regional biochemical changes were observed mainly in the caudate-putamen and the substantia nigra and, to variable extent, in the nucleus accumbens, the olfactory tubercle, the ventral tegmental area, the locus coeruleus and the hypothalamus (21). Inconsistently, monoaminergic changes were found in the cerebral cortex.

Acute increases in striatal levels of dopamine were observed in monkeys (37) and mice (38) with marked reduction in dopamine's main metabolites, homovanilic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) soon after the administration of the toxin. The observations that MPTP causes acute release of dopamine was subsequently

confirmed in live animals by microdialysis in rats (39). This massive release of dopamine seems to be mediated by MPTP's toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+), as pretreatment with monoamine oxidase-B (MAO-B) inhibitor preventing it. This acute effect also stimulated the release of norepinephrine and serotonin (37,38). Unexpectedly, the effect does not seem to be restricted to the monoaminergic systems, since acute rise in striatal acetylcholine has been also demonstrated in mice after MPTP administration (40). It is surprising that while this phenomenon has been recognized for so many years, so little is still known about the pathophysiology of this acute neurotransmitter response to MPTP.

Chronic effects of MPTP on monoaminergic systems have been more extensively studied. In the monkey, striatal dopamine and metabolite levels are dramatically reduced after 10 days and remain low even after one year (37,41-43). A similar situation has been reported in other animals species, although in both cat and mice some studies have documented striking recovery of striatal dopamine levels over time (44,45). In addition to caudate-putamen, MPTP-induced dopamine depletion occurs also in substantia nigra (46,47) and to a lesser extent in nucleus accumbens (46,48) and olfactory tubercle (46,48). Dopamine depletion has been occasionally found in regions such as the hypothalamus, frontal cortex or ventral tegmental area (49). Chronic effects on norepinephrine and serotonin are more controversial. Striatal norepinephrine levels have been reported to be reduced as much as dopamine at 5 weeks in mice (50). A different image is found in the cat where initially norepinephrine levels were dramatically reduced in the caudate-putamen and the nucleus accumbens, but after 5 months, striatal levels were increased while the levels in the accumbens remained down (30). In the monkey (21) and the dog (51) MPTP produces a chronic depletion of serotonin in the caudate-putamen, while no chronic effects on this monoamine have been reported in mice (44). It is interesting to note that an effect on the serotonergic system appears to parallel a species

susceptibility to MPTP. Increases in number of striatal dopamine D2 receptors (52,53) and in enkephalin mRNA (54) have also been observed in monkeys and in rodents after MPTP administration. Because these elements are located post-synaptically to dopaminergic cells, these changes can be explained by a secondary adaptative mechanism to the deficit in dopamine. No similar increases were seen in striatal dopamine D1 receptors (52,55,56), cholecystokinin receptors (55) or substance P concentrations (57), although these elements are also post-synaptically located. Finally, no consistent changes in glutamic acid decarboxylase or choline acetyltransferase activities or muscarinic receptor densities have been observed, suggesting that MPTP most likely does not affect GABAergic or cholinergic neurons (58,59).

Neuropathological effects of MPTP

In the MPTP-treated monkey, the major neuropathological finding is a profound loss of pigmented neurons within the substantia nigra (1,37,60) with some gliosis (17,61). The tyrosine hydroxylase (TH)-positive neurons of the substantia nigra are extensively destroyed or damaged, as demonstrated by immunohistochemistry (1,37,60). The pattern of substantia nigra cell death in MPTP-treated monkeys is similar to that following knife cuts in the nigrostriatal pathway. Cells in the centrolateral area of the pars compacta are damaged more extensively than those in the medial portion (62) which resembles the pattern seen in PD. In the mouse neuronal loss in the substantia nigra after MPTP injection is not uniformly obtained as it depends on the regimen used. Following the administration of four times 20 mg/kg of free base MPTP in one day at 2 hours apart, there is a massive loss of the nigral neurons. Degenerating neurons are already detectable by 12 hours post-MPTP, reach a maximum between 24 and 48 hours, and are no longer detected by five days (63). With this regimen, the morphology of dying neurons is non-apoptotic (63). Conversely, upon administration of 30 mg/kg once a day for five

