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This four year grant proposal investigates the role of nitric oxide (NO) in dopaminergic (DA) neuron death in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's Disease (PD). The PI's work suggests that both superoxide radicals and nitric oxide (NO) contribute to MPTP toxicity. Using this mouse model of PD, **Specific Aim (SA) I** identifies which nitric oxide synthase (NOS) enzyme is the main culprit in the death of DA neurons in the substantia nigra of MPTP-treated mice. **SA II** (1) characterizes the upregulation of iNOS following MPTP in wild-type mice, and (2) assesses the effects of MPTP on the nigrostriatal DA pathway of iNOS.ko mice. **SA III** quantifies brain levels of dityrosine and nitrotyrosine, main markers of the deleterious effects of peroxynitrite on brain proteins while **SA-IV** assesses several candidate proteins and identifies others that are at risk of oxidative/nitrative attack following MPTP administration. All of our data confirm that both the superoxide radical and NO are indeed involved in the MPTP neurotoxic process in the SNpc of mice and point to peroxynitrite, the product of the superoxide radical/NO reaction, as the agent most likely damaging these DA neurons.

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INTRODUCTION

Parkinson's Disease is a common progressive neurodegenerative disorder characterized mainly by resting tremor, slowness of movement, rigidity and postural instability (1), all the result of a severe loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and a dramatic loss of dopaminergic fibres in the caudate-putamen (2). The prevelance of PD in North America alone is estimated at about 1,000,000 individuals with 50,000 newly diagnosed cases each year (1). The most potent treatment for PD still remains the administration of a precursor of DA, levodopa (L-DOPA), which replenishes the brain in DA, thus relieving almost all PD symptoms. However, the chronic administration of L-DOPA brings its own set of baggage as it often causes motor and psychiatric side effects which can be as debilitating as PD itself (3). Therefore, without undermining the importance of L-DOPA for the control of PD symptoms, it is urgent that we acquire a better and deeper understanding of the cause(s) of PD not only to prevent the disease but also to develop therapies aimed at halting the progression of the disease in those newly diagnosed patients who may not require the use of L-DOPA during the early phases of PD. Of the varied theories as to the causes of PD, the oxidative stress hypothesis is the most investigated theory. Consequently, of the various models used in PD research, the MPTP model is by far the model of choice to investigate the mechanisms involved in PD neurodegeneration (4). In human, non-human primates, and in various mammalian species, MPTP causes a severe parkinsonian syndrome that replicates almost all of the hallmarks of PD including tremor, rigidity, slowness of movement, postural instability and freezing. Both the responses and the complications to traditional PD medications are remarkably identical to those seen in PD. Mounting evidence such as the production of reactive oxygen species like the superoxide radical and nitric oxide (NO) following MPTP administration support this oxidative stress hypothesis. Using transgenic mice that overexpress human copper/zinc superoxide dismutase (SOD1), the enzyme responsible for ridding the cell of the superoxide radical we demonstrated previously that the superoxide radical is indeed involved in the MPTP neurotoxic process (5). The superoxide radical has also been shown to be increased in various stroke models and in other neurodegenerative situations. Beckman et al (6, 7) suggested that NO may be the other culprit involved in the oxidative stress hypothesis and that the superoxide radical and NO, only modestly reactive by themselves, react with each other to form the highly reactive, tissue damaging peroxynitrite, which severely damages DNA, proteins, polypeptides, monoamines and enzymes (8). It is the nitric oxide synthase (NOS) enzyme that produces NO. Three distinct isoforms of the NOS enzyme exist. Neuronal NOS (nNOS) is the principle NOS isoform in the brain and is constitively expressed throughout the central nervous system (9) whereas endothelial NOS (eNOS) is found mainly in the endothelial layer of blood vessels and in very low concentrations in the brain (9). The third isoform of NOS, inducible NOS (iNOS), is not expressed at all or only minimally expressed in the brain (9). iNOS expression in the brain has been shown to be increased in pathological conditions such as stroke, AIDS and amyotrophic lateral sclerosis (10-12). In fact, iNOS expression has been demonstrated in the substantia nigra pars compacta (SNpc) of post-mortem brain tissues from PD patients (13) indicating that an inflammatory response may be part of the progressive nature of PD. From previous experience, since we and others have found that nNOS knockout mice

are only partially protected against MPTP's toxic effects (14), we surmised that other NOS isoforms might indeed take part in MPTP-induced neurotoxicity. Therefore, based on the above information, it seemed germaine to our investigations into the causes of PD, to investigate the role of NO in the MPTP neurotoxic process.

STATEMENT OF WORK

Our overall long-term goal is the study of the pathogenesis of PD. To accomplish this, work in this project centers around the roles of the superoxide radical and NO in the MPTP neurotoxic process. The basis of our work is the oxidative stress hypothesis of PD which supports free radical involvement in the generation and progression of this debilitating disorder. Both the superoxide radical and NO are proposed as contributors to DA neuron death here, however, each is only modestly reactive, but can combine to produce peroxynitrite, which damages proteins, DNA, polypeptides, enzymes and monoamines. Therefore, the overall objective of this project is to better understand the actual cascade of events that take place within the DA neuron following MPTP administration and which are ultimately responsible for the death of said neurons.

Research Plan

Specific Aim I: Determine the contribution of superoxide, NO or both to MPTP neurotoxicity by administering MPTP to different lines of mice which are genetically engineered to enhibit a greater capacity for detoxifying superoxide (transgenic copper/zinc superoxide dismutase {SOD1} mice) and/or a lower neuronal capacity for synthesizing NO (neuronal NO synthase {nNOS} knockout mice) and by assessing the status of the nigrostriatal DA pathway in these different types of animals following MPTP administration using high performance liquid chromatography (HPLC) and immunostaining with quantitative morphology.

Specific AimII: Determine the contribution of inducible NOS (iNOS) to MPTP neurotoxicity by assessing iNOS protein expression and enzyme activity in different brain regions, at different time points and at different toxin concentrations in wild-type mice following MPTP administration. MPTP will also be administered to mice deficient in iNOS activity the status of their nigrostriatal DA pathway will be assessed by HPLC and immunostaining with quantitative morphology.

Specific Aim III : Assess peroxynitrite effects on protein tyrosine residues following MPTP administration by quantifying the two main products of peroxynitrite oxidation of tyrosine, dityrosine and nitrotyrosine by gas chromatography with mass spectrometry. Quantification will be performed in different brain regions, at different time points and at different toxin concentrations in wild-type mice and in transgenic SOD1, iNOS and nNOS knockout mice.

Specific Aim IV: Examine the potential biological consequences of protein tyrosine nitration by assessing whether candidate proteins, mitochondrial electron transport chain polypeptides and manganese superoxide dismutase (MnSOD) are nitrated. This will be tested in PC-12 cells after exposure to different concentrations of and lengths of time of peroxynitrate and MPTP's active metabolite, MPP+ as well as in wild-type and in transgenic SODI, iNOS and nNOS knockout mice after MPTP administration. Tyrosine nitration will be ascertained by immunoprecipitation, Western blot analysis and amino

acid analysis. The catalytic activity of these enzymes in both PC-12 cells and in mice experimemts will be done using spectrophotometric enzymatic assays.

RESEARCH ACCOMPLISHMENTS

Year One of the Award.

During year I of the award, we addressed Specific Aim II which was to determine the source of NO in the SNpc following MPTP administration. Our major findings here were that **(1) MPTP produces a robust glial response**. To demonstrate this robust glial response, we used the macrophage antigen-1 (MAC-1) and glial fibrillary acidic protein (GFAP) as markers to gauge the responses of microglia and astrocytes, respectively in the SNpc of C57/bl mice following MPTP administration. In saline-treated mice, only faint immunostaining for both MAC-1 and GFAP was observed. In the MPTP-treated animals, however, a strong glial response was observed. Alterations in MAC-1 were evident as early as 12 hours after the last dose of our acute MPTP regimen (18 mg/kg x 4 doses over 8 hours), peaked between 24 and 48 hours and was no different from saline-treated mice at 7 days after MPTP administration. Conversely, GFAP changes were noted at 24 hours, reached maximum increases between 4 and 7 days and remained above control even at 21 days after MPTP. Striatal responses for both antibodies were similar to those of the SNpc. We noted that **(2) MPTP stimulates iNOS expression in glial cells.** In saline-treated mice, whereas the number of iNOS-positive cells in the SNpc were rare to non-existent, iNOS-positive cell numbers increased to 259% by 24 hours after MPTP treatment and returned to control levels by 48 hours. Simultaneous staining techniques for iNOS and MAC-1 or GFAP were used to determine the nature of these iNOS-positive cells. Twenty-four hours after the last dose of MPTP at a time when the number of iNOS-positive cells reached their peak in the SNpc, MAC-1-positive activated microglia exhibited iNOS mmunoreactivity. No iNOS-positive staining was found in GFAP-positive cells nor were they found in the striatum. Also noted was the fact that **(3) iNOS mRNA levels and enzymatic activity increased dramatically after the acute regimen of MPTP administration.** Here, saline-treated ventral midbrain, which contains the SNpc, showed very little iNOS mRNA, whereas iNOS mRNA levels in ventral midbrain from MPTP-injected mice were detected as early as 12 hours ,reached maximum levels by 48 hours and was undetectable at 4 days after our acute regimen of MPTP administration. Striatal mRNA levels were low throughout the entire time course study. In agreement with mRNA levels, iNOS enzyme activity increases were evident as early as 12 hours after MPTP injections and peaked at 48 hours then slowly returned to control activity by 7 days. iNOS striatal enzymatic activity like iNOS mRNA levels was unaffected by MPTP throughout the entire time course. In this study, nNOS enzyme activity was consistently higher than iNOS enzymatic activity as well as unchanged following MPTP administration. Since MPTP does increase iNOS expression and does up-regulate iNOS mRNA levels in normal wild-type mice after MPTP, absolute proof that iNOS is indeed the primary source of NO in the MPTP neurotoxic process was obtained using mice deficient in the iNOS enzyme. Administering the same regimen of MPTP to iNOS knockout mice and examining the same time points, we found that while 29% of the tyrosine hydroxylase (TH)-positive neurons and 46% of the Nissl-stained SNpc survived the toxic assault of MPTP in wild-type mice, about twice as many TH-positive and Nissl-stained neurons in iNOS deficient mice survived the MPTP onslaught

indicating that **(4) iNOS is indeed the source of MPTP-induced NO production.** Interestingly, striatal fibres in the iNOS deficient mice exhibited the same level of loss as the MPTP-treated wild-type littermates. To make sure that the decreased loss of TH-positive neurons in the iNOS deficient mice was not related to alterations in MPTP uptake and metabolism due to the lack of the iNOS gene, we measured striatal MPTP and MPP+ levels in our iNOS knockout mice compared to their wild-type littermates. We observed **(5) no differences in MPTP and MPP+ levels in striata were noted between these two groups of mice.** Finally, it is known that NO can damage DNA and nitrate the tyrosine residues in the phenolic rings of proteins and that nitrotyrosine (NT) is the indicator that NO has indeed reacted with the tyrosine residues of these proteins. In MPTP-treated wild-type littermates following MPTP, a significant presence of NT was noted in striatum and ventral midbrain. In contrast, in the iNOS deficient mice, whereas NT did increase, these increases were significantly less than those observed in the wild-type littermates. Thus, **(6) iNOS deficient mice have much less NT following our acute regimen of MPTP administration.** This entire work was published in the journal Nature Medicine in 1999 (Volume 5 (12), pp. 1403-1409 (see attached publication).

During the first year of this award, we also addressed the question put forth in Specific Aim III, that of assessing peroxynitrite effects on protein tyrosine residues following MPTP administration by quantifying the two main products of peroxynitrite oxidation of tyrosine, dityrosine and nitrotyrosine using gas chromatography with mass spectrometry. Analyses of oxidized amino acids were performed on freshly isolated tissues from ventral midbrain which contains the SNpc, striatum, cerebellum and frontal cortex and the compounds of interest had retention times that were identical to authentic 3-nitrotyrosine (3-NT), ortho-tyrosine and o,o-dityrosine. In these studies we found that **(1) 3-NT was elevated in ventral midbrain and striatum of mice as early as 24 hours after MPTP treatment.** Levels of 3-NT in ventral midbrain (+110%) and striatum (+90%) were markedly elevated following MPTP administration compared to saline-injected controls. It was noted that the observed increases were selective for regions of the brain that are susceptible to the neurotoxic effects of MPTP. Those regions that were not damaged by MPTP, cerebellum and frontal cortex, showed no changes in 3-NT levels. That **(2) o,o-dityrosine was elevated in ventral midbrain (+120%) and striatum (+170%) 24 hours after our acute regimen of MPTP administration was also noted.** These results were strikingly similar to the increases found in 3-NT and were found only in those regions of the brain that were affected by a toxic insult from MPTP. In contrast to the changes in 3-NT and o,o-dityrosine found in ventral midbrain and striatum of these MPTP-treated mice, **(3) no changes in ortho-tyrosine were observed in any of the analyzed brain regions.** Since our theory is that the observed altered proteins were the result of exposure to peroxynitrite, we exposed, *in vitro*, homogenates prepared from ventral midbrain, striatum, cerebellum and frontal cortex to peroxynitrite, tyrosyl radical and the hydroxyl radical ($HO^{\cdot}$). **(4) Peroxynitrite exposure of the brain proteins for, 30 minuets caused both significant and similar increases in 3-NT (80 fold) in all brain regions** indicating that peroxynitrite does indeed damage brain proteins. Ortho-tyrosine and o,o-dityrosine levels, however, increased only 2-3 fold. To generate the tyrosyl radical, we used an *in vitro* myeloperoxidase-tyrosine-H_2O_2 generation system, then exposed the various brain region homogenates to the generated tyrosyl radical. **(5) The**

major product of this reaction was o,o-dityrosine and all homogenates from the different brain regions exhibited similar increases in this compound. There were no changes in 3-NT or in ortho-tyrosine in any region. Recent evidence suggests that the myeloperoxidase-H_2O_2 system will convert tyrosine into 3-NT in a reaction that requires nitrite, a degradation product of NO. Addition of nitrite to this system caused the appearance of 3-NT levels that were similar to those 3-NT levels found in mice following MPTP administration. To complete our studies here, we exposed our brain region homogenates to a $HO^{\cdot}$-generating system that contained copper and H_2O_2. In this system, **(6) while levels of 3-NT remained unchanged, levels of ortho-tyrosine and o,o-dityrosine increased significantly with ortho-tyrosine levels being 10-fold higher than those of o,o-dityrosine.** All three compounds can be considered markers of proteins damaged by exposure to NO in its various forms. This study was published in 1999 in the Journal of Biological Chemistry, Volume 274, pp. 34621-34628.

Year Two of the Award

During year II of this award, since endothelial NOS (eNOS) is an isoform of the NOS enzyme, as part of Specific Aim I, we assessed the contribution of this isoform of NOS to the production of NO in the MPTP neurotoxic process. Our reasoning was that since the single main determining factor of the MPTP neurotoxic process is its conversion to MPP+ in glial cells in the brain, the absence of the eNOS gene could affect striatal blood flow thus compromising striatal MPP+ levels and the MPTP neurotoxic insult itself. For these studies, eNOS deficient mice and in C57/bl mice from Charles River Laboratories were given our acute regimen of MPTP (18mg/kg, i. p.) or saline at 2 h intervals and sacrificed at selected time points after the last injection. Brains were quickly removed and striatum, ventral midbrain, frontal cortex and cerebellum were dissected out, frozen on dry ice and stored at -80°C for Western blot analyses and monoamine and striatal MPP+ levels. Mice treated similarly were also sacrificed for fresh-frozen and perfused brains. **1) Analysis of striata from eNOS deficient mice and their wild-type littermates revealed no significant differences in MPP+ levels between the two groups of mice.** Examination of ventral midbrain tissue optical density for Western blot analyses from eNOS deficient control, saline and MPTP-treated mice **2) showed a specific band at 135 kDa indicating eNOS expression and that at no time during the time course study (0, 1, 2, 4, 7 days after MPTP administration) did MPTP alter eNOS protein expression levels.** Total mRNA was extracted from ventral midbrain from saline and MPTP-injected mice at the same time points as before for RT-PCR amplification and quantification of eNOS and GAPDH. Following amplification, electrophoresis, and exposure to radioactivity, quantification by optical density **3) showed that the expression of eNOS mRNA remained unchanged throughout the entire MPTP time course.** To determine whether any change in eNOS immunostaining occurred in the SNPc, we performed immunohistochemistry for eNOS. **4) In saline-injected mice, there was a dense network of eNOS positive blood vessels. Positive immunostaining showed a homogenious distribution of blood vessels of varying sizes over the entire midbrain. No alternation in eNOS intensity of staining was seen in the midbrain after MPTP intoxication. In striatal sections, eNOS immunostaining was not as intense as in the midbrain. Also, no differences in eNOS immunoreactivity were noted among saline-injected and MPTP-treated mice at any timepoint. Results for**

NADPH histochemistry were similar to eNOS immunohistochemistry. In any experiment involving MPTP, it is necessary to count the total number of TH-positive neurons that remain following the MPTP toxic insult. In this case, the total numbers of TH-positive and Nissl-stained neurons in the SNpc were counted using stereology. TH- and Nissl-stained neurons were counted in the right SNpc of every fourth section throughout the entire extent of the SNpc. **5) In wild-type and eNOS deficient mice, there was a large number of TH- positive cell bodies intermingled with a dense network of TH-positive fibers within the SNpc and there was no significant difference in the number of neurons between the two groups of saline-injected controls. In wild-type mice, 37 % of the SNpc neurons and 62% of the Nissl-stained SNpc neurons survived at 7 days after the 18 mg/kg acute MPTP regimen; the loss of both TH and Nissl-stained neurons in the eNOS deficient group was not statistically different from the wild-type group following MPTP dministration.** Thus, our conclusion from these experiments was that while nNOS and iNOS both played a role in the MPTP neurotoxic process, eNOS has no such role.

Since we have demonstrated that nNOS and iNOS are both involved in the MPTP neurotoxic process, that we can partially block the NOS enzyme with 7-nitroindazole, and that iNOS is the principal here, a logical extension of Specific Aim II would be the pharmacological blockade of iNOS upregulation. For this, we used minocycline a second-generation semi-synthetic tetracycline antibiotic, that is a potent inhibitor of microglial activation independent of any anti-microbial action. Its effectiveness as a neuroprotective agent was demonstrated againat experimental brain ischaemia and disease progression in the R6/2 mouse model of Huntington's disease. In the MPTP mouse model pf PD, minocycline **(1) attenuated MPTP-induced SNpc dopaminergic neurodegeneration.** Varying doses of minocycline 1.4-45 mg/kg x 2 daily) effectively increased significantly the number of surviving TH-positive neurons in the SNpc of mice given our acute regimen of MPTP (18 mg/kg x 4 doses over 8 hours). This protection was dose dependent in that while neuroprotection was not seen with 1.4 mg x 2 daily dosing, slight neuroprotection was seen with 5.625 mg/kg x 2 daily dosing and maximal protection (50%) occurred at 11.25 mg/kg x 2 daily and higher. Sparing of SNpc dopaminergic neurons does not always correlate with the sparing of their corresponding fibres which are essential for maintaining dopaminergic neurotransmission. Thus, we examined the striatal fibres for a neuroprotective effect using both the 18mg/kg and the 16 mg/kg acute dosing regimen dosage of MPTP and the varying doses of minocycline. **We found that whereas minocycline offered striatal fibres no protection against the higher dose of MPTP, it did protect these fibres against the 16 mg/kg dose of MPTP.** A significant part of the MPTP neurotoxic process is mediated by NO-related oxidative damage the extent of which can be evaluated by assessing nitrityrosine formation. As before, we found that nitrotyrosine levels were significantly increased in ventral midbrain following MPTP administration. We also noted that these increases in nitrotyrosine levels were significantly smaller when minocycline in the presence of minocycline. Thus, **2) in the presence of minocycline, MPTP-induced nitrotyrosine level increases were significantly smaller than in the non-minocycline-treated mice.** One can not establish with certainty that a compound is neuroprotective unless one demonstrates that the metabolism of the offending agent is not interfered with at any time along its metabolic

pathway. In comparing striata from MPTP only with MPTP-minocycline treated mice, **3) 90 mins after MPTP administration, results showed that striatal levels of MPP+ were not different between these two groups of mice.** In experimental brain ischaemia, the neuroprotective effect of minocycline is reported to be due to its inhibition of microglial activation and proliferation. Part of the MPTP neurotoxic insult is a microglial response. Thus, we examined whether the neuroprotection afforded to SNpc dopaminergic neurons in the MPTP mouse model by minocycline is due to its inhibition of microglia activation. Mice were treated as per usual with the 18 mg/kg acute regimen of MPTP only or in combination with minocycline (45 mg/kg x 2 daily). As in previous experiments, MPTP alone elicited a robust microglial activation and a significant GFAP upregulation in the ventral midbrain 24 hours after the last injection. In mice treated with the minocycline-MPTP combination, **4) although GFAP mRNA and immunostaining in the ventral midbrain and striatum remained as high and as intense as the MPTP only group, ventral midbrain MAC-1 immunostaining was similar to saline injected control mice.** Since minocycline attenuated the MPTP-induced microglial activation, we theorized that it should attenuate the production of some of the noxious mediators known to result from microglial activation. We found **5) that the pro-inflammatory cytokine interleukin-1ßeta (IL-1ß) was indeed increased significantly following MPTP administration and that minocycline (45mg/kg x 2 daily) sigmificantly reduced this increase. Furthermore, the MPTP-induced upregulation of iNOS and NADPH oxidase, two prominent enzymes found in activated microglia that produce NO and reactive oxygen species (ROS), was completely abolished in the presence of minocycline.** NO and ROS are both products of MPTP intoxication and can react with each other to produce peroxynitrite which is known to damage proteins, DNA amono acids and even monoamines. The goals of Specific Aim IV were to examine the biological consequences of protein nitration by assessing whether candidate proteins such as MnSOD and mitochondrial electron chain polypeptides as well as any other proteins might be nitrated following MPTP intoxication. Using cell culture involving HEK293 cells transfected to overexpress the human alpha-synuclein presynaptic protein, we found that **6) following exposure to peroxynitrite, the alpha-synuclein in these cells was nitrated as demonstrated by immunoprecipitation techniques.** This situation was replicated in vivo in the MPTP mouse model as we saw **(7) nitration of alpha synuclein as early as 4 hours after the last dose of MPTP. No nitration of other presynaptic proteins such as ß-synuclein and synaptophysin was noted.**

Year Three and Year Four of the Award.

All of the work in wild-type C57/bl mice has been completed. The remainder of the proposed studies uses transgenic mice overexpressing wild-type SOD1, nNOS and iNOS knockout (ko) mice, and crosses between SOD1 and nNOS.ko and SOD1 and iNOS.ko mice. Since we have demonstrated that both the superoxide radical and NO are involved in the MPTP neurotoxic process, we now wished to dampen the effects of the MPTP toxic insult. The first part of our studies were done using wild-type C57/bl mice, thus, we transferred the SOD1 transgene into a C57/bl genetic background by applying the backcross system between hemizygote transgenic SOD1 mice and wild-type C57/bl mice at least seven times to assure that almost all of the alleles from the original strain were replaced followed by brother-sister matings. This breeding system took close to one year

and these animals are viable. Roughly 50% of each litter overexpress the SOD1 human transgene.

The breeding of nNOS knockout mice was not as straightforward as we thought it would be. These mice were originally in a mixed 129 SvEv agouti -C57/bl background thus we first had to transfer the nNOS.ko gene into a C57/bl background using the backcross and brother-sister mating system. We bred the few homozygote nNOS.ko mice that we had with C57/bl females (Jackson Labs) to obtain nNOS heterozygotes. Brother-sister mating of the resulting heterozygotes was then done to obtain nNOS.ko mice. What we found was that this crossing of heterozygote x heterozygote did not respect Mendalian genetics (25/50/25)' Although they were viable, from our breeding program, we were lucky if one mouse per litter was a nNOS.ko mouse. Thus acquiring the numbers needed was not so fruitful. After a number of failed attempts, we decided to try and buy these animals ($338.00 per breeding pair) from Jackson Labs. We spent a number of months going back and forth with them only to be informed that they were having problems with their colonies of nNOS.ko mice and that we would have to wait until they solved their problems with this colony. In April of 2003, Jackson Labs informed us that they had cryopreserved this line of mice. We assumed that this cryopreservation occurred because they did not solve their colony problems. We thus have looked to other investigators who may have these mice and who were maybe more successful in their breeding programs. This approach has been partially successful. Attempts to crossbred SOD1 with nNOS.ko was also a problem in that this $SOD1^{+}/nNOS^{-/-}$ cross was indeed a rarity per litter.

The breeding of iNOS ko mice proved to be almost as problematic as nNOS.ko mice. Litter size was usually 8-10 pups however, mothers in many cases ate their young or did not care for them. Thus, a significant number of iNOS pups died before they reached weaning age. Jackson Labs carries this line of mice also, so we decided to purchase adult iNOS.ko mice from this vender. Production of these mice, already in a C57/bl background, at Jackson Labs is, however, intermittent and one is allowed to purchase only 10 iNOS.ko mice at any one time making it difficult to obtain sufficient numbers for any one experiment. Crosses with SOD1 were problematic as well yielding maybe 1 to none $SOD1^{+}/iNOS^{-/-}$ mouse per litter. While we were conducting the breeding program, we published several articles in peer-reviewed journals relevant to the subject of the role of NO in MPTP neurotoxicity.

KEY ACCOMPLISHMENTS RESULTING FROM THIS 4 YEAR AWARD.

Specific Aim I

Both the iNOS and nNOS enzymes are involved in the MPTP neurotoxic process. The SNpc of both iNOS and nNOS mknockout mice is only partially protected against the damaging effects of MPTP.

eNOS is expressed in blood vessels in the brain, but is not involved in MPTP neurotoxicity in the SNpc as (1) content and peak time of MPP+ levels show no differences between wild-type and eNOS deficient mice; (2) mRNA and protein levels of the eNOS gene are unchanged in the ventral midbrain following MPTP treatment in eNOS deficient mice; (3) the loss of both TH.- and Nissl-stained

neurons between wild-type and eNOS deficient mice were not statistically different following MPTP administration.

Specific Aim II

iNOS seems to be the main NOS enzyme and the main producer of the NO involved in the MPTP neurotoxicity and degeneration of DA neurons ; it is up-regulated in microglia in the Snpc of MPTP-treated mice. iNOS mRNA is also up-regulated here.

MPTP-induced toxicity in the SNpc can be attenuated with minocycline, a second generation semisynthetic tetracycline antibiotic.

Minocycline inhibits the inflammatory response in the SNpc induced by MPTP and characterized by microglial activation.

Minocycline prevents three key microglial-derived mediators of cytotoxicity following MPTPadministration: iNOS upregulation, formation of mature IL-1ß and activation of NADPH oxidase.

NADPH oxidase is induced in the SNpc of MPTP-treated mice, is expressed in activated microglia and is the source of the superoxide radical in MPTP-mediated microglial activation. Subunits of NADPH oxidase such as GP^{91} is also up-regulated in the SNpc in PD and following MPTP; inactivation of NADPH attenuates the MPTP effect by mitigating inflammation..

The cyclooxygenase 2 (COX-2) enzyme expression is induced within SNpc DA neurons in postmortem PD samples and in the SNpc of MPTP-treated mice during their degenerative process. This up-regulation of the COX-2 enzyme occurs through a c-jun kinase (JNK)/ c-jun dependent mechanism. Both ablation and inhibition of COX-2 attenuates MPTP-induced DA neuron degeneration in the SNpc of mice possibly by decreasing the level of damage.

Specific Aim III

Documentation of the existence of peroxynitrite and the regional quantification of protein oxidation markers 3-nitrotyrosine, o,o-dityrosine and orthotyrosine in the MPTP mouse model of Parkinson's disease. This suggests tyrosyl radical and peroxynitrite involvement and may be mediated through the myeloperoxidase heme protein secreted by activated phagocytes.

Demonstration that tyrosine hydroxylase is nitrated in the SNpc and inactivated following MPTP administration.

Specific Aim IV

Examination and demonstration of the inactivation of tyrosine hydroxylase as a result of the nitration of tyrosine residues in HEK293 cells exposed to peroxynitrite.

Demonstrated the up-regulation, nitration and oxidative modification of alpha synuclein protein in neurons in the SNpc of MPTP-treated mice. All synuclein-positive neurons were also TH-positive

Showed that the blockade of the complex I enzyme by MPTP, which increases the production of the superoxide radical, can be overcome by infusion of the ketone body D-ß-hydroxybutyrate (D-ßHB) which enhances the functional operation of complex II thus partially protecting against MPTP-induced DA neuron degeneration, improves mitochondrial respiration and increases ATP production. This suggests that the complex I enzyme, a possible candidate protein for interaction with NO, may be nitrated thus prevented from passing electrons down the mitochondrial chain.

REPORTED OUTCOMES FROM THIS 4 YEAR AWARD.

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SHORTFALLS AND RECOVERY

The work in the genetically engineered mice has not been completed. In fact, because of the breeding problems, we have been forced to investigate other avenues, be it mammalian or pharmacological, to make up for these shortfalls. While SOD1 overexpressers pose no problem, nNOS and iNOS do. We have, however, been promised a limited number of nNOS.ko mice and we can purchase limited numbers of iNOS.ko at

any one time. Thus, to examine our original question on the role of NO in MPTP-induced dopaminergic neuron degeneration for **Specific Aim I**, we will administer a SOD-minetic compound (M40401) to nNOS and iNOS knockout mice with a lower capacity for synthesizing NO and to wild-type mice (all in a C57/bl Jackson Labs background). We will then assess the status of the nigrostriatal dopaminergic pathway in these diffenent lines of mice following MPTP and in the absence of and in the presence of M40401 using high performance liquid chromatography (HPLC) and immunostaining with quantitative morphology. Because of the limited numbers of both iNOS and nNOS knockout mice, we will use ½ of the brain for HPLC and the other ½ for morphological studies. To test the feasibility of this approach, we have experimented with this type of protocol for samplings in normal mice and have found no differences between using whole brain versus using ½ brain. For **Specefic Aim III**, we will assess peroxynitrite effects on protein tyrosine residues in iNOS and nNOS knockout mice and in wild-type C57/bl mice following MPTP and MPTP in combination with the SOD mimetic M40401. We will quantify the two main products of peroxynitrite oxidation of tyrosine, dityrosine and nitrotyrosine in different brain regions (striatum, ventral midbrain, frontal cortex, berebellum) and at different time points using gas chromatography with mass spectrometry. For **Specific Aim IV**, we will examine the consequences of protein tyrosine nitration by assessing whether candidate proteins, mitochondrial electron transport chain polypeptides and manganese-SOD are nitrated. This will be done in transgenic SOD1 mice as well as in nNOS and iNOS knockout mice following MPTP with and without M40401. Tyrosine nitration will be determined by immunoprecipitation, Western blot and amino acid analyses. Any enzymatic analyses will be performed using spectrophotometric enzymatic assays. Aside from manganese SOD, we have identified complexes I, II, and V, ANT, VDAC, creatine kinase, and aconitase, all found in mitochondria, as possible candidate proteins that may be nitrated or oxidized following reaction with peroxynitrite. Because of the breeding problems even though the crosses were viable, we were granted a non-monetary one year extension on this proposal to complete the studies.