consecutive days, not only is the degeneration of dopaminergic neurons more protracted as it is least for 21 days, but also the morphology is apoptotic (64). In rats, the systemic administration of MPTP is rarely used, and the vast majority of studies involve the stereotaxic infusion of MPP^+ (65-69). In contrast to the substantia nigra, there are no noticeable loss of intrinsic neurons in the caudate nucleus and putamen of MPTP-treated monkeys or mice, despite dramatic decrements in TH-activity (70,71), [^{3}H]-dopamine uptake capacity (72), and [^{3}H]-mazindol binding (73), all indicative of a loss of dopaminergic terminals. Other dopamine-containing neuronal nuclei, like the ventral tegmental area, which is adjacent to the substantia nigra, show limited and variable neuronal lesions. In young primates treated with MPTP there is no observable damage to this structure (37,58,60,74), while in the mouse, a small but definite neuronal loss was found after high dose MPTP (49,75). The locus coeruleus has also been the subject of several investigations because of its involvement in PD. Histological studies in monkeys sacrificed during the acute phase of MPTP intoxication demonstrated involvement of locus coeruleus, but in studies conducted in animals investigated six weeks after administration of the toxin, lesions of the locus coeruleus were found only in the oldest monkeys (5-20 years). In addition, a positive correlation between the dose of the toxin injected and the extent of the lesion was seen in the locus coeruleus in mice after administration of MPTP (49). The authors also identified substantial loss in TH-positive neurons in the hypothalamus of these animals. It is also worth noting that both in monkeys and in mice, the nigrostriatal lesion produced by MPTP is consistently detectable earlier and more profound at the level of the fiber terminals than over the cell bodies. In light of this finding several researcher have raised the possibility that the degenerative process in the MPTP model occurs, at least in part, by a dying–back process (43,76). The fact that an intrastriatal delivery of anti-oxidant mitigates the loss of nigral dopaminergic neurons in mice injected systemically with MPTP (76) provides strong supports for the concept that

damage to the terminals does indeed govern to some extent the fate of the intoxicated neurons. It is also important to mention that like in monkeys, the death of dopaminergic neurons in mice is accompanied with a robust glial response made of primarily astrocytes and microglia and whose time course parallels that of neuronal degeneration (77-81). As discussed below (see Secondary Events), this glial reaction appears to be more than a housekeeping event as it seems to play a real pathogenic role in the MPTP model.

Although its significance is unknown, Lewy body is another important feature of the pathology of PD. Eosinophilic intraneuronal inclusion bodies have been found in the brain of aged monkeys (15 to 20 years) after prolonged administration of MPTP (82). The inclusion bodies bear some resemblance to Lewy bodies at the light microscopic level, although they do not exhibit the dense central core that is typical of brainstem Lewy bodies under electron microscope examination. The authors of this description speculated that these MPTP-induced inclusions could represent a form of immature Lewy body. It is of interest to note that these eosinophilic inclusions have been identified in the medulla, nucleus basalis of Meynert, substantia nigra, locus coeruleus and dorsal raphe nucleus, areas in which typical Lewy bodies are found in PD. In only one report was shown the presence of Lewy body-like inclusions in MPTP monkeys containing alpha-synuclein (83). In mice intoxicated with MPTP, it seems that the formation of proteinaceous inclusions can only be elicited by administering the toxin for an extended period, which can be achieved by either serial subcutaneous injections of MPTP in association with probenecide (84) or by chronic infusion using osmotic minipumps (85).

Because of the close relationship between the rate of energy metabolism and local functional activity, it is of interest to report the results on cerebral glucose metabolism study performed in MPTP monkeys (86). The authors of this work have found significant reductions in glucose metabolism in areas of cell loss such as the substantia nigra and the ventral tegmental area. In contrast, they found significant increases in glucose metabolism

in the caudate-putamen, the external segment of the globus pallidus, the subthalamic nucleus, the ventral anterior nucleus of the thalamus and the premotor cortex, regions which are all involved in the production of movement or maintenance of posture.

THE TOXICOKINETICS OF MPTP

Systemic metabolism of MPTP and its penetration in the brain

Following its systemic administration, MPTP, which, as indicated by its octanol/water partition coefficient of 15.6 (87), is highly lipophilic, is able to readily permeate all lipid bilayer membranes and accumulates in virtually all organs. That said, not all organs are capable of either activating this pro-toxin into its actual toxic derivative (see below) or catabolizing it. For instance, organs such as the lung and the kidney seem to play a minor role in the conversion of MPTP into inactive metabolites (88). On the other hand, the biotransformation of MPTP by the liver enzymes like cytochrome *P*-450 and flavin mono-oxygenase (88) seem to be more instrumental in this regard as inhibition of either enzymes enhance the neurotoxicity of MPTP (88,89). Corroborating this view is the demonstration that the anti-convulsivants diphenylhydantoin and phenobarbital, which are known inducer of these liver enzymes mitigates MPTP-induced dopamine depletion in mice (90).

After a certain amount of injected MPTP has been catabolized by the liver and possibly by other organs, the remaining of this pro-toxin quickly gains access to the brain since it readily crosses the blood-brain barrier (Fig. 1). The rapidity upon which MPTP reaches the central nervous system is remarkable and Markey et al. (91) were the first to show that one minute after its intraperitoneal injection, MPTP can be measured in the brain of monkeys and mice by high-performance liquid chromatography (HPLC).

Bioactivation of MPTP by monoamine oxidase

Once in the brain parenchyma, MPTP is rapidly converted into its toxic metabolite MPP^+ by a two step process (Fig. 2). First, MPTP undergoes a two electron oxidation, catalyzed by MAO-B that yields the intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) (92). Second, the produced $MPDP^+$, which is an unstable molecule, readily undergoes spontaneous disproportionation to MPP^+ and MPTP (93,94) as depicted in Fig. 2.

Independent of the MPTP story, MPP^+, presented in the early 1970s as a powerful new herbicide (Cyperquat™; has never been commercialized), was recognized as a potent toxin. Thus, based on the fact that MPP^+, a major metabolite of MPTP and a known potent cytotoxin, was found in the brains of monkeys for several days after MPTP administration when MPTP itself was not longer detectable, led to the idea that MPP^+ or the process of MPP^+ formation had to be linked to MPTP-induced toxicity (91).

Crude mitochondrial preparations from rat brains have been shown to transform MPTP to MPP^+ (92). Since pargyline (a nonselective MAO inhibitor) and deprenyl (a selective MAO-B inhibitor) but not clorgyline (a selective MAO-A inhibitor) block the formation of MPP^+, MAO-B was implicated as the enzyme involved in the metabolism of MPTP. The importance of MAO-B-mediated bioactivation of MPP^+ in the toxicity of MPTP has been established by the demonstration that the inhibition of MAO-B by pretreatment with pargyline, deprenyl or other MAO-B inhibitors not only blocks the formation of MPP^+ but also prevents the dopamine depletion in the brains of mice (91,95) and protects against the development of MPTP-induced parkinsonism in monkeys (96). An important aspect of MPTP-induced toxicity is the demonstration that MAO-B is localized in non-dopaminergic cells, such as astrocytes and serotonergic neurons (97,98). Thus, it appears that MAO-B catalyses MPTP conversion to MPP^+ outside of dopaminergic neurons (Figure 1), a view which is consistent with the findings that animals with substantial loss in serotonergic neurons (99) or astroglial ablation (100) are more resistant to MPTP.

MAO-B has also been identified in blood vessel walls of some species. If MAO-B is present in sufficiently high enough concentrations in capillary or blood vessel walls outside the blood-brain-barrier, then the conversion of MPTP to MPP^+ at this site might limit entry of the toxin into the brain, as MPP^+ does not readily enter the brain. It was found that arterial walls accumulated [^{3}H]-MPTP as densely as did the caudate-putamen 24 hours after its intravenous injection to monkey (91). In other species, MAO-B may have a real protective effect as Harik and colleagues (101) attribute the resistance of rats to the toxic effects of the systemically injected MPTP to an enzymatic barrier resulting from high levels of MAO-B in the capillaries of rat brain. They found that MPTP oxidation by rat cerebral microvessels was 30-fold more rapid than by human microvessels; mouse microvessels were intermediate in their MPTP-oxidizing capacity.