CONCLUSION

Parkinson's disease is a common neurodegenerative disorder that affects a reasonable percentage of our aging population (1). Possessing a tool like MPTP that can replicate almost all of the hallmarks of this disorder has indeed been a gift. Finding the cause of this debilitating disease and elucidating the molecular mechanisms that are involved in the death of dopaminergic (DA) neurons in the SNpc can give us specific targets for therapeutic strategies aimed at abating DA cell loss and the results of this loss. In keeping with our original hypothesis of free radical participation in the death of these specific neurons (8), using the MPTP mouse model of PD, we have found that both the superoxide radical and NO are indeed involved in the death of DA neurons in the SNpc of MPTP-treated mice. In a previous work, we used transgenic mice that overexpressed wild-type SOD1 and found that the nigrostriatal DA pathway in these mice was protected from the damaging effects of MPTP compared to their non-transgenic littermates (4). In elucidating the role of NO in the MPTP-neurotoxic process, it is necessary to sort out which of the three nitric oxide synthase (NOS) isoenzymes actually contribute to the MPTP neurotoxic process in DA neurons in the SNpc. Our research shows that of the

three, nNOS (NOS1) and iNOS (NOS2) are involved here (14, 15), but eNOS (NOS3) is not (personal communication). nNOS (14) and iNOS (15) knockout mice were partially protected against MPTP-induced DA cell loss individually whereas eNOS knockout mice treated with MPTP exhibited the same level of DA neuron death as that displayed by their wild-type littermates. Thus, targeting of either NOS1 or NOS2 or both can interfere with the MPTP neurotoxic process which, in turn, may save DA neurons in the SNpc of the MPTP mice and may be a useful strategy for treating the progressive nature of neuronal loss in the SNpc of PD patients.

Although both nNOS and iNOS are up-regulated, we have demonstrated that the NOS2 enzyme in the SNpc seems to be the main NOS culprit in PD and in the MPTP mouse model and this culprit comes with heavy baggage. First of all, as stated above, MPTP induces an increased expression of the NOS2 enxyme in the SNpc of the treated mice; up-regulation of NOS 2 has also been found in postmortem tissue samples from PD brains (13). Increased expression of NOS2 results in the activation of microglia (15, 16) which is a response to neuronal injury. And it is the neuronal injury that provokes and keeps the inflammatory process going which makes this process circular. Coupled with the up-regulation of NOS2 in microglia following MPTP administration, which increases NO production, we observed that the expression of NADPH oxidase in microglia is also increased after administration of this toxin (17). Since NADPH oxidase promotes the production of the superoxide radical and is up-regulated in microglia (17), microglia are the forum for a superoxide radical/NO clash in the MPTP mouse model and possibly in PD itself. The interaction of these two "radicals" most likely results in the formation of peroxynitrite, a compound that can modify, thus inactivate amino acids, proteins and catecholamines (8). But, microglia are not the only cells in the SNpc that produce the superoxide radical and NO. Neurons themselves are also involved in their own demise in the MPTP model and probably in PD. From our studies as well as from studies by other investigators (18, 19, 20), it is known that the MPTP metabolite, MPP^+, blocks mitochondrial complex I of the mitochondrial electron transport chain within the neuron which kicks out the superoxide radical. Since NO can travel as many as 300 microns from its site of production and can freely travel through membranes (21), we conclude that the superoxide radical and NO can also interact within the neuron to produce peroxynitrite thus causing its own demise. Peroxynitrite can therefore be formed both inside of and outside of the DA neuron. Inhibition of either the superoxide radical (transgenic SOD1 overexpression) or NO formation (minocycline, 7-nitroindazole) can diminish the damage caused by the interaction of these two compounds in the MPTP mouse model and possibly in PD itself.

A quick, cheap and easy way to end-run the MPP^+ inactivation/block of complex I and to overcome the reduction in complex 1 activity seen in PD is to circumvent the block and the reduction of complex 1 possibly through the application of the ketone body, D-ß-hydroxybutyrate (D-ßHB) (18). D-ßHB is producesd by the hepatocytes and, to a lesser extent, by astrocytes (18). It can act as an alternative source of energy in the brain when the glucose supply is depleted such as during starvation. In vitro, D-ßHB prevents neuronal damage following glucose deprivation and exposure to mitochondrial poisons. In our MPTP mouse model, we have documented that the infusion of D-ßHB

protects SNpc DA neurons in a dose-dependent and stereo-specific manner and prevents the development of PD-like motor abnormalities. D-ßHB seems to enhance oxidative phosphorylation by a mechanism dependent on succinate ubiquinone oxidoreductase (complex II).

As stated above, SNpc neurons in PD and in the MPTP mouse may contribute to their own destruction. The superoxide/NO interaction is not the only source of inflammation within the DA neuron. The COX-2 enzyme is thought to play a role in inflammation within the DA neuron as well, for up-regulation of the COX-2 enzyme within the neuron is thought to be one event among a cascade of deleterious events in the neurodegenerative process (22). In the inflammatory process within the DA neuron, it had been suggested that the increases in the expression of the COX-2 enzyme is responsible for the elevated levels of prostaglandins particularly prostaglandin E2 which has been associated with neurodegeneration. In our studies using the MPTP mouse model of PD and SNpc tissue samples from PD brains, we found induction of the COX-2 enzyme and elevation of its catalytic activity in DA neurons of the SNpc of MPTP-treated mice and in the PD tissues. We also noted that this induction was absent in MPTP treated COX-2 knockout mice, in MPTP mice treated with Refocoxib , a specific COX-2 inhibitor as well as in mice treated with the JNK inhibitor CEP11004 (23). What was interesting in these experiments was that neither ablation nor inhibition of the COX-2 enzyme affected the activation of microglia by MPTP in the SNpc (23). From these experiments, we concluded that the JNK/c-jun signaling pathway is instrumental in COX-2 enzyme induction within the DA neuron, that induction of this enzyme is responsible for elevated PGE2 levels, that this sequence of events has a role in the inflammatory process inside of the DA neuron and that these events do not involve activated microglia.

Peroxynitrite is a very short-lived elusive compound that, in reality, cannot be physically measured. It is formed intracellularly within mitochondria and, can diffuse in and out of mitochondria and can undergo targeted molecule reactions with various cellular compounds such as proteins, amino acids and poly peptides as well as react with carbon dioxide to yield secondary radicals which participate in the oxidation, nitration or nitrosation of critical mitochondrial components (6). Specific markers of peroxynitrite interaction attest to the damage it can inflict. In keeping with our original hypothesis about the role of NO in PD and in the MPTP neurotoxic process, and after having shown that during the MPTP neurotoxic process that TH was nitrated (24), we demonstrated *in vitro* that peroxynitrite can nitrate tyrosine residues in the TH molecule and concluded from these studies that tyrosine 423 was the primary tyrosine that was nitrated and that this nitration was sufficient to inactivate the TH enzyme (25). We also noted in another publication that other molecules are nitrated by peroxynitrite. For instance, the synaptic protein, alpha synuclein, whose role in the degeneration of DA neurons has yet to be elucidated, is not only up-regulated in the SNpc of MPTP-treated mice (26), but also is nitrated and oxidatively modified following the administration of this toxin (27). Based on the fact that peroxynitrite has access to many proteins in mitochondria, we have since sought out other mitochondrial proteins or components which may be at risk of nitrative/oxidative damage by peroxynitrite. These are complexes I, II, and V as well as

ANT, creatine kinase, aconitase and manganese SOD (SOD2) (28). We intend to examine the nitration/oxidative modification of some of these compounds.

The free radical hypothesis of PD suggests that oxidative stress is implicated in the death of DA neurons in the SNpc (29). Several reactions within the DA neuron set the stage for this damaging situation. For instance, the reaction between the superoxide radical and NO, which produces peroxynitrite, puts cells in an oxidative/nitrative stress. Furthermore, the fact that the superoxide radical can be overproduced in several situations (Blockade of complex 1, induction and up-regulation of NADPH oxidase) also indicates oxidative stress. Therefore, the superoxide radical, by itself, can exert damaging effects by generating reactive species such as the hydroxyl radical (HO•) whose oxidative properties can ultimately kill cells (29). Superoxide can facilitate HO•production in the metal-catylized Haber-Weiss reaction and can be dismutated by SOD1 to form hydrogen peroxide (H_2O_2) (30). In certain oxidative damage situations, there exists an oxidative pathway that does not require metal ions. This pathway involves myeloperoxidase, a heme protein secreted by activated macrophages (31). Myeloperoxidase uses H_2O_2 to convert the phenolic acid tyrosine into a reactive intermediate that promotes the oxidation of proteins and lipids. Studies indicate that the oxidizing intermediate generated by myeloperoxidase is the tyrosyl radical (31) and suggest that this radical may promote oxidative reactions at sites of inflammation. Stable endproducts of protein oxidation act as indicators that a specific reaction has occurred thus we used gas chromatography with mass spectrometry to measure these reactions first in an *in vitro* situation with myeloperoxidase to determine what these reaction products might be, then *in vivo* following MPTP administration reaction to see if they were indeed part of the MPTP neurotoxic process. We found elevated levels of 3-nitrotyrosine and o,o-dityrosine in the ventral midbrains and striata of MPTP-treated mice as compared to saline control tissues. Cerebellum and frontal cortex showed no evidence of protein oxidation (31). The presence of these compounds in the brain indicate that oxidative modification of proteins took place in brain areas sensitive to the effects of MPTP while the reaction in the test tube confirms that a myeloperoxidase-H_2O_2 oxidative pathway is responsible for the existence of the oxidized proteins found in the brain tissues (31). These findings give further support to the oxidative stress hypothesis of PD and may be relevant to our understanding of the pathogenesis of this debilitating disorder.

We have put together a series of experiments to delve deeper into the mysteries of PD by examining the role of NO in the MPTP neurotoxic process. Our proposal is largely based on the free radical hypothesis of PD. From these studies, we conclude that the superoxide radical and NO are indeed instrumental in the death of DA neurons in the SNpc of MPTP-treated mice. The death of these neurons apparently involves induction of iNOS and the up-regulation of nNOS, production of the superoxide radical, microglial activation with all of its cytotoxic components, peroxynitrite production and protein oxidation and nitration. A number of these same findings have also been found in PD, the human condition that MPTP mimics.

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APPENDICES (SEE ENCLOSED MANUSCRIPTS).

Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease

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MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) damages dopaminergic neurons as seen in Parkinson disease. Here we show that after administration of MPTP to mice, there was a robust gliosis in the substantia nigra pars compacta associated with significant upregulation of inducible nitric oxide synthase (iNOS). These changes preceded or paralleled MPTP-induced dopaminergic neurodegeneration. We also show that mutant mice lacking the iNOS gene were significantly more resistant to MPTP than their wild-type littermates. This study demonstrates that iNOS is important in the MPTP neurotoxic process and indicates that inhibitors of iNOS may provide protective benefit in the treatment of Parkinson disease.

Parkinson disease (PD) is a common neurodegenerative disorder whose cardinal features include tremor, slowness of movement, stiffness and poor balance[1]. Most, if not all, of these disabling symptoms are due to a profound reduction in striatal dopamine content caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and of their projecting nerve fibers in the striatum[2,3]. Although several approved drugs do alleviate PD symptoms, their chronic use is often associated with debilitating side effects[4], and none diminish the progression of the disease. Moreover, the development of effective neuroprotective therapies is impeded by our limited knowledge of the actual mechanisms by which dopaminergic neurons die in PD. So far, however, considerable insights into the pathogenesis of PD have been achieved by the use of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which causes in humans and in nonhuman primates a severe and irreversible PD-like syndrome[5]. In several mammalian species, MPTP reproduces most of the biochemical and pathological hallmarks of PD, including the substantial degeneration of dopaminergic neurons[5]. Furthermore, there is mounting evidence that reactive oxygen species, especially nitric oxide (NO), are pivotal in the MPTP neurotoxic process[6], which supports the hypothesis that oxidative stress contributes to the pathogenesis of PD (ref. 7).

So far, three distinct NO-synthesizing isoenzymes have been purified and molecularly cloned[8]: neuronal NO synthase (nNOS), inducible NOS (iNOS) and endothelial NOS. nNOS is the main NOS isoform in the brain, as its catalytic activity and protein are identifiable throughout the central nervous system[9,10]. In contrast, iNOS normally is not[11] or is minimally[12] expressed in the brain. However, in pathological conditions, iNOS expression can increase in brain glial cells[13] and invading macrophages in response to a variety of injuries[13–15]. Endothelial NOS is mainly localized in the endothelium of blood vessels and to a minimal extent in different discrete regions of the brain[16–18]. Thus, our success in determining the pathogenesis of PD as well as in developing neuroprotective therapies that target the NO pathway is contingent on our elucidation of which of the NOS isoenzymes contribute to the production of the NO involved in dopaminergic neuron degeneration. The pharmacological inhibition of nNOS, produced by 7-nitroindazole and *S*-methylthiocitrulline, substantially attenuates MPTP-induced dopaminergic neurotoxicity in mice and monkeys[19–22]. Although these NOS antagonists are considered selective nNOS inhibitors, it is not certain that, at the dose used in these studies, they retain all of their selectivity. Consistent with this is the demonstration that nNOS-deficient mice with about 10% residual nNOS activity[10] are partially protected against MPTP (ref. 20), whereas mice treated with doses of 7-nitroindazole that cause about 80% nNOS inhibition are completely protected[19,20]. These data indicate that although nNOS is important, other NOS isoforms might also participate in the dopaminergic neurodegeneration that occurs in the MPTP model and in PD. Relevant to this is the demonstration that many cells in the SNpc from postmortem PD samples express considerable amounts of iNOS, whereas those from age-matched controls do not[23]. Although upregulation of iNOS in acute injury may lead to cell death[15,24], most likely through the production of large amounts of NO over a prolonged period of time[25], its involvement in a chronic neurodegenerative process such as in PD is not known. Here we show that iNOS is not only upregulated in the SNpc of MPTP-treated mice, but that its ablation in mutant mice significantly attenuates MPTP neurotoxicity, thus indicating that iNOS is essential in MPTP-induced SNpc dopaminergic neurodegeneration.

Table 1 Number of neurons in the SNpc

	Saline		MPTP	
	Wild-type	*iNOS*$^{-/-}$	Wild-type	*iNOS*$^{-/-}$
Tyrosine hydroxylase	10,188 ± 619	9,680 ± 496	2,940 ± 698*	5,720 ± 539**
Nissl	13,563 ± 1007	12,913 ± 530	6,300 ± 539*	10,417 ± 380**

SNpc neurons (mean ± s.e.m.; $n = 5$ per group) were counted by stereology. *, $P < 0.001$, fewer than both saline-injected groups; **, $P < 0.05$, fewer than both saline-injected groups and more than MPTP-injected wild-type mice; Newman-Keuls post-hoc test.

MPTP produces a robust glial response

In saline-injected mice, ventral midbrain expression of macrophage antigen-1 (MAC-1) and glial fibrillary acidic protein (GFAP), which are specific markers of microglia and astrocytes, respectively, was minimal (Fig. 1*a–c*). This corresponded to only a few faintly immunoreactive resting microglia and astrocytes in the substantia nigra (Fig. 1*f* and *i*). In MPTP-injected mice, ventral midbrain expression of MAC-1 and GFAP was significantly greater (Fig. 1*a–c*) and there were many robustly immunoreactive MAC-1-positive activated microglia and GFAP-positive reactive astrocytes (Fig. 1*d,e,g and h*). Although the MPTP-induced glial response predominated in the SNpc, it spanned the entire substantia nigra (Fig. 1*d* and *g*). Changes in MAC-1 expression were evident by 12 hours, reached a maximum by 24–48 hours, and were no longer different from control samples by day 7 after MPTP injection (Fig. 1*a* and *b*). In contrast to MAC-1 expression, changes in GFAP expression were only noticeable by 24 hours, were maximal by 4–7 days, and showed a trend towards returning to control levels by 21 days after MPTP injection (Fig. 1*a* and *c*). In the striatum, the time course of the MPTP-induced changes in MAC-1 and GFAP expression were similar to those seen in the ventral midbrain (data not shown).

MPTP stimulates iNOS expression in glial cells

In saline-injected mice, there were rarely iNOS-immunoreactive cells in the SNpc (Fig. 2). In MPTP-injected mice, the number of SNpc iNOS-positive cells increased rapidly over time, reaching a 250% increase by 24 hours after MPTP injection (Fig. 2). However, iNOS-positive cell counts were no longer significantly different from controls by 48 hours after MPTP injection (Fig. 2*d*). In the striatum, no iNOS-positive cells were identified after MPTP injection. We confirmed the specificity of the antibody against iNOS by western blot analysis (Fig. 2*e*).

To elucidate the nature of the SNpc iNOS-positive cells, we simultaneously immunostained midbrain sections for iNOS and MAC-1 or GFAP. At 24 hours after MPTP injection, the time with the most iNOS-positive cells, there was iNOS immunoreactivity in MAC-1-positive activated microglial cells (Fig. 2*c*). In contrast, none of the iNOS-positive cells were either GFAP-positive or had a neuronal morphology.

MPTP increases iNOS mRNA levels and enzymatic activity

We further characterized the iNOS response to MPTP by assessing its mRNA level and enzymatic activity. In saline-injected mice, ventral midbrain iNOS mRNA was almost undetectable (Fig. 2). In contrast, in MPTP-injected mice, midbrain iNOS mRNA levels were detected by 12 hours, were maximal by 48 hours, and were no longer detected by 4 days after MPTP injection (Fig. 2*f* and *g*). Striatal iNOS mRNA levels were very low and were unchanged by MPTP injection (not shown). In agreement with the mRNA results, ventral midbrain iNOS enzymatic activity was minimal in saline-injected mice, but rapidly increased after MPTP injection (Fig. 2*h*). Indeed, in MPTP-injected mice, ventral midbrain iNOS activity began to increase by 12 hours, peaked by 24–48 hours (300% increase), and then slowly subsided back to control activity by 7 days after MPTP injection (Fig. 2*h*). As for iNOS mRNA, iNOS catalytic activity in the striatum was low and was unaffected by MPTP injection (not shown). We also measured nNOS activity in ventral midbrain from mice treated with saline or MPTP; nNOS activity was consistently higher than iNOS and was unmodified by MPTP injection (Fig. 2*h*).

iNOS-deficient mice are more resistant to MPTP

Given the MPTP-induced SNpc iNOS upregulation, we determined the involvement of this enzyme in MPTP neurotoxicity by comparing the effects of the toxin in mutant mice deficient in iNOS (*iNOS*$^{-/-}$) and in their wild-type littermates; this approach is more advantageous than pharmacological inhibition of NOS because it allows for the study of iNOS independently of other NOS isoenzymes. Stereological counts of SNpc dopaminergic neurons, defined by tyrosine hydroxylase (TH) and Nissl staining, did not differ between saline-injected *iNOS*$^{-/-}$ mice and their saline-injected wild-type littermates (Fig. 3*a* and Table 1). In wild-type mice, only 29% of the SNpc TH-positive neurons and 46% of the Nissl-stained SNpc neurons survived MPTP injection (Fig. 3*a* and Table 1). In contrast, about twice as many SNpc TH-positive and Nissl-stained neurons survived in *iNOS*$^{-/-}$ mice treated with an identical MPTP regimen (Fig. 3*a* and Table 1). However, there were no significant differences in the extent of loss in striatal levels of dopamine, DOPAC (3-4-dihydroxyphenylacetic acid) and HVA (homovanillic acid) between *iNOS*$^{-/-}$ mice and their wild-type littermates after the administration of MPTP (Table 2).

Microglial responses and MPP$^+$ production in *iNOS*$^{-/-}$

Although iNOS$^{-/-}$ mice lack iNOS expression, they showed increases in MAC-1 expression similar to those seen in wild-type mice in response to MPTP (Fig. 3*b* and *c*). The main determining factor of MPTP neurotoxic potency is its conversion in glia to the 1-methyl-4-phenylpyridinium ion[26] (MPP$^+$). To confirm that the resistance of *iNOS*$^{-/-}$ mice was due to the absence of the iNOS gene and not due to an alteration in the glial production of MPP$^+$, we measured its striatal content at different times after MPTP injection. At no time did the striatal content of MPP$^+$ differ significantly between the *iNOS*$^{-/-}$ mice and their wild-type littermates (Table 3).

Table 2 Striatal monoamine levels (ng/mg tissue)

	Dopamine	DOPAC	HVA
Saline ($n = 6$)	14.7 ± 0.8	1.6 ± 0.3	2.9 ± 0.1
MPTP			
Wild-type ($n = 4$)	2.8 ± 0.5*	0.7 ± 0.2*	1.8 ± 0.2*
iNOS$^{-/-}$ ($n = 5$)	2.4 ± 0.3**	0.5 ± 0.1**	1.7 ± 0.2**

As dopamine, DOPAC, and HVA values did not differ between saline-injected *iNOS*$^{-/-}$ and their saline-injected wild-type littermates, data from both groups were combined. *, $P < 0.01$, different from saline-injected control mice but not MPTP-injected *iNOS*$^{-/-}$ mice; **, $P < 0.01$, different from saline-injected control mice but not MPTP-injected wild-type mice; Newman-Keuls post-hoc test. Data represent means ± s.e.m. for four to six mice per group.

Brain nitrotyrosine levels in *iNOS*$^{-/-}$ and wild-type mice

To assess the extent of NO-related oxidative damage, we determined nitrotyrosine levels by dot–blot analysis in selected brain regions of *iNOS*$^{-/-}$ and wild-type mice after saline or MPTP injection. In saline-injected mice, the distribution of nitrotyrosine was similar between the two groups of mice in that levels were highest in striatum and cerebellum, followed by frontal cortex, and were lowest in ventral midbrain (Table 4). In MPTP-injected *iNOS*$^{-/-}$ and wild-type mice, nitrotyrosine levels were significantly increased in striatum and ventral midbrain and unchanged in the other brain regions studied (Table 4). MPTP produced significantly smaller increases in nitrotyrosine levels in the ventral midbrain of *iNOS*$^{-/-}$ mice than of their wild-type counterparts, whereas it produced similar increases in the striata of the two groups of mice (Table 4).

Table 3 MPP$^+$ levels (μg/g striatum)

	90 min	120 min	180 min	360 min
Wild type	17.75 ± 0.50	23.10 ± 4.30	19.17 ± 0.63	1.61 ± 0.12
iNOS$^{-/-}$	24.24 ± 2.70	20.61 ± 2.23	17.18 ± 0.83	1.91 ± 0.49

Striatal MPP$^+$ levels in wild-type and *iNOS*$^{-/-}$ mice at 90, 120, 180 and 360 min after the last MPTP injection do not differ ($P > 0.05$; Newman-Keuls post-hoc test) between the two groups. Data represent means ± s.e.m. for four mice per group and time point.

Discussion

This study shows that, in addition to the considerable loss of dopaminergic neurons, gliosis is a salient neuropathological feature of the SNpc and the striatum in the MPTP mouse model, as in PD (refs. 27,28). Although gliosis sometimes may be associated with beneficial effects, there are many more situations in which gliosis may be deleterious[29,30], including in PD (ref. 28). Consistent with this, our data indicate that inflammatory-related events, such as gliosis, may contribute to the degeneration of dopaminergic neurons in the MPTP model. For example, activated microglial cells appeared in the SNpc much sooner than reactive astrocytes (Fig. 1) and at a time when only minimal neuronal death has occurred[31]. This supports the contention that the microglial response to MPTP arises early enough in the neurodegenerative process to contribute to the demise of SNpc dopaminergic neurons. In keeping with the deleterious role of microglia, we found that these cells not only increase in number after MPTP injection, but also, more importantly, were the site of iNOS upregulation (Fig. 2). Therefore, activated microglial cells can flood surrounding dopaminergic neurons with large amounts of iNOS-derived NO and other reactive species, such as superoxide radicals[32]. The time course of the response of astrocytes to MPTP was quite distinct from that of microglia (Fig. 1), in that the changes in the density of reactive astrocytes in both striatum and SNpc did not precede but rather occurred at the same time as the active phase of dopaminergic neuron degeneration[31]. This indicates that the astrocytic reaction is secondary to the loss of dopaminergic neurons and not a primary event. Although this diminishes the potential role of reactive astrocytes in initiating the dopaminergic neurodegeneration, it does not undermine the potential role of these cells in propagating the neurodegenerative process.

Both *in vitro* and *in vivo* experiments indicate that iNOS transcription can be induced by various cytokines, including tumor necrosis factor-α, interleukin-1β and interferon-γ (refs. 33–35) as well as by ligation of the macrophage cell surface antigen CD-23 (ref. 36). It is thus particularly relevant to PD that glial cells immunoreactive for those cytokines and CD-23 are detected in the SNpc of PD patients[37]. In agreement with this transcriptional induction model of iNOS, the mRNA levels of iNOS increased in the ventral midbrain of MPTP-injected mice (Fig. 2). However, in the striatum, whereas a strong glial reaction did occur after MPTP injection, there was no detectable induction of iNOS mRNA. Among various possibilities, the discrepancy in the iNOS response between striatum and ventral midbrain may reflect either a differential mode of iNOS regulation between these two brain regions or the existence of a striatal factor that suppresses the induction of iNOS[38].

Our data on microglial iNOS immunoreactivity in the SNpc of MPTP-injected mice are in agreement with results in PD patients, in whom iNOS immunoreactivity has also been found

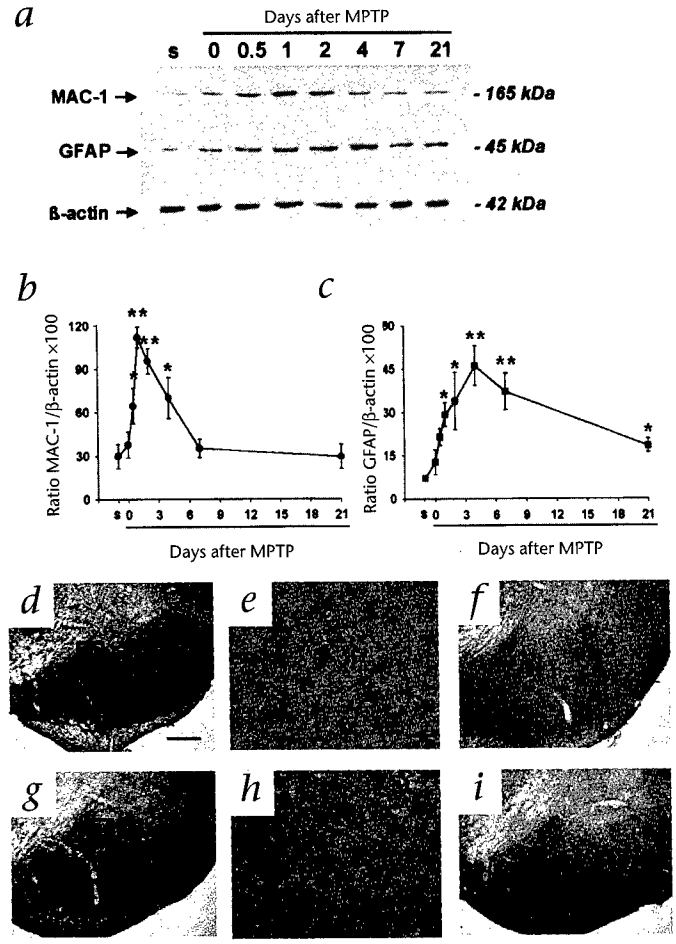

Fig. 1 MPTP-induced glial reaction. ***a–c***, Ventral midbrain MAC-1 (*a* and *b*) and GFAP (*a* and *c*) expression is minimal in saline-injected mice (S), but increases in a time-dependent manner after MPTP injection. Data represent mean ± s.e.m. ($n = 4–5$). **, $P < 0.01$ and *, $P < 0.05$, compared with saline, Newmas-Keuls post-hoc test. ***d–i***, There is a robust MAC-1 (*d*) and GFAP (*g*) immunostaining in the SNpc of MPTP-treated mice compared with that in saline-treated control mice (*f* and *i*) at 24 h 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*).

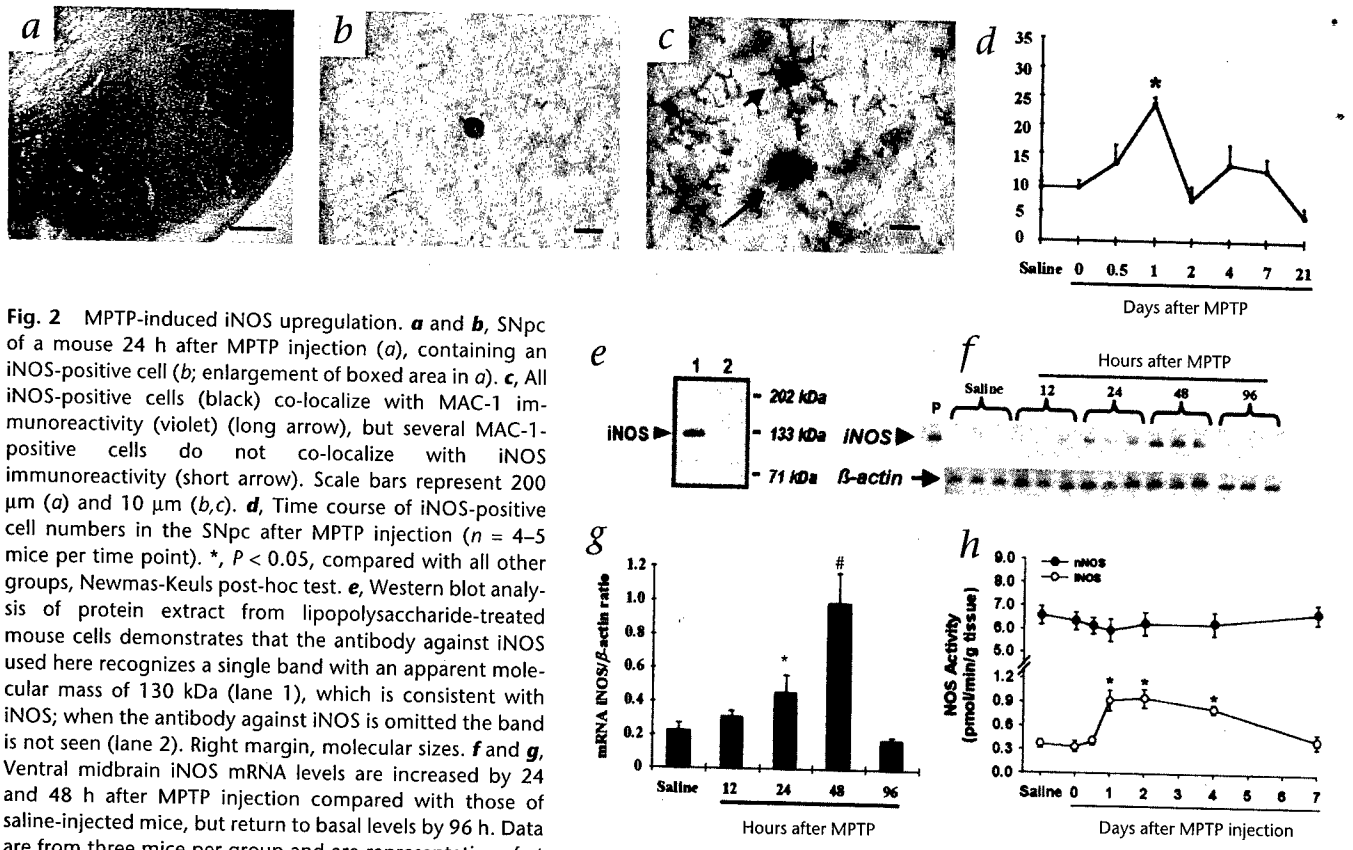

Fig. 2 MPTP-induced iNOS upregulation. ***a*** and ***b***, SNpc of a mouse 24 h after MPTP injection (*a*), containing an iNOS-positive cell (*b*; enlargement of boxed area in *a*). ***c***, All iNOS-positive cells (black) co-localize with MAC-1 immunoreactivity (violet) (long arrow), but several MAC-1-positive cells do not co-localize with iNOS immunoreactivity (short arrow). Scale bars represent 200 µm (*a*) and 10 µm (*b,c*). ***d***, Time course of iNOS-positive cell numbers in the SNpc after MPTP injection (n = 4–5 mice per time point). *, $P < 0.05$, compared with all other groups, Newmas-Keuls post-hoc test. ***e***, Western blot analysis of protein extract from lipopolysaccharide-treated mouse cells demonstrates that the antibody against iNOS used here recognizes a single band with an apparent molecular mass of 130 kDa (lane 1), which is consistent with iNOS; when the antibody against iNOS is omitted the band is not seen (lane 2). Right margin, molecular sizes. ***f*** and ***g***, Ventral midbrain iNOS mRNA levels are increased by 24 and 48 h after MPTP injection compared with those of saline-injected mice, but return to basal levels by 96 h. Data are from three mice per group and are representative of at least three independent experiments. P, iNOS-positive control (lipopolysaccharide-treated mouse cells). *, $P < 0.05$ and #, $P < 0.01$, higher than all other groups, Newmas-Keuls post-hoc test. ***h***, Ventral midbrain iNOS activity is significantly increased 1 d after MPTP injection, is still increased at 4 d although it returns to basal levels by 7 d after MPTP injection, whereas ventral midbrain nNOS activity remains unchanged throughout. *$P < 0.01$, higher than all other groups, Newmas-Keuls post-hoc test.

in SNpc microglia/macrophages[23]. However, our study provides essential insights into these autopsy findings, by indicating that iNOS upregulation is not due to the chronic use of anti-PD drugs such as L-dopa, nor is it an alteration that occurs at the very end of the disease process. Instead, our observation of increased iNOS immunoreactivity and enzymatic activity in MPTP-injected mice (Fig. 2) fits with the idea that iNOS-mediated NO and superoxide production[32] may contribute to the neurodegenerative process in this model and in PD. However, NO is membrane-permeable and can diffuse to neighboring neurons, whereas superoxide cannot readily transverse cellular membranes[39], making it unlikely for microglial-derived extracellular superoxide to gain access to dopaminergic neurons and directly trigger intracellular toxic events. Alternatively, NO could react with superoxide in the extracellular space to form the very reactive tissue-damaging species, peroxynitrite, which can cross the cell membrane and injure neurons. Therefore, microglial-derived superoxide, by contributing to peroxynitrite formation, may be important in this model. The presumed absence of direct involvement of extracellular superoxide in MPTP neurotoxic process, however, does not contradict the instrumental role of intracellular superoxide in this model[6], especially that produced within dopaminergic neurons consequent to the mitochondrial electron transport chain blockade by MPP^+.