Neuronal uptake of MPP^+

Thus far, the mechanism by which MPP^+ is released from serotonergic neurons and astrocytes remains unclear (Fig. 1). MPP^+ has an octanol/water partition coefficient of 0.09 (87), which indicates that, while being a lipophilic cation, it is far less lipophilic than MPTP. Thus, unlike MPTP, MPP^+ is not expected to easily diffuse across cellular lipid bilayer membranes, and there is no evidence that it simply leaks out by killing the cell within which it has been formed. Instead, it is believed that the release of MPP^+ from its intracellular sites of formation and entry into adjacent neurons depend on specialized carriers whose nature remains to be determined (Fig. 1).

A second important event, which in part may explain the selectivity of MPTP for dopaminergic neurons, is that MPP^+ is actively transported into the dopaminergic neurons by the dopamine uptake system (Fig. 1). Javitch et al. (102) have shown that the kinetic parameters for the uptake of MPP^+ into crude preparations of striatal synaptosomes from rats were similar to those for dopamine. These authors (102) as well as others (38,103) have demonstrated that the pretreatment of mice with dopamine uptake inhibitors

prevents MPTP-induced dopaminergic toxicity as does the genetic ablation of dopamine transporter (104). These findings indicate that the dopamine uptake system plays a critical role in MPTP dopaminergic neurotoxicity (Fig. 1). What remains unclear, however, is why other dopaminergic neurons such as the mesolimbic dopaminergic neurons with presumably a similar uptake system, are much less affected by MPTP than nigrostriatal dopaminergic neurons. Nor is it understood why noradrenergic and serotonergic neurons are only minimally or not at all affected by MPTP, even though MPP^+ has an equally high affinity for norepinephrine and serotonin uptake sites and is readily transported into these cells (105,106). As reviewed in reference (107), there are wide variations in the vulnerability of catecholamines in mice to MPTP toxicity. Striatal dopamine levels in C57BL/6 mice were found to be more affected by MPTP than were those of Swiss-Webster mice. Differences in vulnerability of striatal dopamine levels among Swiss-Webster mice obtained from different suppliers have also been reported. Age, route of MPTP administration, and gender have also been demonstrated as additional factors in determining the effect of MPTP in monkeys as well as in mice. Thus, while the uptake of MPP^+ into dopaminergic neurons appears to be necessary for MPTP toxicity, it does not fully explain the specificity for the dopaminergic neurons.

Mitochondrial and synaptic vesicle uptake of MPP^+

AS illustrated in Fig. 3, once inside neurons, MPP^+ rapidly accumulates in the mitochondrial matrix (108), not via a specific carrier but by being passively transported (109,110) by a mechanism relying on the mitochondrial transmembrane potential gradient ($\Delta\Psi$) of -150 to -170 mV (108-111). Like with other lipophilic cations , the higher the concentration of intramitochondrial MPP^+, the lower the $\Delta\Psi$ and, consequently, the slower the uptake of extramitochondrial MPP^+ (109,110). The demonstration that the ion-pairing agent tetraphenylboron anion increases both the rate and the extent of MPP^+

uptake in isolated mitochondria (112) further supports this concept. Remarkably, energized mitochondria incubated with 0.5 mM MPP^+ reach matrix concentrations of more than 24 mM after only 10 minutes (111). This fast and avid uptake suggests that most of the cytosolic MPP^+ would eventually accumulate in the mitochondrial matrix after the systemic injection of MPTP.

A shown in Fig. 3, MPP^+ can also bind to the vesicular monoamine transporters (VMAT), whereby it is translocated into synaptosomal vesicles (113). The vesicular accumulation of MPP^+ appears to protect cells from MPTP-induced neurodegeneration by sequestering the toxin and preventing it from accessing mitochondria, its likely main site of action (Fig. 3). The importance of the vesicular sequestration of MPP^+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells (113). Conversely, mutant mice heterozygous for a VMAT null mutation are significantly more sensitive to MPTP-induced dopaminergic neurotoxicity compared to their wild-type littermates (114). These findings indicate that there is an inverse relationship between the capacity to sequester MPP^+ in synaptic vesicles and the magnitude of MPTP neurotoxicity (Fig. 3).