Consistent with the involvement of iNOS in the MPTP neurotoxic process is our demonstration that approximately twice as many SNpc dopaminergic neurons survived in *iNOS*$^{-/-}$ mice as in their wild-type littermates after MPTP injection (Table 1). As activated microglia can also exert deleterious effects unrelated to NO, it must be emphasized that *iNOS*$^{-/-}$ mice showed no evidence of impaired microglial activation in response to MPTP (Fig. 3) and *iNOS*$^{-/-}$ macrophages, which do not produce NO, remain responsive to interferon-γ and, once activated, preserve their respiratory burst capacity[40] that includes the formation of superoxide[41]. Moreover, ablation of iNOS was not associated with alterations in the formation of the MPTP active metabolite MPP^+ (Table 3), which is the most important modulating factor of MPTP potency[26]. Given these data, the resistance of *iNOS*$^{-/-}$ mice to MPTP may result from the lack of iNOS expression and the consequent reduced NO formation, and not from either a microglial-deficient respiratory burst capacity or an altered MPTP metabolism. Unexpectedly, the resistance of the SNpc dopaminergic neurons in *iNOS*$^{-/-}$ mice was not accompanied by a similar sparing of striatal dopaminergic fibers, given that the levels of dopamine and metabolites after MPTP injection were similarly decreased in *iNOS*$^{-/-}$ mice and their wild-type littermates (Table 2). In the SNpc, MPTP caused significant upregulation of iNOS and, constitutively, there are only a few midbrain nNOS-positive neuronal elements that are not of dopaminergic nature and that do not have a close relationship with SNpc dopaminergic neurons[9,42]. In contrast, in the striatum, MPTP did not cause any detectable iNOS upregulation and, constitutively, there are many nNOS-positive neu-

rons and nerve fibers[9]. Therefore, the substantial preservation of the SNpc dopaminergic neurons coinciding with the loss of striatal dopaminergic fibers in MPTP-injected *iNOS*$^{-/-}$ mice can be explained by the fact that MPTP-mediated damage of striatal dopaminergic nerve fibers, unlike that of SNpc dopaminergic neurons, does not rely much on NO produced by iNOS, but instead on other NOS isoforms such as nNOS (ref. 20).

Peak ventral midbrain iNOS enzymatic activity was about 15% that of nNOS enzymatic activity (Fig. 2), raising the question of how such a non-predominant component of total NOS enzymatic activity could substantially modulate MPTP-induced SNpc injury. The answer may reside in the distance that NO must travel from the site of release to the dopaminergic neurons. Indeed, as described above, ventral midbrain nNOS-positive neuronal elements are located far from dopaminergic structures and thus it is likely that the amount of nNOS-derived NO that succeeds in reaching the target neurons is much less than could be expected, given the nNOS catalytic activity. In contrast, ventral midbrain iNOS-positive microglial cells are in close proximity to dopaminergic structures and thus the amount of iNOS-derived NO that succeeds in reaching the target neurons is conceivably very substantial. Also relevant is the fact that nNOS activity is under the dynamic regulation of calcium, thus it is probable that midbrain nNOS does not produce anywhere near 600% as much NO as iNOS.

Table 4 Nitrotyrosine levels (ng/μg protein)

	Frontal Cortex	Striatum	Ventral Midbrain	Cerebellum
Wild-type				
Saline	18.9 ± 3.7	30.9 ± 2.1	13.3 ± 3.2	21.6 ± 3.2
MPTP	19.1 ± 2.1	67.8 ± 3.1*	31.7 ± 1.7*	23.5 ± 2.2
iNOS$^{-/-}$				
Saline	18.4 ± 3.5	28.3 ± 2.5	14.0 ± 2.5	20.6 ± 1.8
MPTP	19.6 ± 2.5	68.4 ± 2.9**	21.9 ± 1.7***	19.1 ± 2.0

*, $P < 0.01$, higher than saline-injected mice; **, $P < 0.01$, higher than saline-injected mice but not MPTP-injected wild-type mice; ***, $P < 0.05$, higher than saline-injected mice but lower than MPTP-injected wild-type mice; Newman-Keuls post-hoc test. Data represent mean ± s.e.m. for three to four mice per group and treatment.

As for mechanisms, NO is a weak oxidant and thus it is not, by itself, sufficiently damaging to participate directly in MPTP deleterious effects. Alternatively, NO-derived species with stronger oxidant properties, such as peroxynitrite, can unquestionably cause direct injury to the dopaminergic neurons[43]. In addition, peroxynitrite can nitrate tyrosine residues[44], which may serve as a stable biomarker for peroxynitrite actions. Supporting the possibility of involvement of peroxynitrite in MPTP neurotoxic process is our demonstration that nitrotyrosine levels were significantly increased after MPTP injection only in brain regions known to be susceptible to the toxin (Table 4), which is consistent with previous studies[19,22,45]. Even more compelling, MPTP-injected *iNOS*$^{-/-}$ mice, which showed significantly less SNpc dopaminergic neuronal loss (Table 1), also showed significantly smaller increases in ventral midbrain nitrotyrosine levels than their wild-type counterparts (Table 4).

Our data provide evidence for a pivotal role for microglial iNOS-derived NO in the cascade of deleterious events that ultimately leads to SNpc dopaminergic neuronal death in the MPTP mouse model and in PD. Therefore, our study indicates that inhibition of iNOS may be a valuable target for the development of new therapies for PD aimed at attenuating the actual loss of dopaminergic neurons. However, this study also shows that iNOS inhibition may not be efficacious in preserving striatal nerve fibers from MPTP neurotoxicity. This indicates that the ideal therapeutic approach for PD may require the combination of iNOS inhibitors with other agents that have strong abilities in promoting nerve fiber re-growth and in stimulating dopaminergic function as well as in preserving dopaminergic nerve terminals. So far, multi-drug strategies have proved to be successful in fitting other pathological conditions, such as HIV infection and cancer.

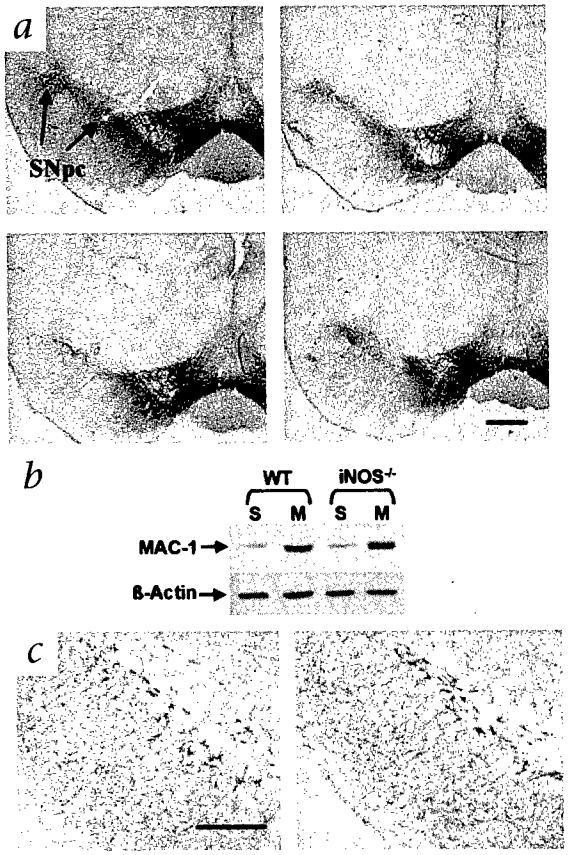

Fig. 3 MPTP-induced neuronal loss and microglial reaction in *iNOS*$^{-/-}$ mice. ***a***, SNpc TH-positive neurons are twice as resistant to MPTP in *iNOS*$^{-/-}$ mice than in wild-type (WT) littermates, 7 d after MPTP injection. In addition, there is no noticeable difference in TH-positive neuron density after saline injection between *iNOS*$^{-/-}$ mice and WT littermates (Table 1, actual neuronal counts). ***b*** and ***c***, Ventral midbrain western blot analysis (*b*) and SNpc immunohistochemical analysis (*c*) for MAC-1, showing the similarity of the microglial response between the *iNOS*$^{-/-}$ mice and their wild-type littermates (WT), 24 h after MPTP injection. Scale bar represents 200 μm (*a* and *c*, shown in *c*).

Methods

Animals and treatment. Eight-week-old male C57/bl mice (Charles River Laboratories, Wilmington, Massachusetts) and iNOS-deficient mice (C57/bl-NOS2^{tm1Lau}; Jackson Laboratories, Bar Harbor, Maine) and their wild-type littermates were used. Mice (n = 4–6 per group) received four intraperitoneal injections of MPTP-HCl (20 mg/kg of free base; Research Biochemicals, Natick, Masssachusetts) in saline at 2-hour intervals in 1 day, and were killed at selected times 0–21 days after the last injection. Control mice received saline only. This protocol was in accordance with the NIH guidelines for use of live animals and was approved by the Institutional Animal Care and Use Committee of Columbia University and Johns Hopkins University School of Medicine.

MAC-1, GFAP, and iNOS immunohistochemistry. These were done as described[46] on cryostat-cut sections (30 μm in thickness) encompassing the entire midbrain, and used the following primary antibodies (at the following dilutions): rat antibody against MAC-1 (1:1,000 ; Serotec, Raleigh, North Carolina), rabbit antibody against GFAP (1:1,000; Dako, Carpinteria, California), and rabbit antibody against iNOS (1:1,000; Transduction Laboratories, Lexington, Kentucky). The double-immunostaining procedure for iNOS and MAC-1 or GFAP was used as described[47] with minor modifications. Because the number of iNOS-positive cells in the SNpc at any given time was very small, we could not use a stereological method[48], but instead have used an assumption-based method[20], following strict guidelines[49] to ensure the validity of our quantification technique.

RNA extraction and RT–PCR for iNOS and β–actin. These were done using methods described[46], using the same pair of primers for iNOS and β-actin, PCR conditions, and a slightly modified reaction mixture (20 μl) consisting of 1 μl cDNA template, 18 μl Supermix (Life Technologies), 10 fmol ^{32}P-dCTP (specific activity, 3,000 Ci/mmol; NEN) and 4–10 pmol of each specific primer. After amplification, the products were separated by 5% polyacrylamide gel electrophoresis. After being dried, gels were exposed to phosphoimager screens (BioRad, Hercules, California), and optical densities were determined using a computerized image analysis system (BioRad, Hercules, California).

Assay of iNOS catalytic activity. nNOS and iNOS catalytic activities were assayed in midbrain samples from four mice per time point and condition, by measuring both the calcium-dependent and calcium-independent conversion of ^{3}H-arginine to ^{3}H-citrulline as described[20,46]. No substantial ^{3}H-citrulline production occurred in the absence of nicotine adenine dinucleotide phosphate, and this represented background counts.

Measurement of striatal dopamine, DOPAC and HVA levels. High-performance liquid chromatography (HPLC) with electrochemical detection was used to measure striatal levels of dopamine, DOPAC and HVA using a method that has been described[20], with minor modifications of the mobile phase. At 7 d after the last MPTP injection, *iNOS*$^{-/-}$ mice and wild-type littermates (four to six per group) were killed, and the striata were dissected out and processed for HPLC measurement. The modified mobile phase consisted of 0.15 M monochloroacetic acid, pH 3.0, 200 mg/l sodium octyl sulfate, 0.1 mM EDTA, 4% acetonitrile and 2.5% tetrahydrofuran.

Measurement of striatal MPP^{+} levels. HPLC with ultraviolet detection (wavelength, 295 nm) was used to measure striatal MPP^{+} levels using a method that has been described[20]. Groups of *iNOS*$^{-/-}$ mice and wild-type littermates (four per time point) were killed at 90, 120, 180 and 360 min after the fourth intraperitoneal injection of 20 mg/kg MPTP, and the striata were dissected out and processed for HPLC measurement as described[20].

***iNOS*$^{-/-}$ TH and Nissl staining and stereology.** The total number of TH- and Nissl-stained SNpc neurons were counted in five mice per group using the optical fractionator method as described[48]; this is an unbiased method of cell counting that is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons)[50]. TH immunostaining was done as described[20], using an affinity-purified polyclonal antibody against TH (1:2,000 dilution; Calbiochem, San Diego, California).

Immunoblots. Mouse brain protein extracts from selected regions were prepared as described[45]. For western blot analysis, 10% SDS–PAGE and transfer of proteins to nitrocellulose membrane were done as described[45], and blots were probed with either antibody against MAC-1 (1:1,000 dilution; Serotec, Raleigh, North Carolina), antibody against GFAP (1:2,000 dilution; DAKO), antibody against iNOS (1:1,000 dilution; Transduction Laboratories, Lexington, Kentucky), or antibody against β-actin (1:5,000 dilution; Sigma). For dot–blot analyses, 100 μg of protein extracts were loaded onto the nitrocellulose membrane, and blots were probed with an affinity-purified polyclonal antibody against nitrotyrosine (1:1000 dilution; a gift from H. Ischiropoulos). For all blots, bound primary antibody was detected using a horseradish-conjugated antibody against IgG (1:2,000 dilution; Amersham) and a chemiluminescent substrate (SuperSignal Ultra; Pierce Chemical, Rockford, Illinois). X-ray films (Kodak BioMax MS) were scanned on a HP-4C Scanjet and bands were quantified using NIH-Image 1.62 software. For all dot–blot analyses, optical densities were converted into nanograms of nitrotyrosine using a standard curve generated from different concentrations of nitrated bovine serum albumin (a gift from H. Ischiropoulos) corresponding to 14–350 μg nitrated protein or 0.4–10 ng nitrotyrosine. In preliminary experiments, we confirmed the specificity of nitrotyrosine immunoreactivity by demonstrating that immunostaining is abolished either by co-incubating the antibody against nitrotyrosine with 10 mM nitrotyrosine or by treating blots with 10 mM sodium hydrosulfite, to reduce nitrotyrosine to aminotyrosine, before incubation with the primary antibody[51].

Statistical analysis. All values are expressed as the mean ± s.e.m. 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, pair-wise comparisons between means were tested by Newman-Keuls post-hoc testing. In all analysis, the null hypothesis was rejected at the 0.05 level.

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α-Synuclein Up-Regulation in Substantia Nigra Dopaminergic Neurons Following Administration of the Parkinsonian Toxin MPTP

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Abstract: Mutations in α-synuclein cause a form of familial Parkinson's disease (PD), and wild-type α-synuclein is a major component of the intraneuronal inclusions called Lewy bodies, a pathological hallmark of PD. These observations suggest a pathogenic role for α-synuclein in PD. Thus far, however, little is known about the importance of α-synuclein in the nigral dopaminergic pathway in either normal or pathological situations. Herein, we studied this question by assessing the expression of synuclein-1, the rodent homologue of human α-synuclein, in both normal and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. In normal mice, detectable levels of synuclein mRNA and protein were seen in all brain regions studied and especially in ventral midbrain. In the latter, there was a dense synuclein-positive nerve fiber network, which predominated over the substantia nigra, and only few scattered synuclein-positive neurons. After a regimen of MPTP that kills dopaminergic neurons by apoptosis, synuclein mRNA and protein levels were increased significantly in midbrain extracts; the time course of these changes paralleled that of MPTP-induced dopaminergic neurodegeneration. In these MPTP-injected mice, there was also a dramatic increase in the number of synuclein-immunoreactive neurons exclusively in the substantia nigra pars compacta; all synuclein-positive neurons were tyrosine hydroxylase-positive, but none coexpressed apoptotic features. These data indicate that synuclein is highly expressed in the nigrostriatal pathway of normal mice and that it is up-regulated following MPTP-induced injury. In light of the synuclein alterations, it can be suggested that, by targeting this protein, one may modulate MPTP neurotoxicity and, consequently, open new therapeutic avenues for PD. **Key Words:** Synuclein—MPTP—Neurodegeneration—Parkinson's disease—Substantia nigra—Dopaminergic neurons.
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Parkinson's disease (PD) is a common disabling neurodegenerative disorder that can present as both a familial and a nonfamilial (i.e., sporadic) condition (Fahn, 1988). Its cardinal clinical features include tremor, stiffness, and slowness of movement, all of which are attributed to the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn, 1988). Although the actual cause of PD remains unknown, a breakthrough on this question emerged from studies on the small brain-specific protein α-synuclein. The first clue linking α-synuclein to PD comes from the observation that point mutations in the α-synuclein gene cause an autosomal dominant parkinsonian syndrome almost indistinguishable from the prominent sporadic form of PD (Polymeropoulos et al., 1997; Kruger et al., 1998). The two missense mutations identified thus far result in a single amino acid substitution in α-synuclein protein, that is, an alanine being replaced by a hydrophobic residue threonine, at position 53, and proline, at position 30. Since the discovery of these mutations, data have been accumulated suggesting that both mutations may alter α-synuclein's normal intracellular distribution, enhance α-synuclein's propensity to interact with other intracellular proteins, and increase α-synuclein disposition to aggregate and consequently to form intraneuronal inclusions (Conway et al., 1998; El-Agnaf et al., 1998; Engelender et al., 1998; Giasson et al., 1999; Narhi et al., 1999). To date, efforts to identify α-synuclein mutations in sporadic PD have failed (Golbe, 1999). On the other hand, in sporadic PD, α-synuclein has been demonstrated to be a major component of the intraneuronal inclusions, Lewy bodies (LB), which are a pathological

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Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LB, Lewy bodies; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NHS, normal horse serum; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

hallmark of the disease (Spillantini et al., 1997, 1998). Furthermore, oxidative stress, which is a leading pathogenic hypothesis of sporadic PD, has been reported to affect wild-type α-synuclein, causing the oxidatively damaged wild-type α-synuclein to mimic some of the abnormal behaviors of mutant α-synuclein (Hashimoto et al., 1999). These observations strongly suggest that both mutant and posttranslationally modified wild-type α-synuclein may participate in the SNpc dopaminergic neuron degeneration in PD, whether it is familial or sporadic.

α-Synuclein is a small ubiquitous protein highly expressed in presynaptic structures of, apparently, all neuronal pathways of the brain (Maroteaux and Scheller, 1991; Clayton and George, 1998; Lavedan, 1998). In light of its presumed role in synaptic function, α-synuclein has been studied especially in the brain of various animal species during development and more specifically during the time frame of synaptogenesis (Withers et al., 1997; Petersen et al., 1999). In humans, α-synuclein has also been studied intensively in brain regions such as cerebral cortex because of the possible role of one of its internal fragments called NAC (i.e., non-β-amyloid component) in the formation of plaques in Alzheimer's disease brains (Iwai et al., 1995, 1996; Irizarry et al., 1996; Masliah et al., 1996). However, although the association of mutant α-synuclein with at least some forms of PD is well established, our knowledge about the role of α-synuclein in normal or even injured SNpc dopaminergic neurons is, to date, quite poor. Therefore, to acquire better understanding about the relationship between α-synuclein and SNpc dopaminergic neurons, we studied the expression and distribution of synuclein-1, the rodent homologue of human α-synuclein (both referred to henceforth as α-synuclein, except when indicated), in normal mice. We also performed these investigations in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD to explore the response of α-synuclein to an injury and examine its contribution to the dopaminergic neurodegenerative process. We elected to use the MPTP model because, to date, it is recognized as the best experimental model of sporadic PD, replicating most of the biochemical and pathological features seen in the clinical condition (Przedborski and Jackson-Lewis, 1998).

MATERIALS AND METHODS

Animals

Eight-week-old male C57/bl mice (22–25 g; Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were used. Animals were housed three per cage in a temperature-controlled room under a 12-h light/12-h dark cycle with free access to food and water. Mice used in this study were treated according to the NIH *Guidelines for the Care and Use of Laboratory Animals* and with the approval of Columbia University's Institutional Animal Care and Use Committee.

MPTP administration

Mice were divided into two groups and received either a chronic or an acute MPTP regimen. For the *chronic regimen* (kills dopaminergic neurons by apoptosis; see Tatton and Kish, 1997), mice received one intraperitoneal injection of MPTP-HCl per day (30 mg/kg/day of free base; Research Biochemicals, Natick, MA, U.S.A.) for 5 consecutive days and were killed at 0, 1, 2, 4, 7, 14, 21, and 42 days after the last injection; control mice received saline injections only. Both saline- and MPTP-treated animals were then divided into two groups. The first group was perfused and the brains used for immunohistochemistry, whereas the second group of mice were killed and the brains quickly removed, dissected (midbrain, striatum, cerebellum, and cortex), snap-frozen on dry ice, and stored at −80°C for western blot and RT-PCR analysis. For the *acute regimen* (kills dopaminergic neurons by necrosis; see Jackson-Lewis et al., 1995), mice received on the day of the experiment four intraperitoneal injections of MPTP-HCl (20 mg/kg) in saline at 2-h intervals and were killed at 0, 2, 4, 7, and 21 days after injection; control mice received saline injections only. Both saline- and MPTP-treated animals were prepared for immunohistochemistry and western blot analysis as described above.

Western blot analysis

Total tissue proteins were isolated in 50 m*M* Tris-HCl, pH 7.0, 150 m*M* NaCl, 5 m*M* EDTA, 1% sodium dodecyl sulfate, 1% Nonidet P-40, and protease inhibitors (Mini Cocktail; Roche Diagnostics, Indianapolis, IN, U.S.A.). Protein concentration was determined using the bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.). After boiling in 1× Laemmli buffer, 50–100 μg of protein was loaded onto 12–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose or polyvinylidene difluoride membrane, and blocked with 5% nonfat dry milk in 1× Tris-buffered saline (TBS), 0.1% Tween 20 for 1 h. Incubation with one of the primary antibodies was performed overnight at 4°C using 1:1,000 anti-synuclein-1 (Transduction Laboratories, Lexington, KY, U.S.A.), 1:1,000 β-synuclein (gift from Dr. S. Nakajo, Tokyo, Japan), 1:2,000 anti-synaptophysin (gift from Dr. Honer, Albert Einstein College of Medicine, Bronx, NY, U.S.A.), or 1:500 anti-tyrosine hydroxylase (anti-TH; Eugene Tech, Ridgefield Park, NJ, U.S.A.). Incubation with a secondary anti-mouse or anti-rabbit-conjugated horseradish peroxidase antibody was performed at room temperature for 1 h. After washing in 1× TBS, 0.1% Tween-20, blots were exposed to Super Signal Ultra chemiluminescence (Pierce) and exposed to Kodak β-Max film. Films were then digitized, each band was outlined with a screen cursor driven by a hand-held mouse, and optical densities were determined using a computerized image analysis system (Inquiry image analyzer, Loats Associates, Westminster, MD, U.S.A.).

Immunohistochemistry

After being anesthetized with pentobarbital (30 mg/kg i.p.), saline- (n = 5) and MPTP-treated mice (0, 1, 2, 4, 7, 14, 21, and 42 days after the last MPTP injection, n = 4–7 for each time point) were perfused intracardially with 24 ml of saline followed by 72 ml of cold 4% (wt/vol) paraformaldehyde in 0.1 *M* phosphate-buffered saline (PBS), pH 7.1. Animals were then decapitated, and brains were removed, immersed for 72 h in the same 4% paraformaldehyde fixative, and cryoprotected in 30% sucrose in 0.1 *M* PBS for 48 h at 4°C. Brains were then frozen on dry ice-cooled isopentane and stored at −80°C until use. Serial coronal sections (30 μm thickness) spanning the entire midbrain and the mid striatum were cut on a cryostat, collected free-floating in PBS, and processed as described below.

For all immunostaining, sections were first rinsed (3 × 5 min) with 0.1 *M* PBS, pH 7.4. Sections were then immersed in a solution of 3% H_2O_2/10% methanol for 5 min, followed by incubation with 5% normal horse serum (NHS) for 60 min. Sections were then incubated with the primary antibody (anti-synuclein-1, 1:1,000) in 0.1 *M* PBS, pH 7.4, containing 2% NHS and 0.3% Triton X-100, for 48 h at 4°C on a shaker. After rinsing in PBS, biotinylated secondary horse anti-mouse IgG (1:200; Vector, Burlington, CA, U.S.A.) in 0.1 *M* PBS, pH 7.4, containing 2% NHS was added, and the sections were incubated for 60 min at room temperature. This was followed by a final incubation in avidin/biotin peroxidase complex (Vector) for 60 min. Visualization was performed by incubation in 3,3′-diaminobenzidine/glucose/glucose oxidase for 10 min. All sections were then washed 3 × 5 min in PBS, mounted on 0.1% gelatin-coated slides, dried, dehydrated in graded ethanols, cleared in xylenes, and coverslipped. To test the specificity of the immunostaining, control sections were processed in an identical manner but with the primary or secondary antibody omitted. Adjacent sections were immunostained for TH (1:1,000; Calbiochem, San Diego, CA, U.S.A.) and counterstained with thionin.

To examine the colocalization of α-synuclein with TH, a double-immunofluorescence technique was used. After washing, sections were blocked in 5% normal goat serum and NHS in 0.1 *M* TBS for 1 h. Incubation with primary antibodies was performed for 48 h at 4°C, with anti-synuclein-1 (1:200) and anti-TH (1:500) antibodies. As the anti-TH antibody was made in rabbit and the anti-synuclein-1 antibody was made in mouse, we used different secondary antibodies for double labeling: anti-rabbit IgG labeled with Texas Red, and biotinylated anti-mouse IgG, followed by avidin D labeled with fluorescein. Sections were examined on green, red, and double (green + red) filters using confocal microscopy. To colocalize α-synuclein staining with apoptotic morphology, additional fluorescent α-synuclein-stained sections were counterstained with the DNA dyes TOTO-3 iodide (2 μ*M* in the secondary antibody solution) or Hoechst 33342 (10 μg/ml in phosphate buffer for 45 min) (both from Molecular Probes, Eugene, OR, U.S.A.).

Quantitative morphology

Early and late in the course of the MPTP neurodegenerative process, the number of α-synuclein-positive cells was minimal or none. This rendered unreliable the use of our stereological method (Mandir et al., 1999) because this approach requires a minimal number of cells being "countable" by applying the random paradigm. If the number is too small, we may miss the rare positive cells and count no cells that may be false. Thus, if this situation occurs, we will return to our assumption-based method (Przedborski et al., 1996), which is not affected by the rarity of the counted cells, following the recommendations of the editors of the *Journal of Comparative Neurology* (Coggeshall and Lekan, 1996) on the question. We are also aware that our conclusions regarding counted cells using this assumption-based method should only imply whether there are significantly more or less counted cells in the experimental cases. By applying these strict criteria, the above-cited editorial (Coggeshall and Lekan, 1996) indicates that our assumption-based method of cell counts is valid. In brief, counts were performed manually and blinded to the treatment received (i.e., MPTP or saline). For each mouse (n = 4–7 per group), eight different stereotaxic planes encompassing the entire substantia nigra and containing the SNpc were analyzed (interaural 0.88 to 0.16 mm; Franklin and Paxinos, 1997) by scanning the entire SNpc on both sides (light microscopy; ×200). The average number of neurons in each plane was added to provide a measure of the total number of SNpc α-synuclein-positive neurons for each animal and then divided by the number of counted sections to provide the number of α-synuclein-positive cells per section.

RNA extraction and RT-PCR

Total RNA was extracted from midbrain, striatal, and cerebellar samples from saline- and chronic MPTP-treated animals (at 0, 4, 7, 21, and 42 days after last MPTP injection, n = 5 for each time point) using a Qiagen RNA isolation kit (Qiagen, Valencia, CA, U.S.A.). The yield and quality of the RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription, using the SuperScript Preamplification System with SuperScript II RNase H-reverse transcriptase (GibcoBRL Life Technologies, Grand Island, NY, U.S.A.). The reaction mixture (20 μl) for PCR consisted of 1 μl of cDNA template, 18 μl of Supermix (GibcoBRL), 0.01 pmol of [^{32}P]dCTP (New England Nuclear, Boston, MA, U.S.A.; specific activity, 3,000 Ci/mmol), and 4–10 pmol of each specific primer. The primer sequences were 5′-GTGGTTCATGGAGTGACAAC-3′ (forward) and 5′-AGGCTTCAGGCTCATAGTCT-3′ (reverse) for α-synuclein and 5′-AGAGAGGACCAGTCAGCCAA-3′ (forward) and 5′-TGACCAGAACCTTCTCTCAAGC-3′ (reverse) for synaptophysin. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was coamplified using primer sequences 5′-GTTTCTTACTCCTTGGAGGCCAT-3′ (forward) and 5′-TGATGACATCAAGAAGTGGTGAA-3′ (reverse). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1.5 min, followed by a final 10-min extension at 72°C. PCR amplification was carried out for 27 cycles for α-synuclein and synaptophysin and 22 cycles for GAPDH using a Perkin–Elmer GeneAmp 9700 thermal cycler. The conditions for each PCR amplification resulted in an exponential amplification range for quantification of each mRNA. After amplification, samples were separated on 5% polyacrylamide gel electrophoresis in 0.5× Tris–borate–EDTA buffer. Gels were dried and exposed overnight to a phosphorimager screen, and then radioactivity was quantified using a computerized analysis system (Bio-Rad Phosphorimager system).

Statistical analysis

For each experiment, four to seven mice per group were studied and all values are expressed as the means ± SEM. Differences were analyzed using one-way ANOVA with the different groups of mice as the independent factor. When ANOVA showed significant differences, pairwise comparisons were tested by Newman–Keuls post-hoc analysis. In all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Increased α-synuclein protein expression in the ventral midbrain after chronic MPTP intoxication

To determine whether α-synuclein may be involved in the deleterious cascade of events induced by MPTP, we assessed α-synuclein protein expression levels in the ventral midbrain of MPTP-intoxicated mice, at different time points after MPTP administration, using western blot analysis. The immunoblot for α-synuclein resulted in a single band of 19 kDa, as previously described

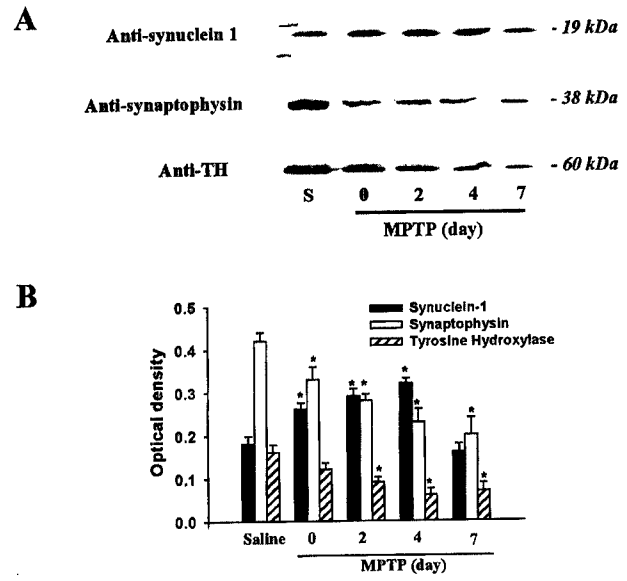

FIG. 1. Expression of synuclein-1, synaptophysin, and TH proteins in ventral midbrain samples of saline-treated (S) and chronic MPTP-intoxicated mice at different time points after intoxication. The immunoblot for synuclein-1 resulted in a single band of 19 kDa (**A**), confirming the specificity of the antibody. Quantitative results (**B**) were obtained by measurement of the optical density of each band using a computerized image analysis system as described in Materials and Methods. After chronic MPTP regimen, α-synuclein protein expression increased progressively in midbrain extracts from 0 to 4 days after MPTP administration, and then returned to the level of controls. Parallel to changes in α-synuclein protein expression, levels of synaptophysin progressively decreased in the ventral midbrain after chronic MPTP administration. Similarly, TH protein expression was also decreased after MPTP intoxication, reflecting the progressive loss of dopaminergic neurons. No changes in α-synuclein protein levels were detected in the striatum or in other cerebral structures, such as cerebellum or cortex (data not shown). $^*p < 0.05$, compared with saline (Newman–Keuls post-hoc analysis).

(Ueda et al., 1993). Quantitative results were obtained by measuring the optical density of each band using a computerized image analysis system as described in Materials and Methods. After the chronic MPTP regimen, α-synuclein protein expression progressively increased in midbrain extracts from 0 to 4 days after MPTP administration (Fig. 1). This increase was already significant at 0 days (+44%), peaked at 4 days (+77%), and then returned to control level. No changes in α-synuclein protein levels were detected in the striatum or in other cerebral structures, such as cerebellum or cortex (data not shown). In addition to α-synuclein, levels of synaptophysin, another presynaptic protein, were determined to test the possibility that α-synuclein alterations might be part of a nonspecific response of synaptic-related proteins to MPTP injury. In contrast to changes in α-synuclein protein levels, synaptophysin protein levels progressively decreased in the ventral midbrain after chronic MPTP administration (Fig. 1). No change of expression was detected in another member of the synuclein family, β-synuclein. TH protein levels were also decreased after chronic MPTP intoxication, reflecting the progressive loss of dopaminergic neurons. In contrast to the chronic regimen of MPTP, the acute regimen did not result in any significant change in α-synuclein protein expression.

Regional and cellular localization of α-synuclein up-regulation

To determine whether the observed changes in α-synuclein protein expression within the ventral midbrain were specific to the SNpc, we performed immunohistochemistry with the same anti-synuclein-1 antibody as used for the western blot. In saline-injected mice, there was a dense network of α-synuclein-positive nerve fibers over the entire substantia nigra that predominated in the pars reticulata (SNpr) and especially in its most medial and ventral parts. Superimposed on this dense α-synuclein-positive neuropil, there were also a few scattered α-synuclein-positive cells with a definite neuronal morphology in the SNpc (Fig. 2). Within α-synuclein-positive neurons, immunostaining was distributed diffusely over the somata with greater immunoreactivity in the cytoplasmic than in the nuclear area, and appeared to extend to proximal neuronal processes. After chronic, but not acute, MPTP intoxication, we did not observe any alteration at the level of α-synuclein-positive neuropil staining in the SNpc and SNpr. In contrast, after this MPTP regimen, the number of α-synuclein-positive neurons in the SNpc increased dramatically as early as 0 days after the last MPTP injection (i.e., 5 days after the first MPTP injection) (Fig. 2); the number of α-synuclein-positive neurons in the SNpc returned to baseline (i.e., saline controls) by 21 days after MPTP administration (data not shown). Of note, in neither saline- nor MPTP-injected mice did we observe (a) any immunostaining in the absence of the primary anti-synuclein-1 antibody, (b) any α-synuclein-positive intraneuronal aggregate, or (c) any glial cells exhibiting α-synuclein immunoreactivity.

Adjacent sections immunostained with TH and counterstained with thionin confirmed the cellular loss induced by MPTP intoxication, as previously described (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). Furthermore, thionin staining revealed the presence of apoptotic morphology in the SNpc of chronic MPTP-intoxicated animals (Fig. 2). None of these apoptotic features could be colocalized with α-synuclein immunoreactivity with absolute certainty.

Double immunofluorescence was performed to identify the phenotype of neurons expressing α-synuclein. Examination with confocal microscopy revealed that all α-synuclein-positive neurons in the SNpc colocalized with TH immunoreactivity (Fig. 3), suggesting that α-synuclein up-regulation after MPTP occurs specifically within dopaminergic neurons, even if not all TH-positive neurons were immunostained with α-synuclein. Adjacent sections were used to colocalize fluorescent α-synuclein staining with apoptotic morphologies, using

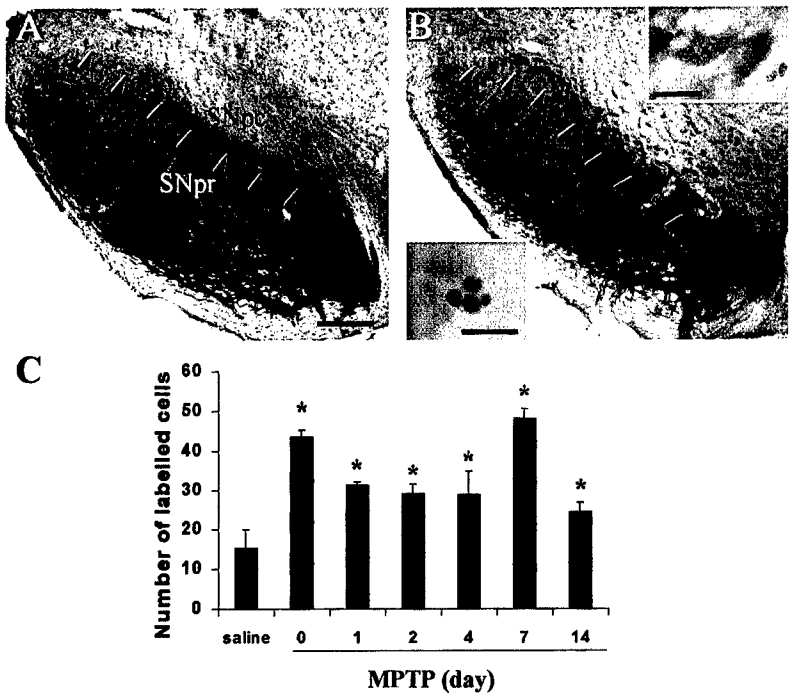

FIG. 2. Representative photomicrographs illustrating synuclein-1 immunoreactivity (brown) in the ventral midbrain of saline-treated (**A**) and chronic MPTP-intoxicated (**B**) mice, counterstained with thionin (blue). Quantitative results (**C**) were obtained by counting α-synuclein-labeled neurons in the SNpc. A strongly immunostained fiber network was observed in the SNpr in both saline-treated and MPTP-intoxicated animals (A and B). A small number of α-synuclein-immunoreactive neuronal bodies were detected in the SNpc of control saline-injected mice (A and C). After chronic, but not acute, MPTP intoxication, the number of α-synuclein-positive neurons in the SNpc increased dramatically (B and C), with peaks at 0 (as shown in B) and 7 days after intoxication. The number of α-synuclein-immunoreactive neurons progressively returned to levels similar to that of controls after 21 days (data not shown). α-Synuclein staining was regularly distributed within the neuronal cytosol, as seen at higher magnification in the upper inset in B, with no detectable intraneuronal inclusions. Up-regulation of α-synuclein expression was parallel to an apoptotic mode of neuronal death in the SNpc, as illustrated in the lower inset in B, with the presence of apoptotic morphology (with chromatin clumps) detected by thionin staining. No colocalization of apoptotic morphology with α-synuclein immunostaining was detected. *$p < 0.05$, compared with saline (Newman–Keuls post-hoc analysis). Scale bars = 200 μm (A), 40 μm (upper inset in B), and 10 μm (lower inset in B).

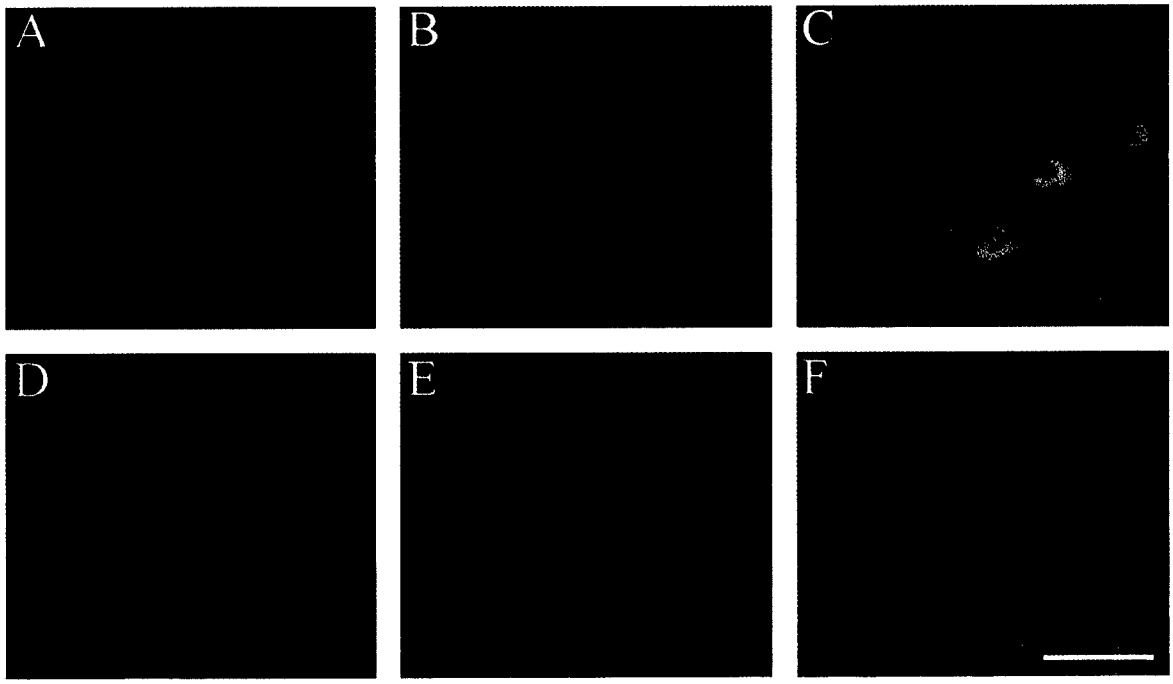

FIG. 3. Representative photomicrographs illustrating double-immunofluorescence staining, visualized with confocal microscopy, of synuclein-1 (green, B and E) and TH (red, A and D) in the SNpc of saline-injected mice (**D–F**) and after chronic MPTP administration (at 0 day after last MPTP injection) (**A–C**). In chronic MPTP-treated animals, α-synuclein immunoreactivity was colocalized with TH, as seen by double filter (red + green) in C. In saline-injected animals, very few α-synuclein-positive neurons were detected by immunofluorescence (E), as illustrated by a predominance of TH (red)-labeled cells using double filter (F). Scale bar = 50 μm.

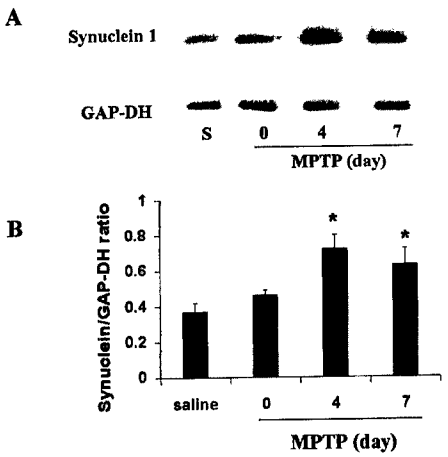

FIG. 4. Midbrain α-synuclein mRNA expression detected by RT-PCR. A photograph of the gel (**A**) and the corresponding bar graph (**B**) show increased α-synuclein mRNA expression after chronic MPTP administration compared with that in saline-injected mice. Levels of α-synuclein mRNA expression progressively decreased to the level of controls up to 21 and 42 days (data not shown). GAPDH mRNA expression levels were determined as an internal control and used to normalize the values for α-synuclein mRNA expression. No changes in α-synuclein mRNA expression were found in either the striatum or the cerebellum, and expression levels of synaptophysin mRNA were unchanged after MPTP intoxication (data not shown). $^*p < 0.05$, compared with saline (Newman–Keuls post-hoc analysis).

TOTO-3 iodide and Hoechst 33342 DNA dyes, failing to reveal any definite colocalization.

Up-regulation of α-synuclein mRNA following MPTP administration

To determine whether changes in α-synuclein protein expression are accompanied by changes in α-synuclein mRNA levels, additional midbrain samples of chronic MPTP-treated mice were used for semiquantitative RT-PCR amplification. Other cerebral structures, such as the striatum and the cerebellum, were also analyzed. In parallel, expression levels of synaptophysin mRNA were also determined. As an internal control, the mRNA levels of expression of the housekeeping gene GAPDH were determined and used to normalize the levels of α-synuclein and synaptophysin mRNA.

Consistent with α-synuclein protein alterations, α-synuclein mRNA was increased in the midbrain following chronic MPTP intoxication in a time-dependent manner (Fig. 4), peaking at 4 days (+94%) after intoxication and then progressively decreasing to the level of controls up to 21 days. No changes in α-synuclein mRNA expression were found in either the striatum or the cerebellum. Expression levels of synaptophysin mRNA in the ventral midbrain were either unchanged or decreased, although not statistically significant, after MPTP intoxication (data not shown).

DISCUSSION

In the present study, we first report the regional and cellular distribution of α-synuclein within the SNpc, which, to date, has not been studied. In normal mice, a striking aspect is the conspicuous α-synuclein-positive nerve fiber network that covers the entire substantia nigra, in the presence of only rare α-synuclein-positive neuronal cell bodies. This remarkable dissociation of α-synuclein immunoreactivity between nerve fibers and cell bodies is consistent with its known normal intracellular distribution in various regions of the mature brain in other animal species and is in keeping with its presumed synaptic function (Maroteaux and Scheller, 1991; Clayton and George, 1998; Lavedan, 1998). At the cellular level, most of the immunoreactivity was located in the cytoplasm of α-synuclein-positive perikarya and proximal processes, with some also present in the nuclei. Although we did not formally study other structures of the basal ganglia, robust α-synuclein immunostaining was also observed in the striatum, which showed high immunoreactivity, followed by the subthalamic nucleus, the globus pallidus, and the thalamus, all of which showed moderate-to-low immunoreactivity. This regional and cellular pattern of α-synuclein immunostaining agrees with that previously reported (Maroteaux and Scheller, 1991; Tobe et al., 1992; Irizarry et al., 1996; Lavedan, 1998), although the originally described nuclear localization of α-synuclein immunoreactivity, which we also observed in neurons of the normal mouse SNpc, has not been observed by all investigators.

This study also defines the topographical and temporal relationship between α-synuclein expression and neuronal degeneration following MPTP administration by using two different regimens of MPTP injections: one, called acute, kills SNpc dopaminergic neurons by necrosis, whereas the other, called chronic, kills SNpc dopaminergic neurons by apoptosis (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). It appeared important to ascertain the α-synuclein response in the context of these two morphologically defined modes of cell death, as they represent the two current schools of thought regarding forms of cell death in PD (Burke and Kholodilov, 1998). Indeed, for many years, the consensus has been that, in PD, as in any pathological brain condition, neurons die by necrosis. However, biochemical and morphological features of apoptosis have been identified in parkinsonian, but not in control, postmortem brain samples (Burke and Kholodilov, 1998). This has raised the possibility that apoptosis may be a mode of neuronal death not only during development and morphogenesis, but also in diseased mature brain as in PD. Thus, it is interesting that α-synuclein expression appeared dramatically altered in the mouse brain following only the chronic, and not the acute, regimen of MPTP. The discrepancy in the α-synuclein response to the acute and chronic regimen cannot be explained by the dose of toxin used because we and others have demonstrated that one injection of 30 mg/kg/day for 5 consecutive days (i.e., chronic regimen) or four injections of 20 mg/kg every 2 h in 1 day (i.e., acute regimen) cause, in both cases, a loss of SNpc dopaminergic neurons that is, in this strain of mice, ~60% (Jackson-Lewis et al., 1995; Tatton and

Kish, 1997). The difference cannot be explained by the time points studied either, as those cover the SNpc dopaminergic neurodegeneration produced by both the chronic and acute regimen (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). Therefore, at this point the most parsimonious explanation may be that α-synuclein alteration in the SNpc is associated specifically with the occurrence of apoptosis. Along this line, it is relevant to point out that the time course of α-synuclein up-regulation paralleled that of neuronal death produced by the chronic MPTP regimen, by either preceding or coinciding with the wave of actual cell degeneration. It is also important to mention that changes in α-synuclein expression followed the regional specificity of MPTP neurotoxic effects because α-synuclein mRNA and protein expression was increased in ventral midbrain, and specifically in dopaminergic neurons of the SNpc, but not in other structures, such as the cortex or cerebellum. The absence of detectable α-synuclein alterations in the striatum, despite definite MPTP-induced damage to SNpc projecting nerve fibers in this structure, likely reflects the small percentage that dopaminergic terminals represent in the striatum relative to the total pool of terminals (Descarries et al., 1996). On the other hand, increased α-synuclein expression does not seem to be part of a common response of synaptic-related proteins to MPTP injury because the expression levels of synaptophysin, another synapse-associated protein, were either unchanged or decreased after chronic MPTP intoxication. It is also noteworthy that the increase in the number of α-synuclein-immunoreactive cells lasted longer than the increases in the levels of α-synuclein mRNA and protein. Indeed, following MPTP injection, levels of α-synuclein protein detected by western blot were close to the levels found in saline-injected controls by day 7, whereas the number of α-synuclein-positive neurons was still increased by this time point and only appeared to return to baseline after day 14. This can be explained by the fact that although neurons may remain unquestionably immunoreactive beyond day 7, their actual α-synuclein protein content may have already decreased significantly. Changes in α-synuclein expression within the dense α-synuclein-positive fiber network in the substantia nigra may also contribute to this discrepancy. It is also important to mention that α-synuclein, which is primarily a synaptic-associated protein, is initially produced in the cell body and then rapidly transported to the nerve terminals (Withers et al., 1997). Accordingly, as MPTP damages terminals first and most severely (Herkenham et al., 1991; Jackson-Lewis et al., 1995), it is possible that the apparent increased content of ventral midbrain α-synuclein protein and number of SNpc α-synuclein-positive neurons result from an impaired anterograde transport of the protein and its subsequent accumulation at the site of synthesis. Although this possibility cannot be excluded with certainty, the observation that levels of α-synuclein mRNA are also increased rather supports the view that following MPTP administration α-synuclein is up-regulated and not solely accumulated in the cell bodies of SNpc dopaminergic neurons.

Altogether our data raise the possibility that α-synuclein up-regulation, which occurs in the specific context of MPTP-induced apoptotic death in SNpc dopaminergic neurons, contributes to the cascade of deleterious events that ultimately kill these cells. For instance, in light of the synaptic location of α-synuclein, it may be envisioned that its up-regulation may affect the normal synaptic machinery, which, in turn, may disturb the trophic support of SNpc dopaminergic neurons originating from the striatum. This hypothesis is of particular relevance to apoptosis because, in many settings, deprivation of the target-derived trophic support triggers a massive apoptotic death among the projecting neurons (Burke and Kholodilov, 1998). By using a two-hybrid system, it has been demonstrated that α-synuclein can bind to intracellular proteins (Engelender et al., 1999). Although, among those, none seemed to belong to the large family of apoptotic-related proteins, it remains plausible that up-regulation of α-synuclein may alter the normal intracellular trafficking of certain proteins that, like many of the Bcl-2 family members, depend on being at a specific intracellular location to exert their regulatory effects on apoptosis (Merry and Korsmeyer, 1997). There is also compelling evidence to indicate that α-synuclein has a significant propensity to aggregate, and that this property can be enhanced by the familial PD-linked mutations or by posttranslational modifications, such as produced by oxidative stress (Conway et al., 1998; El-Agnaf et al., 1998; Giasson et al., 1999; Hashimoto et al., 1999; Narhi et al., 1999). Relevant to this is the fact that α-synuclein is present in high amounts in the intraneuronal inclusion LB, which is regarded by some as a key factor in the demise of SNpc dopaminergic neurons in PD (Spillantini et al., 1997, 1998). In the MPTP mouse model, we failed to identify any evidence supporting the formation of intraneuronal inclusions in SNpc dopaminergic neurons, whether they were α-synuclein-immunoreactive or not. This rules out the possibility that α-synuclein up-regulation, should it play a role in the MPTP-induced neurotoxic process, does so through the formation of LB-like inclusions. However, except for old-aged monkeys (Forno et al., 1988), it is known that MPTP does not stimulate the formation of LB-like inclusions, and thus the MPTP model may not be suitable to study the role of α-synuclein aggregation in the SNpc dopaminergic neurodegenerative process. Arguing against a role of α-synuclein up-regulation in the MPTP neurotoxic process is the fact that although we found a close regional and temporal relationship between α-synuclein up-regulation and induced neuronal death, we did not find any definite association at a cellular level between α-synuclein protein expression and morphological features of apoptosis: chromatin clumps, as evidenced by thionin or fluorescent DNA dyes (Clarke and Oppenheim, 1995; Suzuki et al., 1997), could not be unequivocally colocalized with α-synuclein immunoreactivity in

neurons. We cannot exclude, however, that at this advanced stage of cellular injury, there is not a loss of immunoreactivity, which may account for the difficulty in colocalizing apoptotic features with α-synuclein immunostaining. For example, it is notorious that only a small fraction of the SNpc apoptotic neurons seen during normal development retain their TH immunoreactivity (Oo and Burke, 1997). It should also be mentioned that apoptotic profiles found in a brain section at any given time following the chronic MPTP regimen are few in number and that apoptotic cells undergo a rapid turnover estimated at only a few hours (Oppenheim, 1991), making the likelihood of identifying both apoptotic features and α-synuclein immunoreactivity within the same neuron quite low.

As mentioned above, a main target of MPTP toxicity is the dopaminergic nerve terminals of the striatum (Herkenham et al., 1991; Jackson-Lewis et al., 1995). Therefore, one may speculate that our results, rather than being the expression of a death process, can represent the expression of a synaptic plasticity response. Supporting this view is the observation that the avian α-synuclein homologue synelfin is specifically up-regulated during early stages of song learning in zebra finch, suggesting a role for the synuclein family in shaping up the synaptic network (George et al., 1995). Furthermore, α-synuclein up-regulation in the substantia nigra during postnatal development in rats coincides with the time of maximal synaptogenic activity (Burke and Kholodilov, 1998); similar observations have been reported at the level of the hippocampus and cerebral cortex (Petersen et al., 1999). Conversely, we may argue against this plasticity hypothesis by emphasizing the fact that, in this case, we would have expected to see similar α-synuclein changes after the acute and chronic MPTP regimens, and not only in the levels of α-synuclein, but also in the levels of other synaptic proteins, such as synaptophysin.

Most of the α-synuclein-positive neurons had a healthy morphological appearance following MPTP administration. This observation may suggest a third possibility for α-synuclein up-regulation, that is, it represents a cellular attempt to survive MPTP injury. Consistent with this is the observation that α-synuclein up-regulation also seemed to involve surviving SNpc neurons in the model of programmed cell death induced by developmental striatal target lesion in rats (Burke and Kholodilov, 1998). In the context of Alzheimer's disease, it has been reported that surviving synapses in Alzheimer's brains contain a higher concentration of α-synuclein compared with controls (Masliah et al., 1996). Also relevant to the speculated beneficial role of α-synuclein up-regulation are the observations that α-synuclein is a potent inhibitor of phospholipase D2 (Jenco et al., 1998) and that, by nullification of phospholipase A2, which is a closely related member of this family, mice become resistant to MPTP (Klivenyi et al., 1998).

In summary, this study reports on alterations of α-synuclein expression in the MPTP mouse model of PD and its relationship to the mode of neuronal death, as well as the time course and the regional specificity of these changes. These data provide valuable descriptive information regarding α-synuclein in both the normal and the injured dopaminergic nigrostriatal pathway. In addition, the observed changes in α-synuclein expression, whether they are part of a death or, on the contrary, of a surviving response, bring to light significant alterations within the SNpc neurons that could be targeted for the development of new neuroprotective therapies for PD.

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Nitration and Inactivation of Tyrosine Hydroxylase by Peroxynitrite*

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From the [a]Stokes Research Institute and Department of Biochemistry and Biophysics, Children's Hospital of Philadelphia and The University of Pennsylvania, Philadelphia, Pennsylvania 19104, the [c]Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27106, the [d]Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66047, the [b]Departamento de Bioquimica, Facultad de Medicina and [e]Laboratorio de Enzimologia, Facultad de Ciencas, Universidad de la Republica, Montevideo 11800, Uruguay, the [g]Departments of Neurology and Pathology, Neuroscience Research, Movement Disorder Division, Columbia University, New York, New York 10032, and the [i]Department of Pharmacology and Physiology, M. C. P. Hahnemann School of Medicine, Philadelphia, Pennsylvania 10912

Tyrosine hydroxylase (TH) is modified by nitration after exposure of mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydrophenylpyridine. The temporal association of tyrosine nitration with inactivation of TH activity *in vitro* suggests that this covalent post-translational modification is responsible for the *in vivo* loss of TH function (Ara, J., Przedborski, S., Naini, A. B., Jackson-Lewis, V., Trifiletti, R. R., Horwitz, J., and Ischiropoulos, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 7659–7663). Recent data showed that cysteine oxidation rather than tyrosine nitration is responsible for TH inactivation after peroxynitrite exposure *in vitro* (Kuhn, D. M., Aretha, C. W., and Geddes, T. J. (1999) *J. Neurosci.* 19, 10289–10294). However, re-examination of the reaction of peroxynitrite with purified TH failed to produce cysteine oxidation but resulted in a concentration-dependent increase in tyrosine nitration and inactivation. Cysteine oxidation is only observed after partial unfolding of the protein. Tyrosine residue 423 and to lesser extent tyrosine residues 428 and 432 are modified by nitration. Mutation of Tyr^{423} to Phe resulted in decreased nitration as compared with wild type protein without loss of activity. Stopped-flow experiments reveal a second order rate constant of $(3.8 \pm 0.9) \times 10^3\ M^{-1}\ s^{-1}$ at pH 7.4 and 25 °C for the reaction of peroxynitrite with TH. Collectively, the data indicate that peroxynitrite reacts with the metal center of the protein and results primarily in the nitration of tyrosine residue 423, which is responsible for the inactivation of TH.

Tyrosine hydroxylase (TH)[1] (EC 1.14.16.2) is a non-heme iron, tetrahydrobiopterin-dependent protein that catalyzes the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA) and represents the rate-limiting step in the biosynthesis of catecholamines (1). Loss of ability to synthesize catecholamines is an important step in the development of Parkinson's disease (PD) and other neurodegenerative diseases (2–6). Early loss of TH activity followed by a decline in TH protein is thought to contribute to the dopamine deficiency and phenotypic expression in PD and the MPTP mouse model (4). Tyrosine hydroxylase is a selective target for nitration following administration of the parkinsonian toxin MPTP to mice and following exposure of PC12 cells to either peroxynitrite or 1-methyl-4-phenylpyridiniun ion (7). Nitration of one or more tyrosine residues of TH was temporally associated with loss of enzymatic activity. The magnitude of inactivation was proportional to the number of TH molecules that were nitrated in PC12 cells. In the mouse striatum, the tyrosine nitration-mediated loss in TH activity parallels the decline in dopamine levels whereas the levels of TH protein remain unchanged for the first 6 h post-MPTP injection (7).

However, a recent report indicated that exposure of recombinant purified TH to peroxynitrite *in vitro* results not only in nitration of tyrosine residues but also in the formation of covalently linked dimers and oxidation of cysteine residues (8). The same report also indicated that cysteine oxidation rather than tyrosine nitration is responsible for the loss of TH enzymatic activity (8). Cysteine, methionine, tryptophan, and tyrosine appear to be the principal amino acids in proteins modified by peroxynitrite *in vitro* (9–14). To resolve the apparent differences, the reaction of peroxynitrite with recombinant purified rat TH *in vitro* was re-examined, and no evidence of cysteine oxidation was found. Oxidation of one cysteine residue per molecule of TH was observed only at high peroxynitrite concentrations, and three cysteine residues were oxidized in partially unfolded protein. Amino acid analysis failed to show any

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[1] The abbreviations used are: TH, tyrosine hydroxylase; DAMBI, 4-(dimethylamino)phenylazophenyl-4′-maleimide; L-DOPA, L-dihydroxyphenylalanine; DTNB, 5,5′-dithiobis(2-nitrobenzene); DTPA, diethylenetriaminepentaacetic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydrophenylpyridine; NEM, *N*-ethylmaleimide; PD, Parkinson's disease; GST, glutathione *S*-transferase; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; OPA, *ortho*-phenyldialdehyde; CD, circular dichroism; GdmHCl, guanidinium hydrochloride.

alteration of methionine, tryptophan, or any other amino acid residues. Digestion and sequence analysis of peptides indicated that nitration of tyrosine 423 is the primary residue modified by peroxynitrite, which was further confirmed by a significant decrease in nitration of the $Tyr^{423} \rightarrow Phe$ mutant TH expressed in *Escherichia coli*. In addition, no loss of TH enzymatic activity was detected after peroxynitrite treatment of the $Tyr^{423} \rightarrow Phe$ mutant TH. Stopped flow experiments revealed reactivity with the ferrous iron in TH typical of metalloproteins reacting with peroxynitrite (15–17). The absence of other amino acid modifications at low peroxynitrite concentrations suggests that nitration of tyrosine 423 is responsible for the inactivation of TH by peroxynitrite.