Brain elimination of MPP^+ and role of neuromelanin

MPTP rapidly disappears from the mouse brain. Two hours after a single intraperitoneal injection of 10 mg/kg of MPTP, it can no longer be detected in brain extracts by HPLC, likely because it has been effectively converted into MPP^+. Interestingly, a similar situation is found for MPP^+ after ~8 hours (115), despite the fact that there is no evidence that MPP^+ is further metabolized. Of note, the half-life of MPP^+ in the monkey brain is much longer (± 48-100 hours), which is more than 10-times longer than the half-life determined in mice (~4 hours; (116)). This marked difference in the retention or the disposition of MPP^+ is most likely a major contributing factor in the large interspecies variation in MPTP

susceptibility. Regardless of the species differences of MPP^+ biodisposition, it remains enigmatic how this poorly lipophilic molecule exits the brain. Whether it is secreted into the CSF or succeeds to cross the blood-brain barrier with the help of an organic cation transporter is a possibility that needs to be investigated.

Neuromelanin is the pigment that is responsible for the dark color of the substantia nigra in primates and several other vertebrates and whose formation relies on the oxidation of dopamine and other catechols (117). MPP^+ was found to bind to neuromelanin with relative high affinity (*Kd* = 28-32 nM) (118), and thus it can be imagine that a slow release of MPP^+ into the cytoplasm from this intracellular depot might maintain toxic levels in the neuron sufficiently long enough to cause irreversible neuronal damage and death. This hypothesis is further supported by the observation that inhibition of the binding of MPP^+ to neuromelanin by the anti-malarial drug chloroquine partially protects monkeys against MPTP toxicity (119). No similar effect was seen in mice (119), which lack brain neuromelanin. The view that neuromelanin might be instrumental in MPTP-induced dopaminergic neurotoxicity has been challenged, however, by Herkenham et al. (43) who failed to find in monkeys any significant relationship between the content of neuromelanin in neurons, the amount MPP^+ accumulated in neurons, and their respective vulnerability.

HOW DOES MPTP KILL NEURONS?

The prevailing view of the macular basis of MPTP-induced dopaminergic neurotoxicity is that following MPTP administration and its accumulation in dopaminergic neurons, a cascade of multiple deleterious events is set in motion. Based on a large body of literature, those pathogenic events can be divided into early and late neuronal

perturbations and secondary alterations. Each of these, to a variable degree and at different stages of the degenerative process, participates in the ultimate demise of dopaminergic neurons.

Early Events

Energy crisis

As discussed above, soon after its entry into dopaminergic neurons, MPP^+ accumulates in the mitochondria (Fig. 3). Once in the mitochondria, MPP^+ impairs the oxidative phosphorylation by inhibiting NADH-ubiquinone reductase activity (120) which, as shown by photoaffinity labeling, is due to the binding of MPP^+ to the PSST subunit of complex I (121). In so doing MPP^+ interrupts the flow of electrons along the chain of cytochromes and provokes an acute failure in ATP formation. It appears, however, that complex I activity should be reduced by >70% to cause a significant depletion in ATP in non-synaptic mitochondria (122) and that, in contrast to *in vitro*, *in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (123). Still, it is likely that this transient perturbation does contribute to the cell death process since different strategies aimed at boosting the cell energy stores such as beta-hydroxybutyrate or creatine do attenuate MPTP-induced dopaminergic neurodegeneration in mice (34,124). Nonetheless, it is legitimate to wonder whether MPP^+-mediated ATP deficit is a sole factor underlying MPTP-induced dopaminergic neuronal death or rather one of the contributors to a multifactorial pathogenic cascade.

Oxidative stress

Consistent with this multifactorial concept is the fact that another consequence of complex I inhibition by MPP^+ is an increased production of reactive oxygen species

(ROS), especially of superoxide radical (125,126). It seems that early ROS production can also occur in the MPTP model from the autooxidation of dopamine resulting from an MPP^+-induced massive release of vesicular dopamine to the cytosol (127). The importance of MPP^+-related ROS production in the dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1), a key ROS scavenging enzyme, are significantly more resistant to MPTP-induced dopaminergic toxicity than their non-transgenic littermates (128). However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury. Instead, superoxide is believed to exert many or most of its toxic effects through the generation of secondary oxidants with much stronger reactivity such as hydroxyl radical, whose oxidative properties can ultimately kill cells. MPTP does stimulate the formation of hydroxyl radicals *in vivo*, as evidenced by the increase in the hydroxyl radical-dependent conversion of salicylate into 2,3- and 2,5-dihydroxybenzoates (129,130). However, there is no compelling evidence that the produced hydroxyl radical actually contributes to the oxidative stress in the MPTP model (131).