MATERIALS AND METHODS

Purification and Activity Assay of TH—Recombinant tyrosine hydroxylase was isolated from BL21(DE3)pLysS *E. coli* expressing the full-length cDNA clone isolated from a rat pheochromocytoma library (18). *E. coli* were grown in LB broth in the presence of 0.1 mM $FeSO_4$ to midlog phase and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for another 2 h. Addition of $FeSO_4$ is essential to retain functional protein, because in the absence of ferrous iron salt, only 60% of the purified GST-TH fusion contained metal (19). The *E. coli* pellets were suspended in a 10-fold excess of 0.05 M Tris-HCl, pH 7.2 (w/v), and homogenized according to the method of Wang *et al.* (20). This mixture was sonicated with four 30-s pulses at 20% power. After centrifugation, the supernatant was discarded and the pellet was resuspended in the same volume. The mixture was then sonicated with ten 30-s pulses. The supernatant was collected by centrifugation and removed. The pellet was resuspended and sonicated once again. The combined supernatants were precipitated with ammonium sulfate. The fraction between 30 and 42% was purified further by column chromatography on heparin-Sepharose CL-6B. Tyrosine hydroxylase was eluted with a KCl gradient (0–0.7 M) in 0.05 M phosphate buffer, pH 6.5. The purity of peak fractions was evaluated by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue and was found to be greater than 95%.

Tyrosine hydroxylase was assayed by the release of $[^3H]H_2O$ from [^{3}H]tyrosine in the presence of catalase (21, 22). Approximately 5 μg of purified tyrosine hydroxylase was assayed in a volume of 30 μl containing the following additions: 50 mM MES, pH 5.5, 1 mM 6(*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (Alexis), 1500 units/ml catalase, 5 mM dithiothreitol, 5 mM $FeSO_4$, 0.10 mM tyrosine, 500,000 dpm of L-[*ring*-3,5-^{3}H]tyrosine (specific activity 50 Ci/mmol, PerkinElmer Life Sciences). The reaction was incubated for 2 min at 37 °C. At the end of the incubation, 300 μl of a cold suspension containing 7.5% activated charcoal in 1 N HCl was added to each tube. After vortexing and centrifugation, 100 μl of supernatant was counted by liquid scintillation spectrometry. Blanks contained buffer instead of enzyme.

Treatment of TH with Peroxynitrite—Peroxynitrite was synthesized from sodium nitrite and acidified hydrogen peroxide, as previously described (23), and treated with manganese dioxide to remove residual hydrogen peroxide. The concentration of peroxynitrite was determined spectrophotometrically by the measurement of absorption at 302 nm in 1 N NaOH ($\epsilon = 1670\ M^{-1}.cm^{-1}$). Dilutions in 0.1 N NaOH were made immediately before use to achieve the desired concentrations.

The protein was first dialyzed against 50 mM phosphate buffer at pH 7.4, and protein concentration was determined by the Bradford assay. Tyrosine hydroxylase was then diluted to a final concentration of 18 μM in 0.1 M phosphate buffer containing 0.1 mM DTPA, pH 7.4, and finally treated with one bolus of 25–500 μM peroxynitrite (1% v/v) at room temperature while stirring. In reverse order experiments, peroxynitrite was added to the buffer before TH addition.

Analysis of TH Sulfhydryl Content—The effect of peroxynitrite on TH sulfhydryl content was determined by ThioGlo-1 assay (24), by 5,5′-dithiobis(2-nitrobenzene) (DTNB) reaction (25) and by labeling with 4-(dimethylamino)phenylazophenyl-4′-maleimide (DAMBI) (26). A 26 mM solution of ThioGlo-1 (Covalent Associates Inc., MA) was prepared in acetonitrile. The protein (0.2 μM) was incubated with 7 M guanidine HCl overnight in phosphate buffer, 50 mM, pH 7.4, before ThioGlo-1 addition. ThioGlo-1 (10 μM) was added to the denatured TH, and the incubation was continued for another 7 h at room temperature. The reaction was monitored by fluorescence spectroscopy with the excitation wavelength set at 384 nm and the emission at 513 nm. The emission values of a series of *N*-acetyl-L-cysteine standards modified with ThioGlo-1 were used to establish a standard curve of fluorescence.

Amino Acid Analysis—Amino acid analysis of TH was performed by hydrolyzing the protein either in the gas phase (HCl) or with methanesulfonic acid. Gas phase acid hydrolysis was achieved with 6 N HCl and 1% (w/v) phenol under vacuum (~40 millitorr) at 115 °C for 22 h, as described by Meltzer *et al.* (27). Protein hydrolysates were analyzed by reverse-phase HPLC (Varian 9012) on an Inertsil C18 ODS3 column (250 × 4.6 mm) using pre-column derivatization with phenylisothiocyanate (PITC) and absorbance detection at 254 nm (Varian 9050). Samples were dissolved in mobile phase, injected onto the column equilibrated with 10 mM potassium phosphate buffer, pH 6.5 (phase A) at 40 °C, and eluted at 1 ml/min by an increase of mobile phase B (75% acetonitrile/25% phase A) to 11% at 15 min, to 14% at 17 min, to 36% at 39 min, and to 45% at 48 min. Amino acids were quantified using the chromatographic peak area, compared with both an amino acid standard (Pierce), and hydrolysis of a known concentration of bovine serum albumin hydrolyzed under similar conditions. Methane sulfonic acid hydrolysis of protein samples was used for the determination of tryptophan, methionine, and methionine sulfoxide, which are usually unstable under conditions of HCl hydrolysis. Samples were hydrolyzed with 4 M methane sulfonic acid at 115 °C for 22 h in an evacuated chamber (~40 millitorr). After hydrolysis, samples were neutralized by the addition of an equal volume of 3.5 M NaOH. They were analyzed by reverse-phase HPLC on a Supelcosil LC-18T column (150 × 4.6 mm), following pre-column derivatization with *ortho*-phenyldialdehyde (OPA), and fluorescence detection (330 and 450 nm, excitation and emission wavelength, respectively) as described previously (28). Samples were injected onto the column equilibrated at 35 °C with mobile phase A, containing 95% 25 mM sodium acetate buffer (pH 5.8) and 5% tetrahydrofuran, and eluted at 0.7 ml/min by the following gradient of mobile phase B (95% methanol and 5% tetrahydrofuran): 0–15% B between 0 and 0.5 min, 15–45% B from 10 to 20 min, and 45–100% B between 30 and 40 min. Amino acids were quantified as described above for the PITC method.

Circular Dichroism (CD)—CD spectra were obtained using a Jasco J720 instrument at room temperature in a 0.2-mm path length cell with the concentration of protein being ~0.2 mg/ml. Curves were baseline-corrected and smoothed by the algorithm provided by Jasco. Mean residue ellipticity, $[\Theta]_{MR}$, is expressed in deg × cm^2/dmol using mean residue masses of 110.

Analysis of 3-Nitrotyrosine Content—After treatment with peroxynitrite, TH (0.5 μg) was subjected to a 10%-SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membrane, and probed with either a polyclonal anti-TH (Calbiochem) or an affinity-purified polyclonal anti-3-nitrotyrosine antibody. The blots were blocked with 10% dry milk in Tris-buffered saline containing 0.5% Tween 20 for 1 h, prior to antibody addition. The polyclonal anti-TH antibody was used at 1:10,000 dilution in 1% dry milk for 2 h, and the anti-3-nitrotyrosine antibody was used at 1:5000 dilution for 3 h. The anti-3-nitrotyrosine antibody was pre-conjugated with the secondary antibody in 3% dry milk overnight at 4 °C. After several washings, probed membranes were incubated with the horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution. After washing, immunoreactive signals were revealed using a chemiluminescence assay (ECL kit, Amersham Pharmacia Biotech). Samples containing untreated or peroxynitrite-treated TH were incubated with freshly prepared solution of *Streptomyces griseus* protease (Pronase, Sigma Chemical Co.) to yield 0.1 mg of protease/mg of protein. Pronase-treated samples were incubated for 18 h at 50 °C and centrifuged through a 10,000 molecular weight cut-off filter. Samples were then dried down, re-suspended in 40 μl of water of HPLC quality, and frozen at −80 °C until analysis. HPLC with electrochemical detection was performed on an ESA model 5600 CoulArray instrument equipped with eight-channel detector operating in the oxidative mode at specified potentials (channel/potential (in mV) = 1, 400; 2, 450; 3, 500; 4, 640; 5, 660; 6, 680; 7, 810; and 8, 810). Analyte separation was conducted on a TOSOHAAS (Montgomeryville, PA) reverse-phase ODS 80-T_M C-18 analytical column (4.6-mm inner diameter × 25 cm; 5-μm particle size) using isocratic elution consisted of 50 mM sodium acetate, 50 mM citric acid, pH 4.7, with 10% methanol. The concentration of 3-nitrotyrosine was determined from the area under the curve using a standard curve of authentic 3-nitrotyrosine from the seventh electrode.

Stopped-flow Experiments—The kinetics of peroxynitrite reaction with TH were studied with or without removing the iron from TH. Accordingly, the same preparation of the enzyme was divided in two parts. One fraction was incubated with ferrous ammonium sulfate (1 mM) for 60 min at 4 °C to obtain the holoenzyme with full complement of iron, and the other was dialyzed against 1,10-phenantroline (1 mM) in 100 mM potassium phosphate buffer, pH 7.35 at 4 °C, to obtain the apoenzyme (29). Moreover, fractions of the TH holoenzyme were incu-

bated either with β-mercaptoethanol (10 mM) or with *N*-ethylmaleimide (NEM, 10 mM) for 30 min at 4 °C. Enzyme preparations were then extensively dialyzed against 100 mM potassium phosphate buffer, pH 7.35 at 4 °C. Protein concentration was determined by measuring the absorbance at 280 nm ($A^{1\%}$ = 10.4 cm^{-1}) (29), which correlated well with Bradford protein measurements. The cysteine content in the enzyme was determined by the reaction of 4 μM protein with 0.2 mM of 5,5′-dithiobis(2-nitrobenzene) (DTNB) in the absence or presence of 6 M guanidine at pH 7.8 and room temperature for 90 min. The cysteine concentration was determined by the absorbance at 412 nm (ϵ = 13,600 $M^{-1}cm^{-1}$) (25). The rate constant at 37 °C could not be evaluated due to protein aggregation, which significantly interfered with the absorbance determinations.

The kinetics of peroxynitrite decay in the presence of TH at pH 7.4 and 25 °C were followed in a stopped-flow spectrophotometer (Applied Photophysics, SF17MV) at 302 nm. An initial rate approach was used to analyze the data, the first 1 s was fitted to a linear plot, and the apparent rate constant of peroxynitrite decay was determined as the ratio between the slope ($-dA/dt$) and the difference between the initial and final absorbance ($A_o - A_f$). After subtracting the apparent rate constant of peroxynitrite decay in the absence of enzyme and dividing by the enzyme concentration, we determined the second order rate constant of peroxynitrite reaction with the enzyme. To assure the accuracy of the determination, 200 absorbance measurements were acquired during the first 1 s of the reaction, and 200 points were acquired until more than 99.9% peroxynitrite had decomposed (1–20 s) (13, 30).

Identification of the Nitrated Tyrosine Residue(s)—Un-reacted control or nitrated TH (18 μM) was exhaustively dialyzed against 100 mM ammonium bicarbonate, pH 7.8. The TH samples were then treated with endoproteinase AspN from *Pseudomonas fragi* (Calbiochem, La Jolla, CA) or sequencing grade-modified trypsin (Promega, WI) at a ratio 1:100 w/w (proteinase/TH) overnight at 37 °C. The samples were dried down and resuspended in 0.1% trifluoroacetic acid. The peptides were analyzed by a Hewlett Packard HPLC system with a diode array detector using an octadecyl silica gel reverse-phase column (5 μm, 4.6 × 250 mm, Jupiter, Phenomenex, Torrance, CA) and 0.1% trifluoroacetic acid in ultra pure water (solvent A) and acetonitrile as solvent B. Peptides were eluted using an increasing linear gradient of B from 0% to 45% in 60 min with a flow rate of 1 ml/min. The HPLC detector was set at 210, 275, and 365 nm. The peptides with absorbance at 365 nm were collected and subjected to conventional Edman degradation at the Protein Core Facility at the Wistar Institute (Philadelphia, PA).

Expression of Mutant TH and Treatment with Peroxynitrite—To elucidate a possible role for Tyr^{423} as a substrate for nitration, the mutant Tyr^{423} → Phe were created with the QuikChange site-directed mutagenesis kit from Stratagene. Wild type TH (25 ng) was used as the DNA template to create Tyr^{423} → Phe. The conditions utilized are outlined in previous studies (31, 32). The primers used (but not previously reported) were as follows: TH Y423F Sense, 5′-GCA GCT GTG CAG CCC TTC CAA GAT CAA ACC TAC C-3′; Antisense, 5′-G GTA GGT TTG ATC TTG GAA GGG CTG CAC AGC TGC-3′. The underlined nucleotides highlight the mutated codons for amino acid 423. The DNA Sequencing and Gene Analysis Facility of the Molecular Genetics Program (Wake Forest University School of Medicine) performed a complete DNA sequencing by using a PerkinElmer Life Sciences/Applied Biosystems 377 Prism automated DNA sequencer. Complete DNA sequencing was done to verify the presence of the appropriate mutation in the coding sequences of all recombinant proteins. This also established that the non-polymerase chain reaction-based mutagenesis (Tyr^{423} → Phe) did not introduce extraneous mutations. All recombinant proteins were expressed using the approach detailed above for the wild type TH. After ammonium sulfate precipitation, the partially purified wild type and Tyr^{423} → Phe mutant TH (2 mg/ml) were reacted with several concentrations of peroxynitrite under identical conditions, in phosphate buffer (0.1 M) containing DTPA (0.1 mM) at pH 7.4. For some experiments, partially purified wild type and Tyr^{423} → Phe mutant TH were treated with 5 mM NEM for 2 h at 37 °C. The proteins were then dialyzed overnight against 50 mM phosphate buffer, pH 7.4, and the activity was determined. The activity of TH was assayed by the release of $[^3H]H_2O$ from [^{3}H]tyrosine in the presence of catalase. The specific activity of the partially purified Tyr^{423} → Phe mutant TH was 20% of the wild type TH.

RESULTS

Lack of Cysteine Oxidation after Exposure of TH to Peroxynitrite—Purified recombinant rat TH was reacted with different concentrations of peroxynitrite, and fractions of the treated and control protein were analyzed for activity and for the concentration of reduced cysteine residues. Exposure to peroxynitrite resulted in a dose-dependent inactivation of TH activity (Table I). The concentration of cysteine residues in TH was determined by three different methods: ThioGlo-1, a maleimide-derivatized naphthopyranone, 5,5′-dithiobis(2-nitrobenzene) (DTNB), and 4-(dimethylamino)phenylazophenyl-4′-maleimide (DAMBI) labeling (24–26). Data in Table I show that in the unreacted protein seven cysteine residues are detected per TH monomer as predicted from the rat TH sequence. No evidence for cysteine oxidation was observed after treatment with up to 250 μM peroxynitrite whereas the TH enzymatic activity was already inhibited by more than 50%. With higher peroxynitrite concentration (500 μM), one cysteine residue per monomer is oxidized. The same results are obtained with DTNB and DAMBI labeling (not shown). These data suggest that TH activity was inhibited after exposure to peroxynitrite without evidence for oxidation of cysteine residues. Moreover, in addition to cysteine oxidation, the reaction of proteins in simple buffers with peroxynitrite has been shown to modify methionine, tryptophan, and tyrosine residues (10–14). Hydrolysis using methane sulfonic acid to analyze tryptophan and methionine as well as methionine sulfoxide failed to show any evidence for tryptophan and methionine oxidation (Table II). Complete amino acid analysis of peroxynitrite exposed TH, using gas phase HCl hydrolysis and PITC pre-column derivatization, did not detect any significant modification of any other amino acid residue (data not shown).

TABLE I
Cysteine oxidation and tyrosine hydroxylase activity

TH (18 μM) was reacted with peroxynitrite for 10 min at room temperature. Aliquots from each reaction were removed to measure TH activity and cysteine content as described under "Materials and Methods."

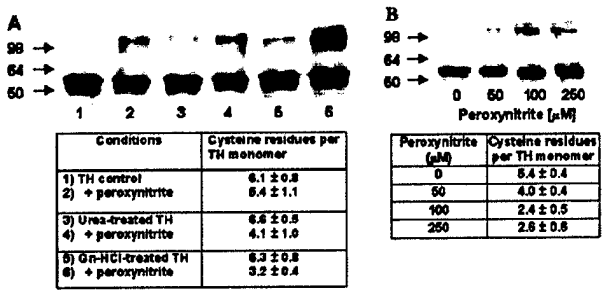
Peroxynitrite	Cysteine residues per TH monomer	Tyrosine hydroxylase activity
μM		% of control
0	6.8 ± 0.3	100
25	6.7 ± 0.4	79 ± 11
50	5.8 ± 1.2	71 ± 11
100	6.2 ± 0.8	57 ± 6
250	6.5 ± 0.6	38 ± 10
500	5.4 ± 1.1	25 ± 11

Peroxynitrite-induced Cysteine Oxidation in Partially Unfolded Tyrosine Hydroxylase—Tyrosine hydroxylase was partially unfolded with either 7 M urea or 6 M of guanidinium hydrochloride (GdmHCl) for 1 h at room temperature before peroxynitrite treatment. Exposure of the urea or GdmHCl partially unfolded protein to 500 μM peroxynitrite resulted in the oxidation of approximately three cysteine residues as 4.1 ± 1 and 3.2 ± 0.4 cysteine residues are detected in the reacted protein, respectively. Another fraction of the protein was analyzed by Western blotting with anti-TH antibodies, which revealed formation of SDS and heat-stable TH polymers (Fig. 1*A*). The polymerized TH was hydrolyzed, and the hydrolysates were monitored by fluorescence (λ_{ex} = 283 nm and λ_{em} = 410 nm) to determine the presence of dityrosine as reported previously (33). The hydrolysates of TH treated with 6 M GdmHCl and reacted with 500 μM peroxynitrite contain 1 ± 0.3 μM dityrosine indicating that one tyrosine residue in TH was oxidized to form dityrosine. The degree of cysteine and tyrosine oxidation in the unfolded protein was proportional to the peroxynitrite concentration (Fig. 1*B*). There was a significant degree of TH unfolding upon treatment with GdmHCl as revealed by the changes in the CD spectrum of the protein; the spectrum of the untreated protein and protein reacted with 1 M GdmHCl

TABLE II
Methionine and tryptophan analysis

Three different tyrosine hydroxylase preparations reacted with peroxynitrite were analyzed for the formation of methionine sulfoxide (MetSO), methionine, and tryptophan content as described under "Materials and Methods." The number indicates the number of residues measured per TH monomer. The reverse order experiment represents the addition of previously decomposed peroxynitrite to TH.

	TH						
	2 mg/ml			3 mg/ml		5 mg/ml	
	Control	$ONOO^-$ (100 μM)	Reverse order	Control	$ONOO^-$ (100 μM)	Control	$ONOO^-$ (100 μM)
MetSO/protein	0.9	1.08	0.45	0.8	0.9	0.9	0.8
Met/protein	4.1	4.8	4.1	4.8	4.9	4.2	5
Trp/protein	2.7	2.4	2.8	2.9	2.4	2.2	2.6



Conditions	Cysteine residues per TH monomer
1) TH control 2) + peroxynitrite	6.1 ± 0.8 5.4 ± 1.1
3) Urea-treated TH 4) + peroxynitrite	6.6 ± 0.5 4.1 ± 1.0
5) Gn-HCl-treated TH 6) + peroxynitrite	6.3 ± 0.8 3.2 ± 0.4

Peroxynitrite (μM)	Cysteine residues per TH monomer
0	5.4 ± 0.4
50	4.0 ± 0.4
100	2.4 ± 0.5
250	2.6 ± 0.8

FIG. 1. **Polymerization and cysteine oxidation of partially unfolded TH.** Tyrosine hydroxylase was treated with urea or guanidinium hydrochloride (GdmHCl) for 1 h at room temperature, centrifuged on a 10,000 molecular weight filter and diluted (18 μM) in 0.1 M phosphate buffer containing 0.1 mM DTPA, pH 7.4. *A*, the native and the partially unfolded TH was then reacted with 500 μM peroxynitrite and analyzed by Western blot analysis, using anti-TH antibodies. *Lanes 1, 3,* and *5* are control, urea-treated, and GdmHCl-treated protein; *lanes 2, 4,* and *6* are control, urea-treated, and GdmHCl-treated protein reacted with peroxynitrite. The *inset table* gives the cysteine content. *B*, tyrosine hydroxylase treated with 6 M GdmHCl was reacted with different concentrations of peroxynitrite and the degree of protein cross-linking and cysteine oxidation was evaluated as in *A*.

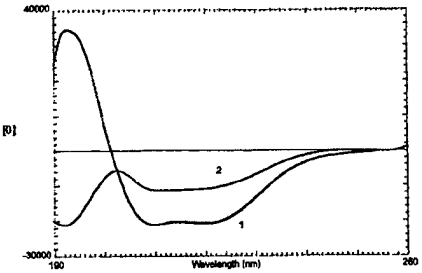


FIG. 2. **CD spectrum of TH (*1*) and of TH-treated with 1 M GdmHCl (*2*).** The spectrum of the untreated control protein is identical to published spectra of TH (34), and treatment with GdmHCl significantly alters the secondary structure of the protein. The cysteine content after exposure to 100 μM peroxynitrite was 6.2 ± 0.8 and 2.3 ± 0.5 for the control and the GdmHCl-treated protein, respectively.

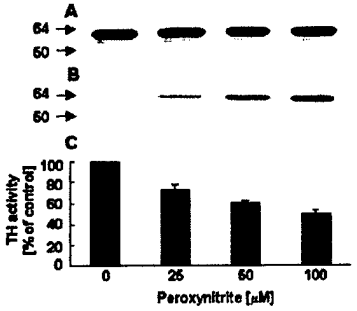


FIG. 3. **Exposure of TH to peroxynitrite results in tyrosine nitration and inhibition of catalytic activity.** *A*, Western blot analysis of 0.5 μg of protein using a polyclonal anti-TH and anti-3-nitrotyrosine antibody (*B*). *C*, inhibition of TH enzymatic activity (mean and standard deviation for three different preparations of TH) as a function of peroxynitrite concentration.

is shown in Fig. 2. Computer-assisted analysis of the CD spectra for the composition of different secondary structural elements indicated the presence of ~58% α-helical conformation in the monomeric TH similar to values reported previously (34, 35). The α-helical content of TH decreased to 11% upon GdmHCl treatment with a concomitant increase in random structure from 30 to 54%. Collectively these data suggested that the degree of cysteine oxidation and the formation of stable dityrosine-containing oligomers appeared to be facilitated by the unfolding of the protein.

Tyrosine Nitration following Exposure of TH to Peroxynitrite—Exposure of TH to a range of peroxynitrite concentrations induced a dose-dependent increase in the nitration of the protein, which correlated with the loss of enzymatic function (Fig. 3). The proportional loss of TH activity and nitration is similar to the data described in PC12 cells exposed to peroxynitrite (7). Assuming that one tyrosine residue is nitrated per TH molecule, then exposure of TH at 1, 2, and 3 mg/ml to 100 μM peroxynitrite resulted in the nitration of 59, 22, and 20% TH molecules, which correlated with 52 ± 0.1, 29 ± 2, and 18 ± 1.3% loss of TH enzymatic activity, respectively.

Identification of the Nitrated Tyrosine Residues—To identify the site of nitration, TH reacted with peroxynitrite was digested with either AspN or trypsin. The AspN-digested peptides were separated by reverse-phase HPLC, and two major peaks with absorbance at 275 and 365 nm eluted at retention times of 29.1 and 47.7 were collected and sequence analysis of the first 20 amino acids was performed. The following sequence, Asp-Thr-Ala-Ala-Val-Gln-Pro-*X*-Gln (*X* represents an unknown amino acid) was obtained for the 29.1-min peak. This sequence corresponds to the expected AspN peptide residues 416–424 (Asp^{416}-Thr-Ala-Ala-Val-Gln-Pro-Tyr-Gln^{424}), which contains a tyrosine residue in position 423. The peak with a retention time of 47.7 min contained two sequences, the major sequence (more than 90% abundance of amino acid residues) corresponding to peptide between residues 44 and 77, which does not contain any tyrosine residues. The minor sequence (less than 10%) was *X*-Gln-Thr-*X*-Gln-Pro-Val-*X*-Phe-Val-Ser-Glu-Ser-Phe, which corresponds to residues 425–439 (Asp^{425}-Gln-Thr-Tyr-Gln-Pro-Val-Tyr-Phe-Val-Ser-Glu-Ser-Phe-Asn^{439}), which includes tyrosine residues 428 and 432. The site of nitration was also confirmed by digestion of nitrated TH with trypsin. The first 15-amino acid sequence of a peak eluted at 73 min of the tryptic digestion was Ala-Phe-Asp-Pro-Asp-Thr-Ala-Ala-Val-Gln-Pro-*X*-Gln-Asp-Gln, which corresponds to the expected peptide spanning residues 412 to 442 that includes tyrosine residues 423, 428, and 432. Collectively, these data suggest that the primary site of nitration is tyrosine residue 423 and to a lesser extent tyrosine residues 428 and 432. To further confirm the site of nitration, Tyr^{423} was mu-

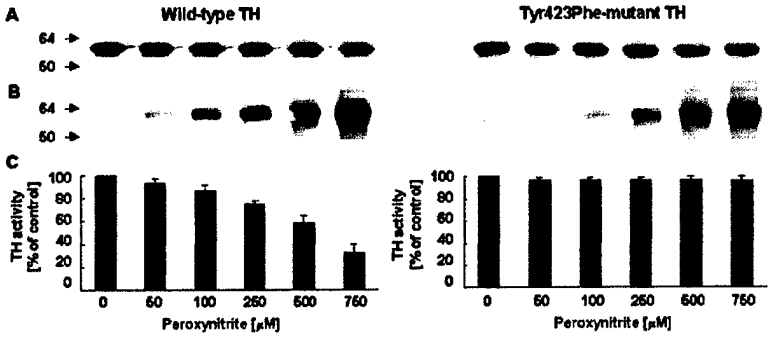


FIG. 4. **Exposure of the partially purified wild type and Tyr^{423} → Phe mutant TH to peroxynitrite.** *A*, wild type and Tyr^{423} → Phe mutant TH analysis by Western blot using a polyclonal anti-TH antibody. *B*, wild type and Tyr^{423} → Phe mutant TH analysis by Western blot using an anti-3-nitrotyrosine antibody, after treatment with a range of peroxynitrite concentrations. *C*, TH enzymatic activity (mean and standard deviation for three different preparations of TH) as a function of peroxynitrite concentration.

tated to Phe in TH, and the sequence of the mutant TH (Tyr^{423} → Phe) expressed in *E. coli* was confirmed. Wild type and mutant proteins expressed in *E. coli* were partially purified and exposed to peroxynitrite under identical conditions. Fig. 4 shows that there was less nitration of the partially purified Tyr^{423} → Phe mutant as compared with the wild type protein under the same protein and peroxynitrite concentrations. However, the activity of the Tyr^{423} → Phe mutant TH was not affected by nitration as compared with the wild type protein. The activity of the Tyr^{423} → Phe mutant TH, similar to the wild type was decreased by >99% after heat inactivation or removal of the ferrous iron for the active site (Table III). In the semi-purified preparation the K_m for tyrosine was 37.7 ± 2.1 μM for the wild type and 14.3 ± 2.6 μM for the Tyr^{423} → Phe mutant TH, which are similar to values reported for purified rat TH (34). The cysteine residues in the wild type and Tyr^{423} → Phe mutant TH appear to be equally reactive toward the alkylating agent *N*-ethylmaleimide (NEM). Treatment of the wild type and mutant proteins with NEM resulted in a significant inhibition of activity as shown in Table III. Moreover, NEM treatment also inhibited the activity of both the wild type and Tyr^{423} → Phe mutant TH proteins that had been reacted with peroxynitrite prior to NEM exposure (Table III). Collectively these data suggest that the cysteine residues were not modified by peroxynitrite exposure and that nitration of Tyr^{423} is the principal reason for the loss of enzymatic activity.

Determination of the Second Order Rate Constant for the Reaction of Peroxynitrite with Tyrosine Hydroxylase—Stopped-flow experiments were performed using TH treated either with ferrous ammonium sulfate to ensure full complement of iron (holoenzyme), or with 1,10-phenantroline, to remove the iron (apoenzyme). Peroxynitrite decomposition profiles at 302 nm were obtained by mixing peroxynitrite (75 μM) with 20 μM of either the holoenzyme or the apoenzyme. The second order rate constant determined for the holoenzyme was (3.8 ± 0.9) × 10^3 M^{-1} s^{-1} and (1.6 ± 0.8) × 10^3 M^{-1} s^{-1} for the apoenzyme, at pH 7.45 and 25 ± 1 °C. Moreover, no differences were detected between the second order rate constants obtained for TH reacted with mercaptoethanol, which had 5.3 ± 0.2 cysteine residues per monomer, and TH treated with NEM, which had 1.06 ± 0.05 cysteine residue per monomer (data not shown).

These data suggest that the contribution of the cysteine residues in the reaction of peroxynitrite with TH is relatively small, and the second order rate constants are typical of the reactions of peroxynitrite with metal centers (15–17, 30).

DISCUSSION

Tyrosine hydroxylase is the rate-limiting step in catecholamine synthesis, and thus the activity of this protein is critical for maintaining dopamine production. Inactivation of TH has been observed in early stages of PD as well as the mouse MPTP model of this disease (4). In the MPTP model of Parkinson's disease (6) previous data revealed that TH is a

TABLE III
Inactivation of wild type and Tyr^{423} → Phe mutant

Dialyzed partially purified wild type and Tyr^{423} → Phe mutant TH (2 mg/ml total protein) were reacted with peroxynitrite or NEM under identical conditions, in 0.1 M phosphate buffer containing 0.1 mM DTPA at pH 7.4. For heat inactivation, the proteins were boiled at 45 °C for 10 min, and apoproteins were prepared as described under "Materials and Methods." After the different treatments the proteins were dialyzed, and the activity was determined as described in detail under "Materials and Methods."

	Wild type TH	Tyr^{423} → Phe TH mutant
	% inhibition	
500 μM peroxynitrite	44 ± 3	3 ± 3
5 mM NEM	96	96
500 μM peroxynitrite + 5 mM NEM	93	96
Heat-inactivated	99.9 ± 0.1	99.8 ± 0.1
Apo-protein	99.8 ± 0.2	100 ± 0.1

protein specifically modified by nitration of tyrosine(s) residues. In addition, a temporal association between the number of TH molecules modified and loss of activity was observed (7). Amino acid analysis and fluorescence spectrometry of purified TH had failed to detect any other amino acid modification after nitration of the protein, and thus we proposed that tyrosine nitration is responsible for the inactivation of the protein (7). However, a publication by Kuhn and co-workers (8) indicated that cysteine oxidation and not tyrosine nitration was responsible for the inactivation of purified TH by peroxynitrite *in vitro*. Although cysteine residues are well-recognized targets for peroxynitrite (9, 13, 26), this study failed to detect oxidation of cysteine residues after exposure of purified recombinant rat TH to peroxynitrite as determined by three independent methods. Cysteine oxidation was evident only after exposure of the protein to large excess of peroxynitrite or when the protein is partially unfolded (Table I and Figs. 1 and 2). It is possible that the protein used by Khun *et al.* (8) was partially unfolded during purification or removal of the glutathione *S*-transferase (GST) tag. It has been reported that nearly 40% of TH purified with the GST tag has no metal in the active site (19), and we have observed that metal free TH apoprotein readily aggregates after dialysis and during storage suggesting some degree of protein unfolding (not shown). Therefore, partial protein unfolding and exposure to high peroxynitrite concentration can account for the oxidation of cysteine residues reported previously (8). Consistent with the observation of Khun *et al.* (8) we also observed formation of SDS and heat-stable TH dimers after exposure to relative high peroxynitrite concentrations and in partially unfolded protein before and after exposure to peroxynitrite (Fig. 1). The dimers appear to be the result of cross-linking via oxidation of tyrosine residues, because dityrosine was detected in the unfolded and peroxynitrite treated TH. Dityrosine cross-linking has been reported for proteins

with random secondary structure in solution (33) or globular proteins exposed to high peroxynitrite concentrations (36). Although protein cross-linking may be also responsible for the inactivation of TH, appreciable inactivation of the enzymatic activity is observed in the absence of protein dimers (Fig. 3) and other amino acid modifications suggesting that under these conditions tyrosine nitration is responsible for the loss of function (Tables I and II).

Peroxynitrite reactivity with proteins in simple buffers is determined by kinetic factors. The stopped-flow data suggest that the contribution of the ferrous iron in the reaction of peroxynitrite with TH is quite important, accounting for nearly 60% of the rate constant. The rate constant of the apoenzyme is typical of peroxynitrite reaction with protein amino acids (13) but precludes the existence of rapidly reacting cysteine residues, similar to those present in glyceraldehyde-3-phosphate dehydrogenase (37) and peroxiredoxins (38). Regarding the polypeptide chain, cysteine, methionine, and tryptophan are the primary amino acids that react with appreciable second order rate constants with peroxynitrite (13). In the case of TH, these amino acids were poorly oxidized after peroxynitrite exposure, similar to data reported previously for sarcoplasmic reticulum Ca-ATPase (39). Instead, at low peroxynitrite concentration, only tyrosine nitration was observed. At high peroxynitrite concentration, tyrosine nitration and oxidation were observed, which could be derived by the formation of hydroxyl and nitrogen dioxide radicals from peroxynitrite or by direct electrophilic substitution at the *ortho* position of the aromatic ring catalyzed by transition metals. However, the role of the ferrous iron of tyrosine hydroxylase in the nitration of the protein awaits further investigation.

The primary site of nitration was identified by digestion of the nitrated protein and peptide sequencing and further confirmed by mutational analysis (Fig. 4). The primary site of nitration was the tyrosine residue 423 and to a lesser extent tyrosine residues 428 and 432. Mutation of Tyr^{423} to Phe resulted in an appreciable decrease in tyrosine nitration and, more importantly, no loss of activity was observed after exposure to peroxynitrite. The specific activity of partially purified $Tyr^{423} \rightarrow$ Phe mutant was ~20–25% of the wild type protein suggesting that this tyrosine residue is critical for the enzymatic activity of the protein. However, similar to wild type protein, the mutant TH activity is completely lost upon heat inactivation and removal of the metal. Kinetic parameters such as the K_{Tyr} of the $Tyr^{423} \rightarrow$ Phe mutant were similar to the wild type, and the cysteine residues of both the wild type and $Tyr^{423} \rightarrow$ Phe mutant were sensitive to alkylation by NEM before or after treatment with peroxynitrite. Therefore, nitration of tyrosine 423 and not cysteine oxidation appears to be responsible for the inactivation of TH by peroxynitrite. However, it remains unclear how nitration of tyrosine residue 423 results in the inactivation of the protein. We speculate that it may relate to the critical positioning of this tyrosine residue near the active site of the protein. The active site of TH is located in the center of the catalytic domain (residues 188–456) and consists of a 17-Å deep cleft. The active site cleft is 30 Å long and 15 Å wide, and within the active site 10 Å below the enzyme surface His^{331}, His^{336}, and Glu^{376} residues bind ferrous iron needed for the catalysis of tyrosine hydroxylation to L-DOPA (35). The entrance to the active site is guarded by two loops (residues 423–428 and 290–296), which come within 12 Å of each other. Proline residues in either side of the loop break the α-helices, and Tyr^{423} starts the five-residue loop that ends with Tyr^{428}. The aromatic ring of Tyr^{423} is oriented toward the opposite loop on the plane of the entrance to the active site whereas the aromatic rings of Tyr^{428} and Tyr^{432} are oriented away from the entrance to the active site. The narrow point entering the active site can be viewed as a size selection process by which only small substrates may enter into the active site. The addition of the bulky nitro (NO_2) in the *ortho* position of the tyrosine 423, but not the tyrosine residues 428 and 432, will narrow the distance between the two loops by more than 2.5 Å, which may be sufficient to prevent the entry of the substrate tyrosine in the active site of the protein.

Overall, the data reported herein are consistent with the view that tyrosine nitration is responsible for the inactivation of TH. It is now apparent that nitration and oxidation of proteins is a widespread event in the affected areas in the brain of PD patients (40–43) as well as in the mouse and baboon MPTP models of this disease (2, 7, 45). More importantly, specific proteins such as TH and α-synuclein that may play a key role in the pathogenesis of PD are targets for modification by nitrating agents (7, 42). Efforts to limit the formation of nitrating agents by limiting the production of nitric oxide and superoxide have been successful in protecting mice and baboons from MPTP-induced neuronal death (45–47). Recently, Pong *et al.* (48) showed that EUK 134, a superoxide dismutase and catalase mimetic, prevented nitration of TH in cultured dopaminergic neurons after 1-methyl-4-phenylpyridinium challenge. Therefore, development of therapeutic agents that can prevent formation of nitrating agents without interfering with normal neuronal function or compounds that will specifically remove nitrating species may protect proteins from inactivation and provide means of limiting neuronal injury in PD.

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RAPID COMMUNICATION

Oxidative post-translational modifications of α-synuclein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease

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Abstract

Structural and functional alterations of α-synuclein is a presumed culprit in the demise of dopaminergic neurons in Parkinson's disease (PD). α-Synuclein mutations are found in familial but not in sporadic PD, raising the hypothesis that effects similar to those of familial PD-linked α-synuclein mutations may be achieved by oxidative post-translational modifications. Here, we show that wild-type α-synuclein is a selective target for nitration following peroxynitrite exposure of stably transfected HEK293 cells. Nitration of α-synuclein also occurs in the mouse striatum and ventral midbrain following administration of the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Conversely, β-synuclein and synaptophysin were not nitrated in MPTP-intoxicated mice. Our data demonstrate that α-synuclein is a target for tyrosine nitration, which, by disrupting its biophysical properties, may be relevant to the putative role of α-synuclein in the neurodegeneration associated with MPTP toxicity and with PD.

Keywords: dopaminergic neurons, MPTP, neurodegeneration, Parkinson's disease, substantia nigra, synuclein.
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Parkinson's disease (PD) is a common neurodegenerative disorder that can be of either familial or non-familial (i.e. sporadic) etiology (Fahn and Przedborski 2000). Cardinal clinical features of PD include tremor, stiffness and slowness of movement, all of which are attributed to the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski 2000). Mutations in the presynaptic protein α-synuclein (α-syn) are associated with a familial form of PD (Polymeropoulos *et al.* 1997) that is clinically and pathologically indistinguishable from the most common sporadic form of this disabling neurodegenerative disorder. Mutant α-syn cytotoxicity is likely related to the fact that both of the identified point mutations may enhance the propensity of α-syn to interact with other intracellular proteins and increase its tendency to aggregate (Conway *et al.* 1998; El-Agnaf *et al.* 1998; Engelender *et al.* 1999; Giasson *et al.* 1999; Narhi *et al.* 1999). Although similar α-syn mutations are not found in sporadic PD (Golbe 1999), mounting evidence indicates that α-syn may also play a deleterious role in sporadic PD (Spillantini *et al.* 1997, 1998). Relevant to these observations, we have recently reported widespread nitration of proteins, which is a consequence of the reaction of nitrating agents such as peroxynitrite with proteins (Ischiropoulos and al Mehdi 1995), in Lewy bodies (LBs) in sporadic PD, in dementia with LBs (DLB) and in the LB variant of Alzheimer's disease (Duda *et al.* 2000a). Moreover, using specific monoclonal antibodies that recognize only nitrated α-syn, we have also demonstrated that α-syn is the major protein that is modified by nitration in the LBs of the above neurodegenerative disorders as well as in neuronal and glial cytoplasmic inclusions (GCIs) in multiple system atrophy (MSA) and in Hallervorden–Spatz disease (HSD) (Giasson *et al.* 2000a). These data provide, for the first time, compelling evidence for the presence of nitrative stress and formation of nitrating agents in human neurodegenerative disorders. They also substantiate the hypothesis that oxidative stress is a leading pathogenic mechanism in neurodegenerative disorders including PD (Przedborski and Jackson-Lewis 2000). In addition, exposure of wild-type human α-syn to nitrating agents *in vitro*

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Abbreviations used: α-syn, α-synuclein; β-syn, β-synuclein; LBs, Lewy bodies; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 3-NT, 3-nitrotyrosine; PD, Parkinson's disease; SNpc, substantia nigra pars compacta.

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causes nitration and cross-linking via the formation of dityrosine, recapitulating some aspects of the α-syn extracted from the brains of humans with α-syn aggregates (Duda *et al.* 2000b; Galvin *et al.* 2000; Giasson *et al.* 2000a). Extending these observations and consistent with the oxidative-nitrative stress hypothesis, we now present compelling evidence that α-syn is a specific target for tyrosine nitration in stably transfected cells challenged with peroxynitrite, and in mice intoxicated with the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tertahydropyridine (MPTP) (Przedborski *et al.* 2000a).

Experimental procedures

Stable transfection of HEK 293 cells

Syn plasmids were constructed by inserting human α- or β-syn cDNAs (Jakes *et al.* 1994) into the mammalian expression vector pcDNA 3.1+ (Invitrogen, Carlsbad, CA, USA). HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in 90% Dulbecco's modified Eagle's medium (high glucose), 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. Cells were transfected with α-syn/pcDNA3.1+ or β-syn/pcDNA3.1+ using calcium phosphate precipitation buffered with N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) (Chen and Okayama 1997). One day following transfection, the cells were re-plated on 10-cm dishes and selection with Geneticin (500 μg/mL) (Life Technologies, Rockville, MD, USA) was initiated 24 h later. Individual stable clones were isolated with glass cylinders and detached from the dish with trypsin. Stable clones were re-plated and maintained in culture medium with Geneticin. Clones expressing high levels of syn were screened by western blot analysis.

Exposure of cells to peroxynitrite

Cells re-plated on six-well dishes in fresh culture medium were exposed to peroxynitrite by the addition of small drops above the cell surface and followed by rapid mixing to give a final concentration of 1 mM. The concentration of peroxynitrite was measured by the increase in absorbance at 302 nm in 1.2 M NaOH as described previously (Ara *et al.* 1998). The pH of the buffer was measured at the end of the exposure and was the same as before each addition of peroxynitrite. For all experiments, at the end of the incubation period, cells were washed with Earle's balanced salt solution, scraped-off of the plates and centrifuged at 8000 *g* for 5 min. The pellet was solubilized with lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 4 mM EGTA, 10% glycerol, 1% Triton-X 100]. As a control, peroxynitrite was allowed to decompose in cell media prior to exposure to the cells. This experiment controlled for hydrogen peroxide and nitrite, which are found in peroxynitrite solutions.

Mice and MPTP administration

Eight-week-old male C57/bl mice (22–25 g, Charles River Breeding Laboratories, Wilmington, MA, USA) were used. Animals were housed with three per cage in a temperature-controlled room under a 12-h light/12-h dark cycle with free access to food and water. Mice used in this study were treated according to the NIH guidelines for *Care and Use of Laboratory Animals* and with the approval of Columbia University's Institutional Animal Care and Use Committee. On the day of the experiment, mice received four i.p. injections of MPTP-HCl (20 mg/kg) in saline at 2 h intervals and were killed at 4 and 24 h post-injection; control mice received saline injections only. MPTP use and safety precautions were as described previously (Przedborski *et al.* 2000b). Frozen mouse striatal and ventral midbrain samples were homogenized (Polytron) in 5.0 mL of extraction buffer (phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and aprotinin at 10 mg/mL) at 4°C.

Immunoprecipitation and western blotting

Solubilized extracts from cells and mice were briefly sonicated and centrifuged (325 *g* for 5 min) to remove cellular debris. Protein concentration was determined using a BCA kit (Pierce, Rockford, IL, USA). Solubilized proteins (2 mg) were precleared (45 min, 4°C) with 15 μL of Gamma bind plus Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the supernatant was incubated (16 h, 4°C) with a rabbit anti-3-nitrotyrosine (3-NT) polyclonal antibody previously characterized (Beckman *et al.* 1994; Ye *et al.* 1996), or a mouse anti-α-syn monoclonal antibody (Syn-1) (Transduction Laboratories, Lexington, KY, USA). Immune complexes were absorbed (1.5 h, 4°C) to 50 μL of Gamma Bind plus Sepharose, extensively washed with extraction buffer by sequential sedimentation and resuspension, eluted in sample loading buffer by heating to 95°C for 5 min, and resolved by SDS–PAGE on 12–15% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose or PVDF membranes, which were blocked with 5% non-fat dry milk in 1 × TBS, 0.1% Tween 20 for 1 h. Incubation with one of the primary antibodies was performed overnight at 4°C using either Syn-1, anti-β-syn (a gift from Dr S. Nakajo, Tokyo, Japan), Syn 207 (Giasson *et al.* 2000b), anti-synaptophysin (a gift from Dr Honer, Albert Einstein College of Medicine, NY, USA) or anti-3-NT antibody. Incubation with a secondary anti-mouse- or anti-rabbit-conjugated horseradish peroxidase antibody was performed at 25°C for 1 h. After washing in 1 × TBS, 0.1% Tween-20, blots were developed with Super Signal Ultra chemiluminiscence (Pierce) and exposed to Kodak BetaMax film.

Results and discussion

In the present study, we demonstrate that α-syn is a specific target for tyrosine nitration in a cell model as well as in the mouse brain after MPTP intoxication. First, non-transfected HEK293 cells and HEK293 cells overexpressing human α-syn or β-syn were exposed to

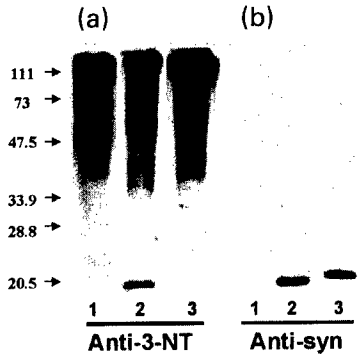

Fig. 1 Exposure of HEK293 cells to peroxynitrite results in the nitration of cellular proteins. All of the cells were exposed, under the same conditions, to 1 mM chemically synthesized peroxynitrite. (a) Western blot analysis using an anti-NT polyclonal antibody: lane 1 – non-transfected cells; lane 2 – cells expressing human α-syn; lane 3 – cells expressing β-syn. (b) The same as (a) but developed using an anti-α-syn antibody (lane 1 and 2) or using the antibody Syn 207, which specifically recognizes β-syn. Fifty μg of total protein was loaded onto each lane of the polyacrylamide gel. The molecular masses of markers are indicated to the left of the blots.

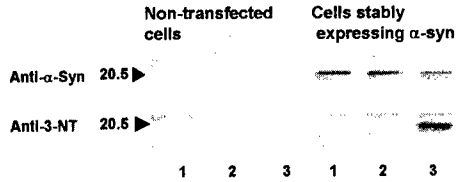

Fig. 2 Immunoprecipitation of α-syn followed by western blotting with antibodies to α-syn or NT reveals nitration of the protein after the exposure of HEK293 cells to peroxynitrite. Cells were untreated (lanes 1), or exposed to decomposed (lanes 2) or fresh (lanes 3) peroxynitrite.

peroxynitrite, an oxidizing and nitrating agent (Ischiropoulos and al Mehdi 1995). This treatment resulted in the nitration of a number of proteins as demonstrated by the western blot analysis using an anti-NT antibody (Fig. 1a). However, only HEK293 cells transfected with α-syn showed a nitrated protein band with the molecular mass corresponding to α-syn. To demonstrate that α-syn was indeed nitrated, the total protein extract was subjected to immunoprecipitation using an anti-α-syn antibody and then the recovered immunoprecipitated protein was probed with the anti-3-NT antibody. This experiment confirmed that a significant fraction of the immunoprecipitated α-syn was definitely nitrated in the cells exposed to peroxynitrite (Fig. 2, lane 3), but not in untreated cells or in cells exposed to decomposed reagent (Fig. 2, lanes 1 and 2).

Given these results, we then proceeded to assess whether α-syn was nitrated in the MPTP mouse model of PD. The use of this particular experimental model has been motivated by the fact that, thus far, significant insights into the pathogenesis of PD have been achieved using this neurotoxin, which replicates in humans and in non-human primates a severe and irreversible PD-like syndrome, with concomitant degeneration of dopaminergic neurons (Przedborski *et al.* 2000a). Moreover, several studies have indicated that reactive nitrogen species and tyrosine nitration not only occur in this model but also participate in the MPTP neurotoxic process (Schulz *et al.* 1995; Przedborski *et al.* 1996; Ara *et al.* 1998; Mandir *et al.* 1999; Pennathur *et al.* 1999).

Immunoprecipitation of α-syn was performed as above from striatum and ventral midbrain, the two main targets of MPTP neurotoxicity (Przedborski *et al.* 2000a). Immunoprecipitated α-syn from striatum and ventral midbrain was selectively nitrated 4 h after the MPTP administration (Fig. 3). Conversely, immunoprecipitation of α-syn from striatum and ventral midbrain of saline-injected mice, also at 4 h post-injection, did not reveal any detectable nitration of the protein (Fig. 3). In contrast to the robust tyrosine nitration of α-syn (Figs 3a and b), no tyrosine nitration was detected in two other presynaptic proteins, β-syn and synaptophysin, following a similar MPTP administration (Figs 3c and d). This observation is consistent with our previous finding that only selected proteins are tyrosine-nitrated after MPTP exposure (Ara *et al.* 1998; Ischiropoulos 1998) and with the observation that α-syn, but not β-syn, is nitrated after exposure of cells to the same peroxynitrite challenge. Moreover, this observation is consistent with the demonstrations that nitrated α-syn is present in the hallmark lesions in a number of human neurodegenerative synucleinopathies (Duda *et al.* 2000a; Giasson *et al.* 2000a).

The higher efficiency of α-syn nitration is likely to be caused by the unstructured conformation of the protein in aqueous solution, which exposes all four tyrosine residues to the solvent phase and increases the probability of the reaction with nitrating agents. Moreover, glutamate residues, a structural conformation associated with enhanced susceptibility of tyrosine to nitration, are near all three tyrosine residues (125, 133 and 136) in the carboxy terminal domain of α-syn. Indeed, purified human α-syn exposed to the nitrating agent *in vitro* and analyzed by

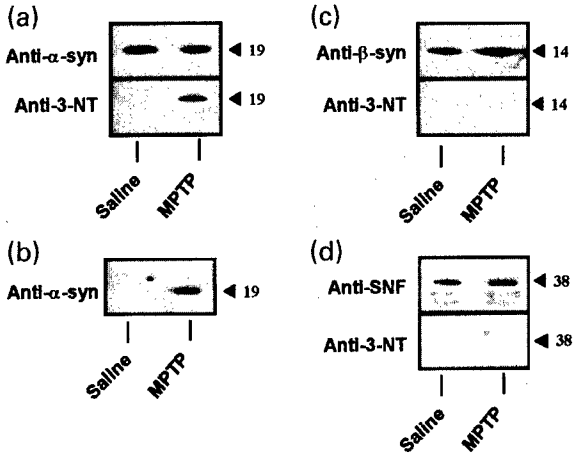

Fig. 3 Tyrosine nitration of striatal α-syn, but not of β-syn or synaptophysin, following MPTP injection to mice. Following administration of MPTP (4 h after the last injection) or vehicle (saline), striatal proteins were immunoprecipitated (IP) using: anti-α-syn (a); anti-NT (b); anti-β-syn (c); or anti-synaptophysin (d) as described in Experimental procedures. After SDS–PAGE and transfer of proteins onto nitrocellulose membrane, immunoblots were probed with anti-NT (lower panels of a, c and d), anti-α-syn (a – top panel and b), anti-β-syn (c – top panel) and anti-synaptophysin (d – top panel) as described in Experimental procedures.

electrospray mass spectrometry, revealed that nitration of α-syn occurs at all four tyrosine residues (Souza *et al.* 2000). In addition to nitration, exposure of α-syn to nitrating agents also results in the stable cross-linking of the protein via the formation of dityrosine (Souza *et al.* 2000). In contrast, β-syn is nitrated to a lesser extent than α-syn *in vitro* and does not form stable *O-O'*dityrosine crosslinks after exposure to nitrating agents, despite the presence of all four conserved tyrosine residues in both proteins (Souza *et al.* 2000). More significantly, we show here that in two *in vivo* models, α-syn is selectively nitrated, whereas nitration of β-syn is below detectable limits. The preferential nitration and oxidation of tyrosine residues in α-syn could be caused by the accessibility of tyrosine residues to nitrating agents and by the presence of the protein in close proximity to the site(s) of generation of the nitrating agent. Our results raise the possibility that both syn proteins may have different confirmations, or that β-syn may be protected from oxidation, perhaps by different interacting partners *in vivo*. We have previously argued that proximity to sites of superoxide generation may be important in determining proteins modified by nitration, as overexpression of superoxide dismutase and superoxide mimetics have been shown to prevent the nitration of proteins *in vivo* and in cell models (Ara *et al.* 1998; Cuzzocrea *et al.* 2000; Pong *et al.* 2000).

The significance of the tyrosine nitration of α-syn remains unclear. Tyrosine nitration induces secondary and tertiary structural alterations, which may critically modify protein functions (Ischiropoulos 1998). The change in the ionization state of the modified protein induced by a local shift in the pKa from 10.01 of tyrosine to 7.5 of 3-NT, and the consequent changes in hydrophobicity and conformation, may facilitate interactions with other proteins, thereby promoting protein aggregation. Preliminary data indeed indicate an increased adherence of mouse brain extracts to nitrated α-syn compared with the unmodified wild-type protein (Chen *et al.*, unpublished observation).

Collectively, the data indicate that α-syn is a preferential target for oxidative stress-mediated post-translational modifications. These

alterations may trigger abnormal protein compartmentalization and aggregation, two phenomena that are potential culprits for the neurodegeneration process in PD.

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Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) damages dopaminergic neurons in the substantia nigra pars compacta (SNpc) as seen in Parkinson's disease. Here, we show that the pro-apoptotic protein Bax is highly expressed in the SNpc and that its ablation attenuates SNpc developmental neuronal apoptosis. In adult mice, there is an up-regulation of Bax in the SNpc after MPTP administration and a decrease in Bcl-2. These changes parallel MPTP-induced dopaminergic neurodegeneration. We also show that mutant mice lacking Bax are significantly more resistant to MPTP than their wild-type littermates. This study demonstrates that Bax plays a critical role in the MPTP neurotoxic process and suggests that targeting Bax may provide protective benefit in the treatment of Parkinson's disease.

Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal clinical features include tremor, slowness of movement, stiffness, and postural instability (1). These disabling symptoms are primarily due to a profound deficit in striatal dopamine content that results from the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibers in the striatum (2, 3). Although several approved drugs do alleviate PD symptoms, their chronic use often is associated with debilitating side effects (4), and none seem to dampen the progression of the disease. Moreover, the development of effective neuroprotective therapies is impeded by our limited knowledge of the mechanism by which SNpc dopaminergic neurons die in PD. Thus far, however, significant insights into the pathogenesis of PD have been achieved by the use of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which replicates in humans and nonhuman primates a severe and irreversible PD-like syndrome (5). In several mammalian species, MPTP reproduces most of the biochemical and pathological hallmarks of PD, including the dramatic degeneration of dopaminergic neurons (5).

Mounting evidence indicates that highly regulated cell death-associated molecular pathways could participate in the relentless demise of neurons in degenerative diseases (6, 7), including PD (8). In keeping with this, Bax (9) has emerged as a pro-cell death driving force within the central decision point constituted by the Bcl-2 family that modulates the activation of downstream effectors of cell death such as caspases (7). It is also clear that Bax is required for the death of several types of neurons in the peripheral and central nervous systems during both normal development and pathological situations (10–18). In light of its critical role within the programmed cell death machinery and its importance in neuronal death, Bax appears as a particularly appealing target for therapeutic interventions aimed at hampering neurodegeneration. Consistent with a potential pivotal role for Bax in SNpc neuronal death, here we show that Bax is highly expressed in the SNpc and that its ablation attenuates SNpc developmental neuronal apoptosis. We demonstrate that there is a dramatic up-regulation of Bax mRNA and protein in the SNpc of adult mice after MPTP administration. These changes parallel the time course of MPTP-induced dopaminergic neurodegeneration. We also show that mutant mice lacking Bax are resistant to MPTP compared with their wild-type littermates, thus indicating that Bax is a key factor in MPTP-induced SNpc dopaminergic neurodegeneration.

Materials and Methods

Animals and Treatment. C57/bl mice heterozygous for Bax were mated to yield F_1 offspring with $Bax^{-/-}$, $Bax^{+/-}$, and wild-type genotypes. Tail DNA was prepared and screened for both the normal and the mutant allele by using a single PCR. The normal allele was amplified by using an exon 5 forward primer (0.64 μM: 5′-TGATCAGAACCATCATG-3′) and an intron 5 reverse primer (0.64 μM: 5′-GTTGACCAGAGTGGCGTAGG-3′), which together generated a 304-bp product. The mutant allele was amplified with a neo/pgk primer (0.16 μM: 5′-CCGCTTCCATTGCTCAGCGG-3′) and the same intron 5 reverse primer, which together generated a 507-bp product. Cycling parameters were 1 min at 94°C, 55°C, and 72°C each for a total of 30–35 cycles. The primer ratio was adjusted to allow amplification of both products simultaneously with preferential amplification of the wild-type allele to assure correct genotyping of the Bax-deficient animals. All mice used in this study were treated according to National Institutes of Health guidelines for Care and Use of Laboratory Animals and with the approval of Columbia University's Institutional Animal Care and Use Committee. Eight-week-old male mice received one i.p. injection of MPTP-HCl per day (30 mg/kg per day of free base; Research Biochemicals, Natick, MA) 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. Both saline and MPTP animals then were divided into two groups. The first group was perfused and brains were used for immunohistochemistry, whereas the second group of mice were killed, and brains were quickly removed, dissected (midbrain, striatum, cerebellum, and

Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SNpc, substantia nigra pars compacta; PD, Parkinson's disease; TH, tyrosine hydroxylase; QA, quinolinic acid; MPP^+, 1-methyl-4-phenylpyridinium ion.

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cortex), snap-frozen on dry ice, and stored at −80°C for Western blot, immunoprecipitation, and reverse transcriptase–PCR analysis. MPTP use and safety precautions were as described (19).

Immunohistochemistry and Double Immunofluorescence. Immunohistochemistry was performed as described by Vila *et al.* (20) with a polyclonal antibody to Bax (1:500; polyclonal; PharMingen). Immunostained sections then were counterstained with thionin. To examine the colocalization of Bax with tyrosine hydroxylase (TH), a double immunofluorescence technique was performed by using the same polyclonal anti-Bax antibody (1:200 dilution) and a monoclonal antibody to TH (1:200 dilution; Boheringer Mannheim). Sections were examined on green, red, and double (green + red) filters by using confocal microscopy.

Striatal Lesions with Quinolinic Acid (QA). After Metofane inhalation, mouse pups aged postnatal day seven of either sex received an intrastriatal injection of 0.5 μl of a 480 nmol solution of QA dissolved in PBS at pH 7.4 as described (21). One day after the QA injection, animals were perfused and brains were processed for morphological analysis.

Immunoblots and Immunoprecipitation. For Western blot analysis, total tissue proteins were isolated in 50 mM Tris·HCl, pH 7.0/150 mM NaCl/5 mM EDTA/1% SDS/1% Nonidet P-40/protease inhibitors (Mini mixture; Roche Diagnostics, Indianapolis, IN). Incubation with primary antibody was performed overnight at 4°C with monoclonal antibodies to Bax (1:1,500 dilution; Santa Cruz Biotechnology) or Bcl-2 (1:500 dilution, Transduction Laboratories, Lexington, KY) and, as an internal control, a monoclonal antibody to β-actin (1:5,000, Sigma). Films were quantified by using the National Institutes of Health IMAGE analysis system. For immunoprecipitation, frozen samples from saline-injected mice and MPTP-intoxicated animals (at day 4 after the last MPTP injection) were homogenized in 10 vol (wt/vol) of 10 mM Hepes (pH 7.20) containing 0.25% Nonidet P-40, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and one tablet of protease inhibitor mixture. Then, 250 μg of protein was incubated (overnight, 4°C) with 3 μg of a polyclonal antibody to Bcl-2 (N-19, Santa Cruz Biotechnology) and further processed for immunoprecipitation and immunoblotting as described by Ara *et al.* (22). Here, blots were immunostained with either a monoclonal antibody to Bax (1:1,500 dilution; Santa Cruz Biotechnology) or a monoclonal antibody to Bcl-2 (1:1,000 dilution; Transduction Laboratories).

RNA Extraction and Reverse Transcriptase–PCR. Total RNA was extracted from midbrain, striatal, and cerebellar samples from saline and chronic MPTP-treated animals and used for reverse transcriptase–PCR analysis as described by Vila *et al.* (20). The Bax primer sequences were 5′-CTGAGCTGACCTTGGAGC-3′ (forward) and 5′-GACTCCAGCCACAAAGATG-3′ (reverse). As an internal control, β-actin cDNA was coamplified by using primer sequences 5′-CTTTGATGTCACGCACGATTTC-3′ (forward) and 5′-GGGCCGCTCTA GGCACCAA-3′ (reverse). Each PCR cycle consisted of denaturation at 94°C for 5 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, followed by a final 10-min extension at 72°C. PCR amplification was carried out for 30 cycles for Bax and 22 cycles for β-actin by using a Perkin–Elmer GeneAmp 9700 Thermal Cycler.

Stereology and Quantification of Apoptotic Neurons. The total number of TH-positive SNpc neurons was counted in the different groups of animals at 21 days after the last MPTP or saline injection by using the optical fractionator method as described by Liberatore *et al.* (23). This unbiased method of cell counting is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons). Immunostaining was performed with a polyclonal antibody to TH (1:1,000; Calbiochem), and sections were counterstained with thionin. Quantification of the number of apoptotic neurons in the SNpc of MPTP- and saline-injected mice was assessed as described (21). Morphological criteria to identify apoptotic figures included shrinkage of cellular body, chromatin condensation, and the presence of distinct, round, well-defined chromatin clumps, demonstrated by thionin staining (21).

Measurement of Striatal Dopamine, 3,4-Dihydroxyphenylacetic Acid, and Homovanillic Acid Levels. HPLC with electrochemical detection was used to measure striatal levels of dopamine, 3,4-dihydroxyphenylacetic acid, and homovanillic acid by using a method that has been described by Przedborski *et al.* (24), with minor modifications of the mobile phase. At 21 days after the last MPTP injection, animals were killed and striata were dissected out and processed for HPLC measurement. The modified mobile phase consisted of 0.15 M monochloroacetic acid, pH 3.0, 200 mg/liter sodium octyl sulfate, 0.1 mM EDTA, 4% acetonitrile, and 2.5% tetrahydrofuran.

Measurement of Striatal MPP^+ Levels. HPLC with UV detection (wavelength, 295 nm) was used to measure striatal MPP^+ levels as described by Przedborski *et al.* (24). Groups of $Bax^{+/-}$, $Bax^{-/-}$, and wild-type littermates were killed at 90 and 180 min after one i.p. injection of 30 mg/kg MPTP, and the striata were dissected and processed for HPLC.