Superoxide can also react with nitric oxide (NO) to produce peroxynitrite, another potent oxidant. Supporting the relevance of this chemical reaction in the MPTP neurotoxic process is the demonstration that inhibition of neuronal NO synthase (nNOS) attenuates, in a dose-dependent fashion, MPTP-induced striatal dopaminergic loss in mice (129,132). Of note, nNOS has, thus far, not been identified inside dopaminergic neurons in rodents. Yet, dopaminergic structures are surrounded by nNOS-expressing fibers and cell bodies in the striatum, and, to a much lesser extent, in the substantia nigra pars compacta (133,134). NO is uncharged and lipophilic, hence it is able to travel away from its site of synthesis and inflict remote cellular damage without the need for any export mechanism. Because several lines of evidence (135) support the notion that

a molecule of NO can cover a distance many times greater than the diameter of a dopaminergic neuron, it can be speculated that the NO production involved in MPTP toxicity takes place in non-dopaminergic cells present in the vicinity of dopaminergic structures. As discussed elsewhere (135), under physiological conditions, while copious amounts of NO are likely constantly produced, concentration of superoxide is low, hence a minimal formation of peroxynitrite likely occurs. Conversely, in pathological situations as caused by MPTP, superoxide formation increases which consequently may lead to the generation of appreciable amounts of peroxynitrite. Supporting the participation of peroxynitrite in the MPTP model is the demonstration that MPTP significantly increases striatal levels of both free and protein-bound nitrotyrosine in mice (129,131), which is a marker of peroxynitrite damage on proteins. Aside from being a marker, nitrotyrosine exerts toxic effects in its own right as shown by the fact that stereotaxic injection of nitrotyrosine causes striatal neurodegeneration *in vivo* (136). Furthermore, TH (137), alpha-synuclein (138), and parkin (139) are all post-translationally damaged by a NO-based process.

DNA damage and PARP activation

Thus far, the lion's share of attention has been given to the effects on proteins of reactive species produced after MPTP administration. However, most of the reactive species, like peroxynitrite, that are implicated in the MPTP model can damage, through oxidative processes, many vital cellular elements other than proteins. Among these, DNA is of unique importance, because it is the repository for genetic information and is present in single copies. In light of the proposed oxidant species involved in MPTP neurotoxicity, all of the aforementioned DNA modifications can possibly occur in this model, as well as in PD. Consistent with this view is the quantitative PCR finding that MPTP administration to mice produced damage in both mitochondrial and nuclear DNA

of the substantia nigra, while there was no damage in either mitochondria or nuclei in the cerebellum, which was used as a negative control (140). Although all DNA modifications are potentially mutagenic and thus harmful, strand breakage is especially deleterious because of its link to poly(ADP-ribose) polymerase (PARP). The activation of PARP, by synthesizing poly(ADP-ribose) polymer, can rapidly deplete intracellular stores of NAD^+ which may impair glycolysis, mitochondrial electron transport chain activities, and, consequently, ATP formation. In the case of the MPTP model, the production of ATP in substantia nigra pars compacta dopaminergic neurons is already compromised due to the inhibition of the mitochondrial complex I by MPP^+ and thus activation of PARP may exacerbate the energy crisis. Corroborating the significance of PARP activation in the MPTP acute neurotoxic process *in vivo* is our demonstration that PARP is intensely activated following MPTP administration and that mutant mice deficient in PARP are more resistant to MPTP-induced dopaminergic neuronal death (141).

Late Events

Surprisingly, when one compared the time course of ATP depletion, ROS production, and PARP activation to that of SNpc dopaminergic neuronal loss, it clearly appears that the former alterations precede the actual neuronal demise (63). This suggests that the early alterations discussed above probably do not provoke the death of many dopaminergic neurons *per se*, but instead activate molecular pathways, which are the real executioners of the majority of dopaminergic neurons. Among the multiplicity of death-related cellular pathways, mounting evidence implicates the recruitment of the apoptotic molecular machinery in the demise of dopaminergic neurons after MPTP administration. As reviewed in (142), the pro-apoptotic protein Bax appears instrumental in this toxic model (143); in contrast, Bak, which cooperates with Bax to initiate apoptosis in response to activation of cell-surface death receptors, is dispensable in MPTP-