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

Results

High Expression of Bax in SNpc Dopaminergic Neurons. Relevant to the potential role of Bax in PD neurodegeneration, we found that virtually all neurons of the SNpc exhibit conspicuous levels of Bax protein, as evidenced by immunohistochemistry (Fig. 1*a*). SNpc neurons are primarily dopaminergic and secondarily GABAergic (25). Thus, to confirm that dopaminergic neurons do contain Bax protein, we performed double immunohistochemistry for TH, the rate-limiting enzyme in dopamine synthesis, and Bax. Examination by confocal microscopy demonstrated that all TH-positive neurons expressed Bax (Fig. 1 *b* and *c*) and, as expected given the ubiquitous expression of Bax in the brain, that Bax was expressed by both TH-positive and TH-negative neurons. Most Bax-positive SNpc dopaminergic neurons showed a prominent punctate immunoreactivity superimposed onto a diffuse cytoplasmic immunostaining (Fig. 1*d*), which is consistent with the known subcellular distribution of Bax in both mitochondria and cytosol (9, 26).

Bax Modulates Developmental Cell Death in the SNpc. During development, neurons in the SNpc undergo an intense naturally occurring cell death process (21, 27). Dying neurons exhibit the morphological characteristics of apoptosis and their numbers are modulated by the size of striatum (21, 27), the brain structure in which SNpc neuron projections form synapses. Indeed, 24 h after unilateral destruction of the striatum with a local injection of the excitotoxin QA at postnatal day seven, wild-type pups showed four times more apoptotic neurons in the SNpc ipsilateral to the lesion compared with the contralateral side (Fig. 2). Age-matched mutant pups heterozygous ($Bax^{+/-}$) or homozygous ($Bax^{-/-}$) for the *Bax* null mutation showed a gene dosage-

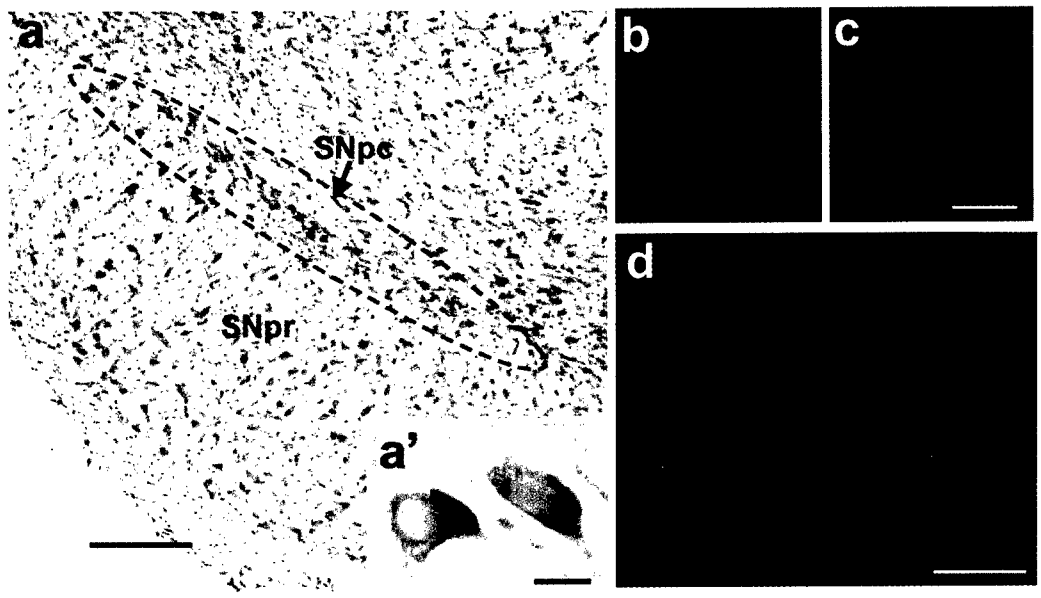

Fig. 1. Bax expression in SNpc dopaminergic neurons of adult mice. (*a*) Bax is highly expressed in SNpc neurons, as assessed by immunohistochemistry; sections are counterstained with thionin. (*a'*) High magnification of Bax-immunostained neurons in the SNpc. (*b* and *c*) Double immunofluorescence with antibodies to Bax and TH confirms that Bax (in green) is expressed in dopaminergic neurons (in red). (*d*) Confocal microscopy analysis of Bax-positive dopaminergic neurons (Bax + TH immunostaining) shows a robust punctate immunoreactivity superimposed onto a diffuse cytoplasmic immunostaining. [Scale bars: 200 μm (*a*), 10 μm (*a'* and *d*), and 30 μm (*b* and *c*).]

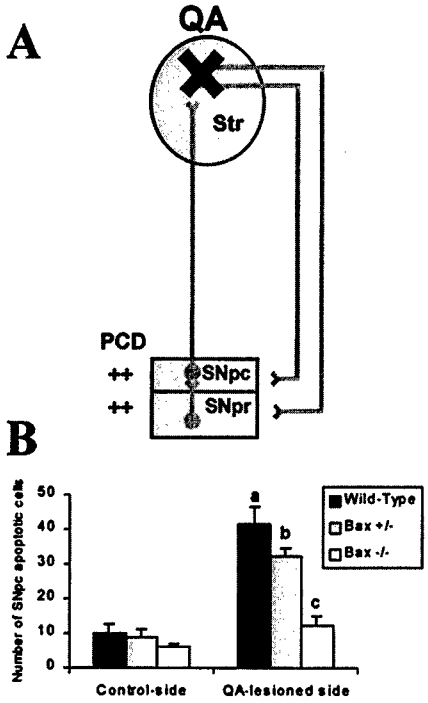

Fig. 2. Bax regulates natural and QA-induced developmental neuronal death in the SNpc. (*a*) Schematic representation of the model of induced apoptotic cell death in the SNpc by unilateral destruction of the striatum (i.e., the target) at postnatal day seven with a local injection of QA. (*b*) 24 h after the lesion, wild-type mice (n = 5) exhibit a substantial number of dying neurons with a definite morphology of apoptosis in the contralateral SNpc and a dramatic increase in this number in the SNpc ipsilateral to the QA lesion. Age-matched mutant mice deficient for Bax ($Bax^{+/-}$ and $Bax^{-/-}$, n = 4 per group) exhibit a striking lower number of SNpc apoptotic neurons after QA administration. *a*, $P < 0.05$, compared with wild-type control-side; *b*, $P < 0.05$, compared with $Bax^{+/-}$ control-side; *c*, $P < 0.05$, compared with wild-type and $Bax^{+/-}$ QA-lesioned sides but not significant when compared with $Bax^{-/-}$ control side; Newman-Keuls post hoc analysis.

dependent reduction of SNpc apoptotic neurons after QA administration (Fig. 2).

MPTP Stimulates Bax Expression in Ventral Midbrain. In saline-injected mice, there was a high constitutive expression of Bax protein in the ventral midbrain (Fig. 3*a*). After systemic MPTP administration, there was a dramatic up-regulation of Bax protein in this brain region (Fig. 3*a*), in agreement with a previous study (28). This change occurred in a time-dependent manner, with protein levels peaking at 4 days after the last MPTP injection (+668%), then progressively returning to control levels (Fig. 3*a*). This alteration was not only time-dependent but was also region-specific as MPTP-intoxicated mice showed no Bax up-regulation, at any of the time points studied, in striatum or in cerebellum, two brain regions devoid of neuronal loss after MPTP administration.

MPTP Increases Bax mRNA Levels in Ventral Midbrain. Given the change in Bax protein after MPTP injections, we also investigated whether this change was associated with Bax transcriptional alterations. In saline-injected mice, there was a constitutive level of Bax transcript in the ventral midbrain (Fig. 3*b*). In MPTP-injected mice, there was a time-dependent increase in the level of Bax transcript, which peaked at 2 days after the last MPTP injection (+364%), then progressively returned to the level of controls by day 7 (Fig. 3*b*). Bax mRNA up-regulation was also region-specific as it was not detected in the striatum nor in the cerebellum of MPTP-intoxicated animals.

Time Course of MPTP-Induced Apoptotic Neuronal Death. Quantification of apoptotic cells in the SNpc of MPTP- and saline-injected mice indicates that apoptotic neuronal death culminated between days 2 and 4 after the last MPTP injection (Fig. 3). Morphological criteria used to identify apoptotic cells were previously validated (21) and included shrinkage of cellular body, chromatin condensation, and presence of distinct, round, well-defined chromatin clumps, demonstrated by thionin staining.

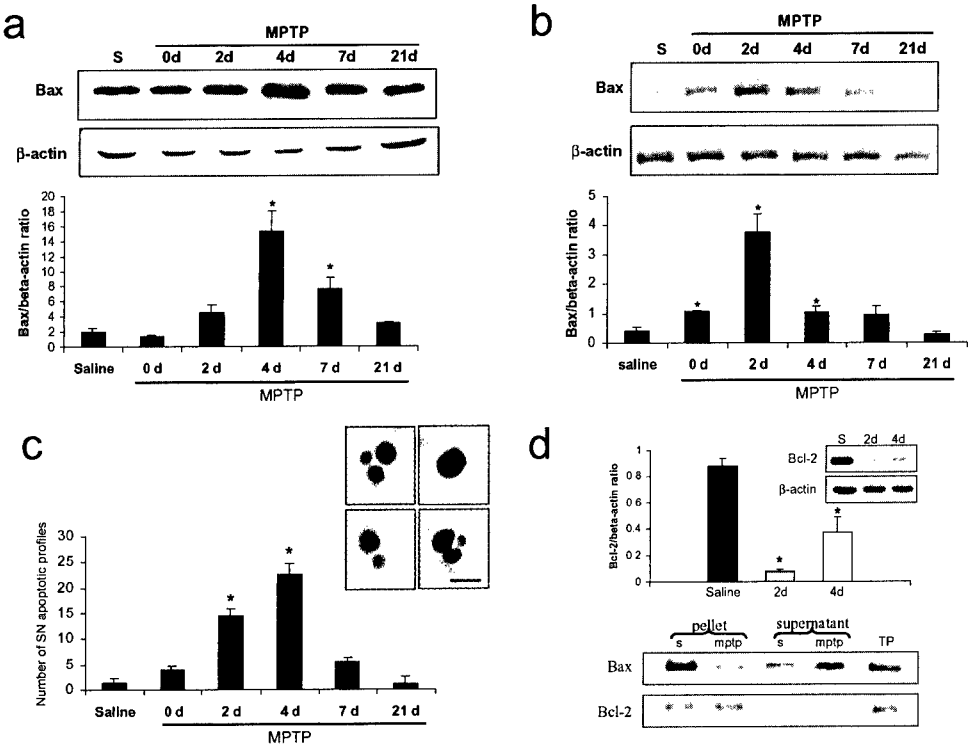

Fig. 3. Bax expression in the ventral midbrain after MPTP intoxication. (*a*) Bax protein levels in the ventral midbrain ($n = 3$ mice per group) were assessed by Western blot analysis. (*b*) Bax mRNA expression in the ventral midbrain was quantified by reverse transcriptase–PCR ($n = 3$–5 mice per group). (*c*) Bax protein and mRNA up-regulation coincide with the time course of apoptotic-induced cell death in the SNpc. Morphological criteria to identify apoptotic figures, as illustrated in photomicrographs, included shrinkage of cellular body, chromatin condensation, and the presence of distinct, round, well-defined chromatin clumps, demonstrated by thionin staining. (Scale bar, 5 μm.) (*d*) Bcl-2 protein expression (*Upper*) and immunoprecipitation (*Lower*) after MPTP intoxication. Bcl-2 protein levels are decreased in the ventral midbrain of MPTP-intoxicated mice at days 2 and 4 after the last MPTP injection ($n = 3$–5 mice per group). At day 4 after the last injection, ventral midbrain proteins ($n = 4$ mice per group) were subjected to immunoprecipitation with a polyclonal antibody to Bcl-2. The amount of Bax coimmunoprecipitated with Bcl-2 appeared less abundant in the pellets of MPTP-intoxicated mice than in those of saline-injected animals. This was associated with increased Bax immunoreactivity in the supernatant. S, saline; TP, total proteins. *, $P < 0.05$, compared with saline-injected animals; Newman-Keuls post hoc analysis. Error bars indicate SEM.

MPTP Decreases Bax:Bcl-2 Heterodimerization in the Ventral Midbrain. Several members of the Bcl-2 family, such as Bcl-2, can bind to Bax to form Bax:Bcl-2 heterodimers, hence antagonizing Bax pro-cell death properties (9). Accordingly, we determined the levels of Bcl-2 protein as well as its capacity to heterodimerize with Bax protein in ventral midbrain of MPTP-intoxicated mice, at the peak of MPTP-induced apoptotic neuronal death. In striking contrast with Bax up-regulation, Bcl-2 protein levels, as assessed by Western blot, were dramatically decreased in ventral midbrain of MPTP-intoxicated mice compared with saline-injected animals, 2 and 4 days after the last injection (Fig. 3*d*). Furthermore, the amount of Bax that coimmunoprecipitated with Bcl-2 at this time point, using an anti-Bcl-2 antibody, was much less in MPTP-intoxicated mice than in saline-injected animals (Fig. 3*d*). Consistent with this finding, the amount of Bax that escaped coimmunoprecipitation using an anti-Bcl-2 antibody was much greater in MPTP-intoxicated mice than in saline-injected animals (Fig. 3*d*). The ratio of these proteins indicates that most of the Bax protein could be inactivated by Bcl-2 in saline-injected mice whereas there is an excess of unopposed Bax in MPTP-injected mice.

Bax-Deficient Mice Are Resistant to MPTP Intoxication. To confirm the involvement of Bax in MPTP-induced neuronal death, we compared the effects of MPTP in $Bax^{+/-}$ and $Bax^{-/-}$ mice and in their wild-type littermates. In saline-injected mice, no significant changes in stereological counts of SNpc dopaminergic neurons, defined by TH immunostaining, were detected between the different groups of mice (Fig. 4*A*). In wild-type mice, MPTP caused a dramatic loss of SNpc TH-positive neurons, which was accompanied by a large number of apoptotic neurons (Fig. 4 *A* and *B*). MPTP can down-regulate phenotypic markers such as TH (29), thus it is important to indicate that the TH/Nissl ratio of neuronal counts did not differ between saline- and MPTP-injected wild-type mice (saline = 1.78 ± 0.05 vs. MPTP = 1.77 ± 0.03; $n = 3$ per group; Student's t test), confirming that the reduction in TH-positive neurons corresponds to an actual loss of neurons. In contrast to the situation in wild-type animals, MPTP failed to affect SNpc TH-positive neuronal counts in $Bax^{-/-}$ and caused only a mild reduction of these numbers in $Bax^{+/-}$ mice (Fig. 4*A*). Similarly, the number of MPTP-induced SNpc apoptotic neurons was significantly smaller in $Bax^{-/-}$ and, to a lesser extent, in $Bax^{+/-}$ than in wild-type animals (Fig. 4*B*). Although less striking than the loss of the SNpc cell body counts, the loss in striatal dopaminergic nerve terminals after MPTP administration, as assessed by measuring the levels of dopamine and its two main metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid, was also markedly attenuated in $Bax^{-/-}$ and $Bax^{+/-}$ mice, compared with their wild-type littermates (Table 1).

MPP^+ Production in Bax-Deficient Mice. The main determining factor of MPTP neurotoxic potency is its conversion in the brain to 1-methyl-4-phenylpyridinium ion (MPP^+) (30). To confirm that the resistance of $Bax^{-/-}$ mice is due to the absence of the *Bax* gene and not to an alteration in the brain's production of

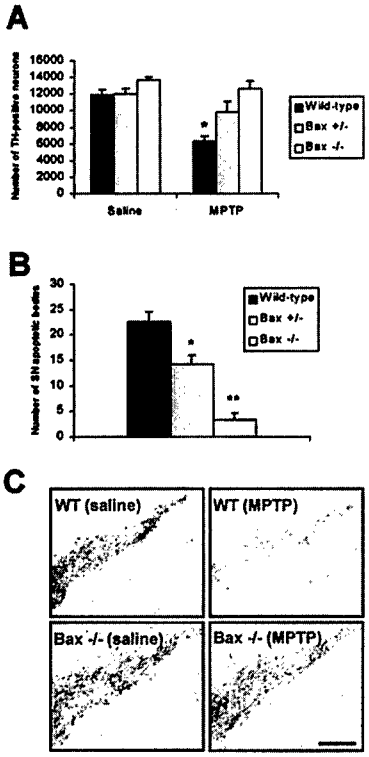

Fig. 4. Bax-deficient mice are resistant to MPTP neurotoxic effect. (*A*) Stereological counts of TH-positive neurons in the SNpc were performed in Bax-deficient mice and their wild-type littermates at day 21 after the last injection (n = 3–5 mice per group). In wild-type mice, only 53% of the SNpc TH-positive neurons survived MPTP administration. In contrast, 81% of SNpc TH-positive neurons survived in $Bax^{+/-}$ mice and no loss of TH-positive cells was found in $Bax^{-/-}$ animals under an identical MPTP regimen. *, $P < 0.05$, compared with saline-injected wild-type animals; Newman-Keuls post hoc analysis. (*B*) At the peak of apoptotic cell death (day 4 after the last MPTP injection), $Bax^{-/-}$ mice (n = 3) presented 85% reduction in the number of apoptotic profiles in the SNpc compared with MPTP-intoxicated control animals (n = 4). In $Bax^{+/-}$ animals (n = 4), these numbers were reduced by 37%. *, $P < 0.05$ compared with MPTP-intoxicated control animals; **, $P < 0.05$ compared with MPTP-intoxicated control animals and MPTP-intoxicated $Bax^{+/-}$ mice; Newman-Keuls post hoc analysis). Error bars indicate SEM. (*C*) Photomicrographs of TH-immunostained sections with thionin counterstain, illustrating the results in *A*. (Scale bar, 400 μm.)

MPP^+, we measured striatal content of MPP^+ at different time points after MPTP administration. At no time point does the striatal content of MPP^+ differ significantly among $Bax^{+/-}$, $Bax^{-/-}$, and wild-type littermate mice (Table 2).

Table 1. Striatal monoamine levels (ng/mg tissue)

Mice	Dopamine	DOPAC	HVA
Saline			
Wild type	12.2 ± 0.2	2.1 ± 0.2	1.8 ± 0.1
$Bax^{+/-}$	13.3 ± 0.3	2.0 ± 0.1	1.7 ± 0.1
$Bax^{-/-}$	13.4 ± 0.4	2.4 ± 0.1	1.6 ± 0.2
MPTP			
Wild type	0.5 ± 0.1	0.3 ± 0.05	0.2 ± 0.01
$Bax^{+/-}$	3.1 ± 0.3*	0.6 ± 0.06	0.6 ± 0.04*
$Bax^{-/-}$	4.1 ± 0.2**	1.3 ± 0.2**	1.0 ± 0.1**

DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid. *, $P < 0.05$, compared to MPTP-injected wild-type mice; **, $P < 0.05$, compared to MPTP-injected wild-type and MPTP-injected $Bax^{+/-}$ mice; Newman-Keuls post hoc test. Data represent means ± SEM for 4–6 mice per group.

Table 2. Striatal MPP^+ levels (μg/g striatum) in Bax-deficient and wild-type mice

Mice	90 min	180 min
Wild type	8.5 ± 1.0	5.7 ± 0.9
$Bax^{+/-}$	7.6 ± 1.9	5.1 ± 0.3
$Bax^{-/-}$	10.0 ± 1.0	5.4 ± 0.8

HPLC measurements of striatal MPP^+ levels in wild-type and Bax-deficient mice were determined at 90 and 180 min after a single i.p. MPTP injection (30 mg/kg). n = 4 animals per group. Values represent the mean ± SEM.

Discussion

Bax is widely expressed in the central nervous system, where it is detected primarily in neurons (9, 31). Herein, we show that almost all neurons of the SNpc, especially all dopaminergic neurons, contain abundant amounts of Bax protein (Fig. 1), likely located both at mitochondria and in cytosol (9). We also demonstrate that Bax controls the apoptotic demise of SNpc dopaminergic neurons during development, because its ablation attenuates SNpc developmental cell death in immature animals (Fig. 2). These findings confirm a key role for Bax in the fate of SNpc neurons, thus setting the stage for Bax being a potential culprit in the degeneration of SNpc dopaminergic neurons in PD.

To test the contribution of Bax in PD neurodegeneration, we used the experimental model produced by the parkinsonian neurotoxin MPTP (5). Because the mode of cell death in PD may be, at least in part, apoptotic (8), we selected a MPTP regimen that kills SNpc dopaminergic neurons by apoptosis (32). This regimen induces a time-dependent apoptotic cell death in the SNpc that is maximal between 2 and 4 days after the last dose of MPTP (Fig. 3*c*). Relevant to the known pro-apoptotic role of Bax, we found that the time course of SNpc apoptotic neuronal death coincides with that of increased levels of Bax mRNA and protein in ventral midbrain after MPTP administration (Fig. 3 *a* and *b*). The opposite image was found for Bcl-2 in that at 2 and 4 days post-MPTP ventral midbrain Bcl-2 protein levels were markedly reduced. These findings suggest that, during the MPTP-induced neurodegenerative process, the finely tuned balance between cell death agonists, such as Bax, and cell death antagonists, such as Bcl-2, is upset in the ventral midbrain, leading to a situation in which molecular pro-apoptotic forces dominate (33). In this context, an aspect related to Bax function is its capacity to form heterodimers with Bcl-2 and homomultimers with itself (34). In saline-injected mice, the amount of Bax can theoretically be neutralized by Bcl-2 as evidenced by the majority of Bax in heterodimers. Whereas, in MPTP-injected mice excess Bax exists free of neutralizing interaction with Bcl-2 (Fig. 3*d*). Taken together, our data suggest that, after MPTP administration, a cascade of deleterious events is set in motion within which Bax up-regulation and Bcl-2 down-regulation are key factors. Consistent with this scenario, the observed neuroprotective effects provided by Bcl-2 overexpression against MPTP (35, 36) may reflect its capacity to counter Bax.

Consistent with the involvement of Bax in the MPTP neurotoxic process is our demonstration that no significant loss of SNpc dopaminergic neurons was observed in $Bax^{-/-}$ mice and that approximately 81% of SNpc dopaminergic neurons survived in $Bax^{+/-}$ mice compared with their wild-type littermates after MPTP administration (Fig. 4). Similarly, there were significantly fewer apoptotic neurons in the SNpc of $Bax^{+/-}$ and $Bax^{-/-}$ after MPTP administration compared with wild-type controls (Fig. 4). The resistance of the SNpc dopaminergic neurons in Bax knockout mice was accompanied by a significant, although less prominent, sparing of striatal dopamine contents (Table 1). The latter suggests that Bax ablation protects against SNpc neuronal death, but still allows some changes in gene expression and/or alter-

ations in dopamine synthesis. Relevant to this, is our previous demonstration that TH, the rate-limiting enzyme in dopamine synthesis, is inactivated by tyrosine nitration after MPTP administration (22).

We also found that ablation of Bax was not associated with alterations in the formation of MPTP active metabolite, MPP^+ (Table 2), which is the most significant modulating factor of MPTP potency (30).

In light of the results reported above, including the resistance of Bax-deficient mice to the neurotoxic effects of MPTP, we argue that Bax is a critical effector molecule in MPTP-mediated cell death. Given the mode of action of MPTP and Bax, it is possible that the mitochondrion is key to the observed neuroprotection. Models of Bax activation indicate its oligomerization may result in a homomultimeric pore (37), a VDAC-containing pore (38), or a permeabilization of mitochondrial outer membrane (39) to release cytochrome *c*. Several lines of evidence indicate that translocation of mitochondrial cytochrome *c* to the cytosol is a critical event in the mitochondrial-dependent activation of effector caspases such as caspase-3 and ensuing cell death (40). Providing credence to this proposed sequence of events in PD is the observation that caspase-3 is indeed activated in postmortem SNpc samples from parkisonian patients (41). Once inside dopaminergic neurons, MPP^+ is actively concentrated within mitochondria, where it inhibits complex I of the electron transport chain (5). This inhibition leads to a deficit in ATP formation and to an increase in reactive oxygen species production (5), which, in turn, cause an energy crisis and oxidative stress. As with other situations, mitochondrial dysfunction seen after MPTP administration ultimately can trigger large amplitude swelling often attributed to the opening of the permeability transition pore complex (PTPC). Alternatively, the opening of the PTPC can lead to several dramatic consequences, including a dissipation of the mitochondrial transmembrane potential and a release to the cytosol of proteins normally confined to the mitochondria, such as cytochrome *c* (42).

Collectively, our results indicate that Bax plays a pivotal role in SNpc dopaminergic neuronal death in the MPTP mouse model likely by acting in injured neurons before the onset of irreversible cell death events. Whether blocking events downstream of Bax also can protect these cells remains to be determined. Ablation of the cell executioner, caspase-3, dramatically decreases neuronal death during development (43). However, whether inhibition of caspases downstream of mitochondria will prove sufficient to interfere with adult-onset pathological stimuli or merely shift the mode of death of severely injured neurons remains uncertain. Because of the striking similarities between the MPTP model and PD, the present study raises the possibility that Bax plays a critical role in the neurodegenerative process of PD and thus that targeting Bax could open new neuroprotective avenues for this disabling neurological disease.

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Blockade of Microglial Activation Is Neuroprotective in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model of Parkinson Disease

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) damages the nigrostriatal dopaminergic pathway as seen in Parkinson's disease (PD), a common neurodegenerative disorder with no effective protective treatment. Consistent with a role of glial cells in PD neurodegeneration, here we show that minocycline, an approved tetracycline derivative that inhibits microglial activation independently of its antimicrobial properties, mitigates both the demise of nigrostriatal dopaminergic neurons and the formation of nitrotyrosine produced by MPTP. In addition, we show that minocycline not only prevents MPTP-induced activation of microglia but also the formation of mature interleukin-1β and the activation of NADPH–oxidase and inducible nitric oxide synthase (iNOS), three key microglial-derived cytotoxic mediators. Previously, we demonstrated that ablation of iNOS attenuates MPTP-induced neurotoxicity. Now, we demonstrate that iNOS is not the only microglial-related culprit implicated in MPTP-induced toxicity because mutant iNOS-deficient mice treated with minocycline are more resistant to this neurotoxin than iNOS-deficient mice not treated with minocycline. This study demonstrates that microglial-related inflammatory events play a significant role in the MPTP neurotoxic process and suggests that minocycline may be a valuable neuroprotective agent for the treatment of PD.

Key words: IL-1β; iNOS; minocycline; microglia; MPTP; NADPH–oxidase; neurodegeneration; Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal clinical features include tremor, slowness of movement, stiffness, and postural instability (Fahn and Przedborski, 2000). These symptoms are primarily attributable to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibers in the striatum (Hornykiewicz and Kish, 1987; Pakkenberg et al., 1991). Although several approved drugs do alleviate PD symptoms, chronic use of these drugs is often associated with debilitating side effects (Kostic et al., 1991), and none seems to dampen the progression of the disease. So far, the development of effective neuroprotective therapies is impeded by our limited knowledge of the pathogenesis of PD. However, significant insights into the mechanisms by which SNpc dopaminergic neurons may die in PD have been achieved by the use of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which replicates in humans and nonhuman primates a severe and irreversible PD-like syndrome (Przedborski et al., 2000). In several mammalian species, MPTP reproduces most of the biochemical and pathological hallmarks of PD, including the dramatic neurodegeneration of the nigrostriatal dopaminergic pathway (Przedborski et al., 2000).

To elucidate PD pathogenic factors, and thus to develop therapeutic strategies aimed at halting its progression, we revisited the neuropathology of this disease in search of putative culprits. Aside from the dramatic loss of dopaminergic neurons, it appears that the SNpc is also the site of a robust glial reaction in PD and experimental models of PD (Vila et al., 2001b). Although gliosis and especially activated microglia may sometimes be associated with beneficial effects, often gliosis appears to be deleterious (Vila et al., 2001b). For instance, microglial cells, which are resident macrophages in the brain, have the ability to react promptly in response to insults of various natures (Kreutzberg, 1996) in that resting microglia quickly proliferate, become hypertrophic, and increase or express *de novo* a plethora of marker molecules (Banati et al., 1993; Kreutzberg, 1996). The multifunctional nature of activated microglia encompasses the upregulation of cell surface markers such as the macrophage antigen complex-1 (MAC-1), phagocytosis, and the production of cytotoxic molecules, including reactive oxygen species (ROS), nitric oxide (NO), and a variety of proinflammatory cytokines such as interleukin-1β (IL-1β) (Banati et al., 1993; Gehrmann et al., 1995; Hopkins and Rothwell, 1995). Given this, there is little doubt that activated microglia, through the actions of aforementioned factors, can inflict significant damage on neighboring cells.

Minocycline, a semisynthetic second-generation tetracycline, is an antibiotic that possesses superior penetration through the

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brain–blood barrier (Aronson, 1980). Minocycline has emerged as a potent inhibitor of microglial activation (Amin et al., 1996; Yrjanheikki et al., 1998, 1999; Tikka and Koistinaho, 2001; Tikka et al., 2001a), an anti-inflammatory property completely separate from its antimicrobial action, and as an effective neuroprotective agent in experimental brain ischemia (Yrjanheikki et al., 1998, 1999), in the R6/2 mouse model of Huntington's disease (Chen et al., 2000), in traumatic brain injury (Sanchez Mejia et al., 2001), and in the 6-hydroxydopamine model of PD (He et al., 2001). In the present study, we report that, in the MPTP mouse model of PD, minocycline (1) mitigates, in a dose-dependent manner, the loss of dopaminergic cell bodies in the SNpc and of nerve terminals in the striatum, (2) reduces the levels of nitrotyrosine, a marker of protein nitrative modification, (3) prevents microglial activation with minimal effects on the astrocytic response, (4) reduces the formation of mature IL-1β and decreases activation of NADPH–oxidase and upregulation of inducible nitric oxide synthase (iNOS), two enzymes implicated in microglial-derived production of ROS and NO, respectively, and (5) protects against MPTP beyond the beneficial effect of iNOS ablation (Liberatore et al., 1999; Dehmer et al., 2000).

MATERIALS AND METHODS

Animals and treatment. All mice used in this study were 8-week-old male C57BL/6 mice from Charles River Laboratories (Wilmington, MA) and iNOS-deficient mice (C57BL/6-NOS2; The Jackson Laboratory, Bar Harbor, ME) and their wild-type littermates weighing 22–25 gm. For MPTP intoxication, mice received four intraperitoneal injections of MPTP-HCl (18 or 16 mg/kg of free base; Sigma, St. Louis, MO) in saline at 2 hr intervals. For minocycline treatment, mice received twice daily (12 hr apart) intraperitoneal injections of varying doses of minocycline-HCl ranging from 1.4 to 45 mg/kg (Sigma) in saline starting 30 min after the first MPTP injection and continuing through 4 additional days after the last injection of MPTP; control mice received saline only. Mice (n = 5–8 per group; saline–saline, saline–minocycline, MPTP–saline, and MPTP–minocycline) were killed at selected time points, and their brains were used for morphological and biochemical analyses. Procedures using laboratory animals were in accordance with the National Institutes of Health guidelines for the use of live animals and were approved by the institutional animal care and use committee of Columbia University. MPTP handling and safety measures were in accordance with our published recommendations (Przedborski et al., 2001b).

Immunoblots. Cytosolic and particulate fractions from selected mouse brain regions were prepared as described previously (Vila et al., 2001a) and used for either one-dimensional Western blot or dot-blot analyses. For Western blots, the following primary antibodies were used: monoclonal anti-p67phox (1:1000; Transduction Laboratories, Lexington, KY), polyclonal anti-calnexin (1:2000; Stressgen, Victoria, British Columbia, Canada). For dot-blot analyses, 25 μg of protein extracts were loaded onto the 0.2 μm nitrocellulose membrane in dot-blot apparatus (Bio-Rad, Hercules, CA), and blots were probed with an affinity-purified polyclonal antibody against nitrotyrosine (1:1000) (Przedborski et al., 2001a) that was preconjugated overnight at 4°C with 1:5000 dilution of horseradish-labeled donkey anti-rabbit IgG. For all blots, bound primary antibody was detected using a horseradish-conjugated antibody against IgG and a chemiluminescent substract (SuperSignal Ultra; Pierce, Rockford, IL). All films were quantified using the NIH Image analysis system.