mediated neuronal death (144). Overexpression of the anti-apoptotic Bcl-2 also protects dopaminergic cells against MPTP-induced neurodegeneration (145,146). Similarly, adenovirus-mediated transgenic expression of the X-chromosome-linked inhibitor of apoptosis protein (XIAP), an inhibitor of executioner caspases such as caspase-3, also blocks the death of dopaminergic neurons in the substantia nigra pars compacta following the administration of MPTP (147,148). Additional caspases are also activated in MPTP-intoxicated mice such as caspase-8 (149), which is a proximal effector of the tumor necrosis factor receptor (TNFr) family death pathway. Interestingly, however, in the MPTP mouse model it is possible that caspase-8 activation is consequent to the recruitment of the mitochondrial-dependent apoptotic pathway and not, like in many other pathological settings, to the ligation of TNFr (150). Other observations supporting a role of apoptosis in the MPTP neurotoxic process include the demonstration of the resistance to MPTP of mutant mice deficient in p53 (151), a cell cycle control molecule involved in programmed cell death, mice with pharmacological or genetic inhibition of c-Jun *N*-terminal kinases (152-154) or mice which received a striatal adeno-associated virus vector delivery of an Apaf-1-dominant negative inhibitor (155). Collectively, these data show that during the degenerative process the apoptotic pathways are activated and contribute to the actual death of intoxicated neurons in the MPTP model.

Secondary events

The loss of dopaminergic neurons in the MPTP mouse model is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes and T-cell (156). In the MPTP mouse model, the astrocyte activation appears secondary to the death of neurons and not the reverse since the blockade of MPP^+ uptake into dopaminergic neurons prevents not only substantia nigra pars compacta dopaminergic neuronal death but also GFAP up-regulation (80). Yet,

activation of microglia, which is also quite strong in the MPTP mouse model (77,78), occurs earlier than that of astrocytes and, more importantly, reaches a maximum before the peak of dopaminergic neurodegeneration (157). In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the substantia nigra pars compacta clearly occurs within a timeframe allowing these glial cells to participate in the demise of dopaminergic neurons in the MPTP mouse model and possibly in PD. Activated microglial cells can produce a variety of noxious factors including ROS, reactive nitrogen species, pro-inflammatory cytokines and prostaglandins. Observations showing that blockade of microglial activation mitigates nigrostriatal damage caused by MPTP (158,159) supports the notion that microglia participate in MPTP-induced neurodegeneration. Among the specific factors that could mediate the deleterious actions of microglia on dopaminergic neurons, NADPH-oxidase and iNOS have emerged as potentially critical. Using mutant mice deficient in these respective enzymes, it was shown that both NADPH-oxidase (76) and iNOS (157) do contribute to MPTP-induced neurodegeneration. However, ablation of either of these enzymes did provide profound protection, thus suggesting that to abate microglial-mediated deleterious effects many factors must be targeted simultaneously. Previous studies have shown that the type of action mediated by neuroinflammation could be modified by vaccination. Based on this findings, it was recently demonstrate that adoptive transfer of copolymer-1 immune cells to MPTP recipient mice led to T-cell accumulation within the substantia nigra pars compacta, suppression of microglial activation, and increased local expression of glial-derived neurotrophic factor (GDNF) (33).

CONCLUSION

Despite some neuropathological shortcomings, the monkey MPTP model is the gold standard for the assessment of novel strategies and agents for treatment of PD symptoms. For example, electrophysiological studies of MPTP monkeys revealed that hyperactivity of the subthalamic nucleus is a key factor in the genesis of PD motor dysfunction (160). This seminal discovery led to the targeting of this structure using chronic high-frequency stimulation procedures (also called deep brain stimulation) to effectively ameliorate the motor function of PD patients whose symptoms cannot be further improved with medical therapy (161). In addition, MPTP-treated monkeys (162,163) were used to demonstrate that the delivery of GDNF both significantly limits MPTP-induced nigrostriatal dopaminergic neurodegeneration and can lead to behavioral recovery when given to previously lesioned animals (163). These studies form the basis for current attempts to use GDNF in PD patients (164). Because of practical considerations, MPTP monkeys have not generally been used to explore the molecular mechanisms of dopaminergic neurodegeneration; the MPTP mouse model is typically used for such studies. As summarized in this chapter, over the year a large number of cellular and molecular investigations have been performed in mice following the administration of MPTP and have led to a wealth of information regarding the mechanisms involved in the death of dopaminergic neurons. Because of the close similarity between PD and the MPTP model, it can be asserted that a cascade of deleterious events similar to that elucidated in animals intoxicated with MPTP underlies the death of dopaminergic neurons in PD.

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Legends

Fig. 1. Schematic Representation of MPTP Metabolism. After systemic administration, MPTP crosses the blood-brain barrier. Once in the brain, MPTP is converted to $MPDP^+$ by MAO-B within nondopaminergic cells, such as glial cells and serotonergic neurons (not shown), and then to MPP^+ by an unknown mechanism (?). Thereafter, MPP^+ is released, again by an unknown mechanism (?), into the extracellular space. MPP^+ is concentrated into dopaminergic neurons via the dopamine transporter (DAT). Source: Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron* 2003; 39:889-909, with permission.

Fig. 2. Proposed mechanism of biotransformation of MPTP in MPP^+. This reaction occurs in two phases. First, MPTP undergoes a two electron oxidation catalyzed by MAO-B that yields $MPDP^+$ (and its conjugated base MPDP). Then, the disproportionation of $MPDP^+$ proceeds through a reaction in which its conjugated base serves as a hybrid donor molecule and $MPDP^+$ as a hybrid acceptor molecule. Based on this event, the reduction of $MPTP^+$, which involves the transfer of one proton from its conjugated base, results in the formation of MPTP while the concomitant oxidation of $MPDP^+$ results in the formation of MPP^+. Modified from Peterson et al., J. Med. Chem. 28:1432-1436, 1985.

Fig. 3Z. Schematic Representation of MPP^+ Intracellular Pathways. Inside dopaminergic neurons, MPP^+ can follow one of three routes: (i) concentration into mitochondria through an active process (toxic); (ii) interaction with cytosolic enzymes (toxic); (iii) sequestration into synaptic vesicles via the vesicular monoamine transporters (VMAT; protective). Within the mitochondria, MPP^+ blocks complex I (X), which interrupts the transfer of electrons from complex I to ubiquinone (Q). This perturbation enhances the production of reactive oxygen species (not shown) and decreases the synthesis of ATP.

Source: Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron* 2003; 39:889-909, with permission.

Table 1 Signs of Parkinson's disease in MPTP patients

Features of idiopathic Parkinson's disease	Severity in untreated MPTP patients						
	1	2	3	4	5	6	7
Bradykinesia	+++	+++	+++	+++	+++	+	++
Rigidity	+++	++	+++	++	+++	+	++
Resting tremor	0	++	0	0	++	+++	+++
Flexion posture	++	++	+++	++	++	++	++
Loss postural reflexes	++	+	+++	++	+++	+	++
Loss of associated movements	+++	++	+++	++	+++	++	++
Shuffling, *petit pas* gait	++	++	+++	++	+++	+	++
En bloc turning	++	++	+++	++	+++	+	++
Difficulty initiating movements	++	++	+++	++	+++	+	++
Cogwheeling	++	++	+	+	+++	+++	+++
Loss of finger dexterity	++	++	+++	++	+++	++	++
Micrographia	++	++	+++	++	+++	+	+
Masked facies	+++	++	+++	++	+++	++	++
Reduced blink rate	+++	+++	+++	++	+++	++	++
Widened palpebral fissure	+++	+++	+++	++	+++	++	+
Limitation of upward gaze	++	+	++	++	0	0	0
Glabellar sign	+++	++	+++	++	+++	++	+
Hypophonia	+++	++	+++	++	+++	++	+
Drooling	++	++	+++	++	+++	++	+
Difficulty swallowing	++	++	+++	+	+++	++	0
Freezing	#	#	#	#	#	0	#
Kinesia paradoxical	#	#	#	#	#	0	0
Seborrhea	+	+++	+	+	+++	++	0
Diaphoresis	0	0	0	0	0	++	0
Hoehn & Yahr score*	V	V	V	IV	V	IV	IV

0, absent; +, mild; ++, moderate; +++, severe; #, present but not rated

*Score: IV = severe disability; still able to walk or stand unassisted.
V = wheelchair bound or bedridden unless aided.

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