RNA extraction and reverse transcription-PCR. Total RNA was extracted from midbrain, striatal, and cerebellar samples from all four groups of mice at selected time points and used for reverse transcription-PCR analysis as described previously (Vila et al., 2001a). The primer sequences used in this study were as follows: for mouse MAC-1, 5′-CAG ATC AAC AAT GTG ACC GTA TGG-3′ (forward) and 5′-CAT CAT GTC CTT GTA CTG CCG C-3′ (reverse); for mouse glial fibrillary acidic protein (GFAP), 5′-CAG GCA ATC TGT TAC ACT TG-3′ (forward) and 5′-ATA GCA CCA GGT GCT TGA AC-3′ (reverse); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GTT TCT TAC TCC TTG GAG GCC AT-3′ (forward) and 5′-TGA TGA CAT CAA GAA GTG GTG AA-3′ (reverse). PCR amplification was performed for 26 cycles for MAC-1 and GFAP and 18 cycles for GADPH. After amplification, products were separated on a 5% PAGE. Gels were dried and exposed overnight to a phosphorimager screen, and then radioactivity was quantified using a computerized analysis system (Bio-Rad PhosphoImager system).

Immunohistochemistry and stereology. Brains were fixed and processed for immunostaining as described previously (Liberatore et al., 1999). Primary antibodies used in this study were as follows: rat anti-MAC-1 (1:200; Serotec, Raleigh, NC), mouse anti-GFAP (1:1000; Boehringer Mannheim, Indianapolis, IN), and a rabbit polyclonal anti-tyrosine hydroxylase (TH) (1:1000; Calbiochem, San Diego, CA). Immunostaining was visualized by using either 3,3′-diaminobenzine (brown) or SG substrate kit (gray blue; Vector Laboratories, Burlingame, CA). Sections were counterstained with thionin.

The total number of TH-positive SNpc neurons was counted in the various groups of animals at 7 d after the last MPTP or saline injection using the optical fractionator method as described previously (Liberatore et al., 1999). This is an unbiased method of cell counting that is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons). Striatal density of TH immunoreactivity was determined as described previously (Burke et al., 1990).

Assay of NOS catalytic activity. Ventral midbrain NOS activity was assessed by measuring both the calcium-dependent and calcium-independent conversion of [^{3}H]arginine to [^{3}H]citrulline as described previously (Liberatore et al., 1999).

Mature IL-1β measurement. Ventral midbrain content of mature murine IL-1β was done as described using an enzyme-linked immunosorbend assay kit specific for this cytokine (R & D Systems, Minneapolis, MN) (Li et al., 2000).

Measurement of striatal levels of 1-methyl-4-phenylpyridinium. This was done in MPTP–saline and MPTP–minocycline mice killed at 90 min after one intraperitoneal injection of 18 mg/kg MPTP using an HPLC method with ultraviolet detection (wavelength, 295 nm) as described previously (Przedborski et al., 1996).

Synaptosomal 1-methyl-4-phenylpyridinium uptake. Naïve mice were killed, and their striata were dissected out and processed for uptake experiments as described previously (Przedborski et al., 1992). The uptake of [^{3}H]1-methyl-4-phenylpyridinium (MPP^+) was assessed in the absence and presence of minocycline (concentration raging from 1 to 330 μM). The assay was repeated three times, each time using duplicate samples.

Mouse tissue slices and lactate measurement. Striatal slices (300 μm) were prepared and processed as described by Kindt et al. (1987) using 50 μM MPP^+ and varying concentrations of minocycline (0–333 μM). At the end of the incubation (60 min; 37°C), media were collected and used for lactate quantification by enzymatic assay based on the formation of NADH, followed by 340 nm in a spectrophotometer. The assay was repeated three times, each time using duplicate samples.

Statistical analysis. All values are expressed as the mean ± SEM. Differences between means were analyzed using a two-tail Student's t test. Differences among means were analyzed using one-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 0.05 level.

RESULTS

Minocycline attenuates MPTP-induced dopaminergic neurodegeneration

As illustrated in Figure 1*G*, the numbers of SNpc TH-positive neurons varied significantly among the various groups of mice ($F_{(9,71)} = 7.045$; $p < 0.001$). MPTP, 18 mg/kg for four injections over 8 hr, caused more than a 55% reduction in the number of SNpc dopaminergic neuron numbers, as evidenced by TH immunostaining (Fig. 1*C*,*G*). In MPTP-treated mice, minocycline increased significantly the number of surviving SNpc TH-positive neurons in a dose-dependent manner (Fig. 1*D*,*G*). Minocycline at a dose of 1.4 mg/kg twice daily had no effect on MPTP neuro-

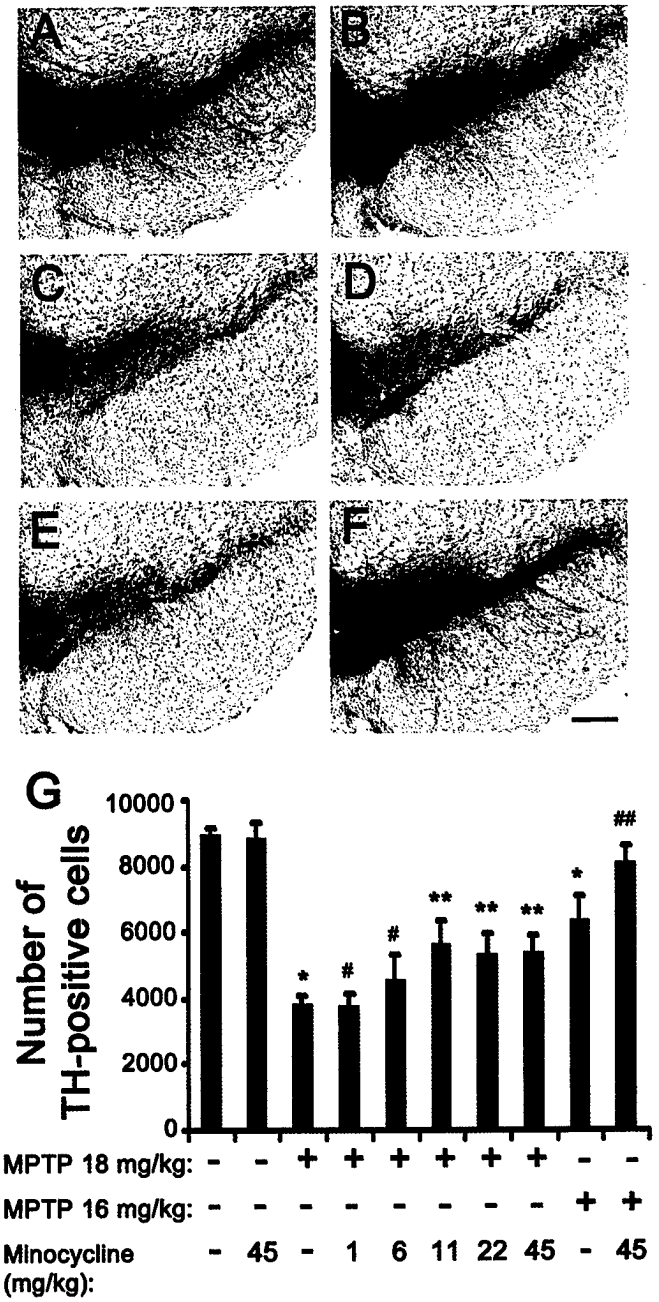

Figure 1. Effect of minocycline on MPTP-induced SNpc dopaminergic neuronal death. In saline-injected control mice treated without (*A*) or with (*B*; 45 mg/kg twice daily) minocycline, there are numerous SNpc TH-positive neurons (*brown*; *A*, *B*). MPTP (18 mg/kg for 4 injections) reduces the number of SNpc TH-positive neurons (*C*) 7 d after the last injection. In mice treated with both MPTP and minocycline, there is a noticeable attenuation of SNpc TH-positive neuronal loss (*D*). At a lower MPTP dosage (16 mg/kg for 4 injections), loss of TH-positive structures is less (*E*) and minocycline protection is more obvious (*F*). Scale bar, 50 μm. Bar graph shows SNpc TH-positive neuronal counts (*G*) assessed under the various experimental conditions. *Minocycline 1, 6, 11, 22, 45*, Mice injected with minocycline at 1.4, 6.1, 11.3, 22.5, and 45.0 mg/kg twice daily. $*p < 0.05$, fewer than saline-injected or minocycline-injected control mice. $\#p > 0.05$, same as MPTP-injected mice. $**p < 0.05$, fewer than control mice but more than MPTP-injected mice. $\#\#p < 0.05$, more than MPTP-injected mice and not different from control mice. Values are means ± SEM (n = 6–8 per group).

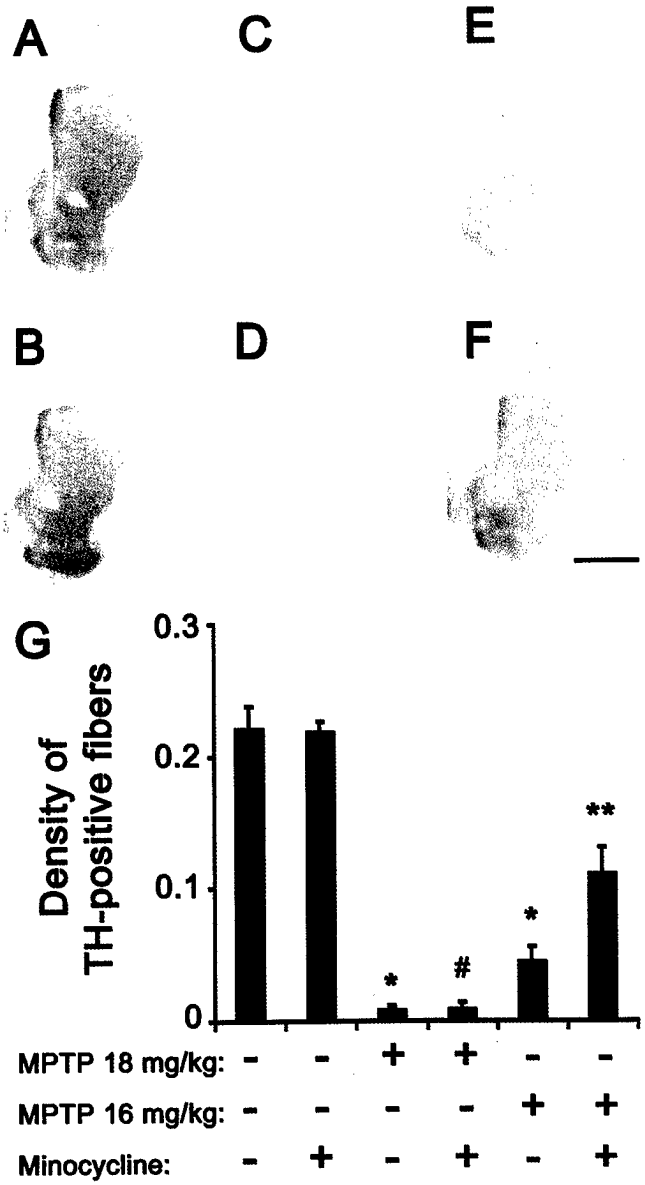

Figure 2. Effect of minocycline on MPTP-induced striatal dopaminergic fiber loss. In saline-injected control mice treated without (*A*) or with (*B*; 45 mg/kg twice daily) minocycline, there are a high density of striatal TH-positive fibers. MPTP (18 mg/kg for 4 injections) reduces the density of striatal TH-positive fibers (*C*) 7 d after the last injection. In mice treated with both MPTP and minocycline, there is also a noticeable striatal TH-positive fiber loss (*D*). At a lower MPTP dosage (16 mg/kg for 4 injections), loss of TH-positive structures is less (*E*) and minocycline protection is obvious (*F*). Scale bar, 1 mm. Bar graph shows striatal TH-positive optical density (*G*) assessed under the various experimental conditions ($F_{(5,33)} = 41.475$; $p < 0.001$). $*p < 0.05$, fewer than saline-injected or minocycline-injected control mice. $\#p > 0.05$, same as MPTP-injected mice. $**p < 0.05$, more than MPTP-injected mice but fewer than control mice. Values are means ± SEM (n = 6–8 per group).

toxicity, whereas at doses of 11.25 mg/kg twice daily and higher, there was significant neuroprotection (Fig. 1*G*). Even at the highest dose tested (45 mg/kg twice daily), minocycline was well tolerated and did not produce any behavioral abnormality. To test whether minocycline could provide complete neuroprotection, we examined another group of mice with less severe SNpc damage by

Table 1. Nitrotyrosine levels (pg/μg protein)

	Saline	Minocycline	MPTP	MPTP–minocycline
Ventral midbrain	16.2 ± 1.3	18.5 ± 1.7	32.2 ± 6.0*	21.8 ± 1.8**
Cerebellum	13.1 ± 0.8	14.0 ± 2.1	13.4 ± 1.0	11.7 ± 1.1

Nitrotyrosine levels are significantly different among groups in the ventral midbrain ($F_{(3,23)} = 4.56; p < 0.05$) but not in cerebellum ($F_{(3,23)} = 0.618; p > 0.05$). *$p < 0.05$, more than saline-injected and minocycline-injected control mice. **$p < 0.05$, less than MPTP-injected mice but not different from both control groups. Saline, Mice injected with saline; Minocycline, mice injected with minocycline only (45 mg/kg twice daily); MPTP, mice injected with MPTP only (18 mg/kg MPTP for 4 injections in one day); MPTP–minocycline, mice injected with both MPTP and minocycline. Values are means ± SEM (n = 6–8 per group).

Table 2. Striatal MPTP metabolism

MPP^+ level				
Treatment	MPTP only	MPTP–minocycline pretreatment	MPTP–minocycline post-treatment	
μg/gm tissue	6.42 ± 0.92	5.21 ± 0.66	6.52 ± 0.59	
$[^3H]MPP^+$ uptake				
Minocycline (μM)	0	10	100	333
% of control	100	98 ± 3	96 ± 3	82 ± 1
MPP^+-induced lactate				
Minocycline (μM)	0	10	100	333
μM/100 mg protein	74 ± 4	71 ± 6	70 ± 6	67 ± 6

For MPP^+ levels, minocycline (45 mg/kg) was given either 30 min before or after MPTP administration. Values are means ± SEM of either six mice per group (MPP^+ levels) or three independent experiments each performed in duplicate ($[^3H]MPP^+$ uptake and lactate levels). None of the presented values differ significantly ($p > 0.05$) from MPTP only (MPP^+ levels) or from 0 μM minocycline ($[^3H]MPP^+$ uptake and lactate levels).

injecting a lower dose of MPTP (16 mg/kg for four injections). In mice that received MPTP only, this lower regimen reduced numbers of SNpc TH-positive neurons by ~30% compared with controls (Fig. 1*E*,*G*). Minocycline at 45 mg/kg twice daily produced >90% protection against MPTP at 16 mg/kg for four injections (Fig. 1*F*,*G*).

Sparing of SNpc dopaminergic neurons does not always correlate with sparing of their corresponding striatal nerve fibers (Liberatore et al., 1999), which is essential for maintaining dopaminergic neurotransmission. To determine whether minocycline can prevent not only MPTP-induced loss of SNpc neurons but also the loss of striatal dopaminergic fibers, we assessed the density of TH immunoreactivity in striata from the different groups of mice (Fig. 2). Four injections of MPTP at 18 and 16 mg/kg reduced striatal TH immunoreactivity compared with controls by 96 and 79%, respectively (Fig. 2*C*,*E*,*G*). Mice that received minocycline (45 mg/kg twice daily) and four injections of 18 mg/kg MPTP (Fig. 2*D*,*G*) showed no protection of striatal dopaminergic fibers, whereas mice that received the same dose of minocycline and four injections of 16 mg/kg MPTP (Fig. 2*F*,*G*) showed significant sparing of striatal TH-positive fibers. These findings indicate that minocycline protects the nigrostriatal pathway against the effects of the parkinsonian toxin MPTP.

Minocycline decreases MPTP-mediated nitrotyrosine formation

A significant part of the MPTP neurotoxic process is mediated by NO-related oxidative damage (Przedborski et al., 2000), the extent of which can be evaluated by assessing nitrotyrosine levels (Liberatore et al., 1999; Pennathur et al., 1999). In saline-injected mice, the levels of nitrotyrosine in ventral midbrain were similar between non-minocycline and minocycline-treated animals (Table 1). In MPTP-injected mice (18 mg/kg for four injections), nitrotyrosine levels were significantly increased in ventral midbrain (brain region containing SNpc) and unchanged in cerebellum (brain region unaffected by MPTP) (Table 1). MPTP

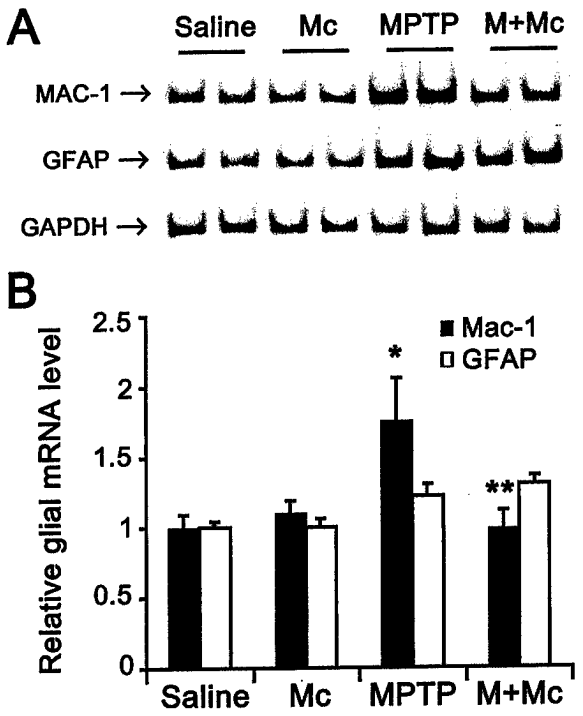

Figure 3. Minocycline prevents MPTP-induced MAC-1 transcription. *A*, *B*, Ventral midbrain MAC-1 mRNA levels but not GFAP mRNA levels are increased by 24 hr after MPTP injection compared with those of saline- or minocycline-injected mice. Minocycline prevents MPTP-induced MAC-1 mRNA increases. MAC-1 and GFAP mRNA values are normalized with GAPDH. Values are mean ± SEM ratios (n = 5–7 mice per group). *Saline*, Saline-treated; *Mc*, minocycline-treated; *MPTP*, MPTP-treated; *M + Mc*, MPTP plus minocycline-treated. *$p < 0.05$, higher than both saline- and minocycline-injected control groups. **$p < 0.05$, lower than MPTP-injected group and not different from both control groups.

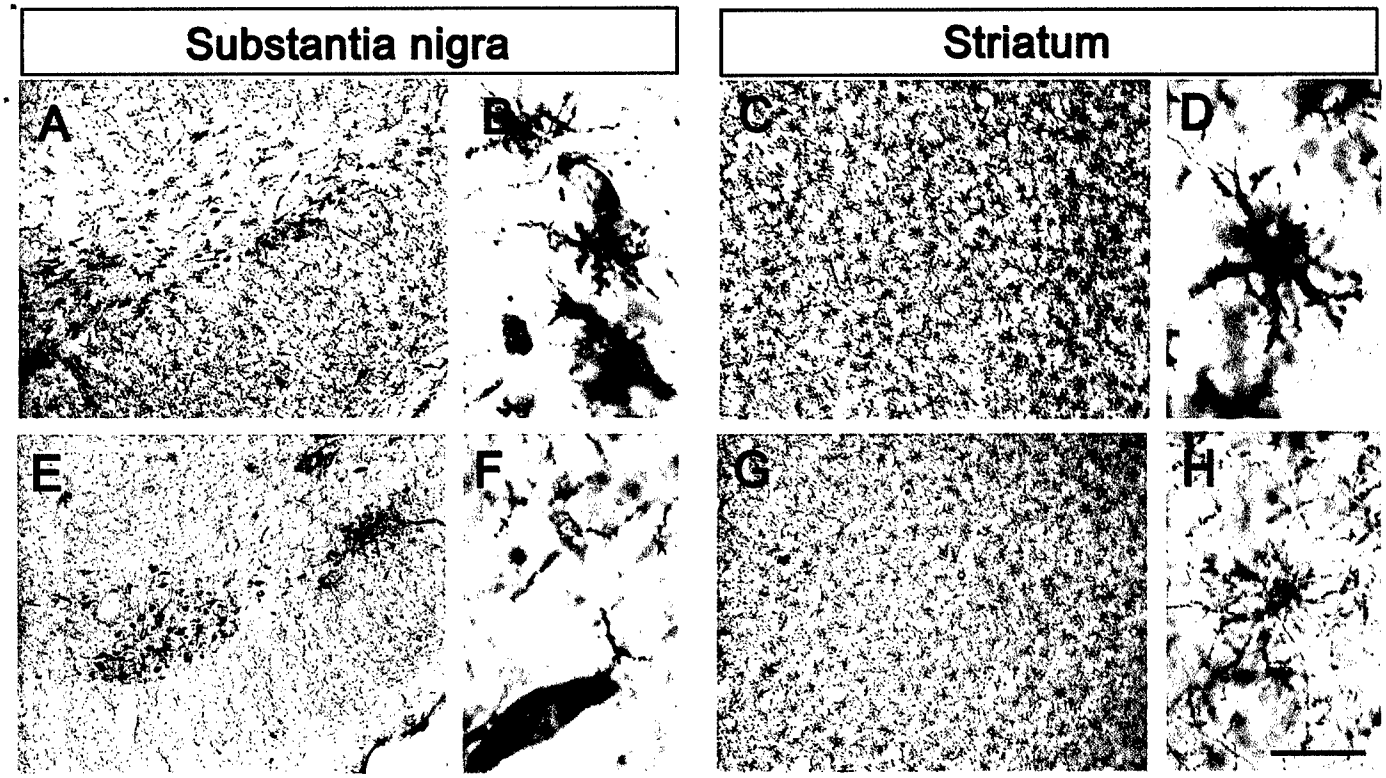

Figure 4. Minocycline prevents MPTP-induced microglia reaction. Microglia cells (*brown*) and TH-positive neurons (*gray blue*) are seen in both SNpc and striatum of all mice. One day after the last MPTP injection, numerous activated microglia (larger cell body, poorly ramified short and thick processes) are seen in SNpc (*A*, *B*) and striatum (*C*, *D*). Mice injected with both MPTP and minocycline show minimal microglial activation in SNpc (*E*) and striatum (*G*); here, microglial cell bodies are small and processes are thin and ramified (*F*, *H*). Scale bar: *A*, *C*, *E*, *G*, 1 mm; *B*, *D*, *F*, *H*, 100 μm.

produced significantly smaller increases in nitrotyrosine levels in ventral midbrains of minocycline (45 mg/kg twice daily)-treated mice than in their non-minocycline-treated counterparts (Table 1). This confirms that minocycline not only attenuates the morphological but also the biochemical impacts of MPTP neurotoxicity.

MPTP metabolism is unaffected by minocycline

The main determining factors of MPTP neurotoxic potency are its conversion in the brain to MPP^+ followed by MPP^+ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (Przedborski et al., 2000). To ascertain that resistance to the neurotoxic effects of MPTP provided by minocycline was not attributable to alterations in any of these three key MPTP neurotoxic steps, we measured striatal levels of MPP^+ 90 min after injection of 18 mg/kg MPTP, striatal uptake of [^{3}H]MPP^+ into synaptosomes, and striatal MPP^+-induced lactate production, a reliable marker of mitochondrial inhibition (Kindt et al., 1987) (Table 2). These investigations showed that striatal levels of MPP^+ did not differ between MPTP-injected mice that either received or did not receive minocycline (45 mg/kg) 30 min after MPTP administration. In addition, minocycline up to 333 μM (maximal solubilizing concentration) did not affect striatal uptake of [^{3}H]MPP^+ or MPP^+-induced lactate production (Table 2).

Minocycline inhibits MPTP-induced microglial activation

To determine whether neuroprotection by minocycline is associated with inhibition of MPTP-induced glial response, we examined the expression of MAC-1, a specific marker for microglia, and GFAP, a specific marker for astrocytes. As shown in Figure 3*B*, MAC-1 mRNA contents ($F_{(3,23)} = 4.252$; $p < 0.05$), but not GFAP mRNA contents ($F_{(3,18)} = 2.843$; $p > 0.05$), varied significantly among the various group of mice. In saline-injected mice, ventral midbrain expression of MAC-1 and GFAP mRNA was minimal (Fig. 3*A*,*B*). In these animals, only a few faintly immunoreactive resting microglia and astrocytes were observed in SNpc and striatum by immunostaining (data no shown). In MPTP-injected mice (18 mg/kg for four injections) without treatment with minocycline, ventral midbrain expression of MAC-1 mRNA was significantly higher, whereas expression of GFAP mRNA, although also higher, was not significantly increased compared with saline controls (Fig. 3). Morphologically, numerous robustly immunoreactive MAC-1-positive activated microglia were observed 24 hr after the last injection of the toxin (Fig. 4*A–D*). Although GFAP immunostaining appeared somewhat increased at 24 hr after the last MPTP injection (Fig. 5*A*,*B*), the strongest GFAP reaction was noted 7 d after the last injection of MPTP (Fig. 5*C*,*D*). Conversely, in MPTP-injected mice treated with minocycline (45 mg/kg twice daily), ventral midbrain MAC-1 mRNA contents (Fig. 3) and SNpc and striatal immunostaining were similar to those seen in saline-injected mice (Fig. 4*E–H*). In contrast, in MPTP-injected minocycline-treated mice, ventral midbrain GFAP mRNA content (Fig. 3) and SNpc immunostaining (Fig. 5*E*,*F*) were almost as high and as intense as in MPTP-only mice. Staining with Isolectin B-4 (Sigma), another marker for microglia, gave results similar to that of MAC-1 (data not shown).

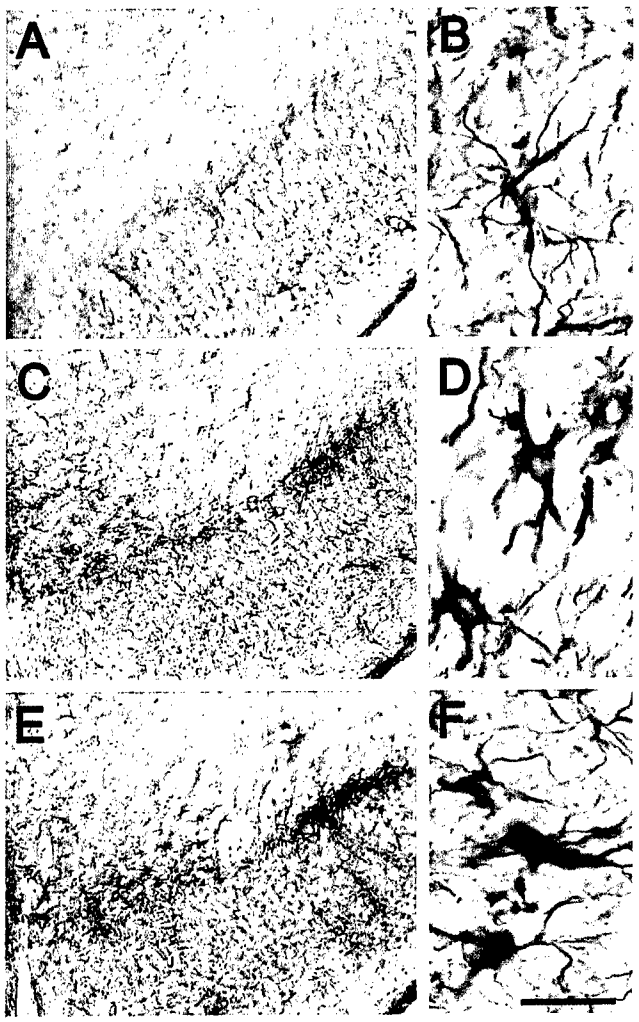

Figure 5. Minocycline does not affect MPTP-induced astrocytic reaction. One day after the last injection of MPTP, there is a mild astrocytic response (*A*, *B*), but 7 d after the last injection of MPTP, it becomes conspicuous (*C*, *D*). Minocycline does not affect the astrocytic response (*E*, *F*) 7 d after MPTP administration. Scale bar: *A*, *C*, *E*, 1 mm; *B*, *D*, *F*, 100 μm.

Minocycline prevents the production of microglial-derived deleterious mediators

Given the effect of minocycline on MPTP-induced microglial activation, we assessed whether the production of known microglial noxious mediators such as IL-1β, ROS, and NO will also be inhibited by minocycline (Fig. 6). The levels of ventral midbrain IL-1β differed significantly among the four group of mice ($F_{(3,21)} = 7.946$; $p < 0.001$) (Fig. 6*A*). Ventral midbrain levels of the proinflammatory cytokine IL-1β in MPTP-injected mice (18 mg/kg for four injections) were significantly increased (Fig. 6*A*). However, MPTP produced significantly smaller increases in IL-1β levels in ventral midbrain of MPTP mice treated with minocycline (45 mg/kg twice daily) (Fig. 6*A*). iNOS activity ($F_{(3,24)} = 9.055$; $p < 0.001$) and the ratio of membrane/total $p67^{phox}$ ($F_{(3,23)} = 4.336$; $p < 0.05$) also varied significantly among the various groups. iNOS and NADPH–oxidase, two prominent enzymes of activated microglia that produce NO and ROS, respectively, exhibited induction patterns similar to those described for IL-1β in that ventral midbrain iNOS activity was increased by 200% (Fig. 6*B*) and NADPH–oxidase activation, evidenced by the translocation of its subunit $p67^{phox}$ from the cytosol to the plasma membrane, was increased by 80% 24 hr after the last injection of MPTP (Fig. 6*C*,*D*). MPTP-induced iNOS activity and NADPH–oxidase were both abolished by minocycline administration (Fig. 6*B–D*).

Minocycline confers resistance to MPTP beyond iNOS ablation

Previously, it has been demonstrated that iNOS ablation attenuates MPTP neurotoxicity (Liberatore et al., 1999; Dehmer et al., 2000). Thus, to demonstrate whether minocycline-mediated blockade of microglial activation protects solely because it inhibits iNOS induction, we compared the effect of MPTP (16 mg/kg for four injections) on the network of striatal dopaminergic nerve fibers between mutant iNOS-deficient mice that received or did not receive minocycline (45 mg/kg twice daily). As shown in Figure 7, MPTP administration reduced by >80% the striatal density of TH-positive fibers both in wild-type and $iNOS^{-/-}$ mice; this is consistent with our previous data that ablation of iNOS protects against MPTP-induced SNpc dopaminergic neuronal loss but not against MPTP-induced striatal dopaminergic fiber destruction (Liberatore et al., 1999). In contrast, striatal TH-positive fiber densities were more than twofold higher in MPTP-treated wild-type and $iNOS^{-/-}$ mice that received minocycline compared with those that did not receive minocycline (Fig. 7). However, there was no difference in the magnitude of the minocycline beneficial effect between MPTP-treated $iNOS^{-/-}$ mice and their MPTP-treated wild-type counterparts (Fig. 7).

DISCUSSION

The main finding of this study is that inhibition of microglial activation by minocycline protects the nigrostriatal dopaminergic pathway against the noxious effects of the parkinsonian toxin MPTP. In mice that received minocycline, MPTP caused significantly less neuronal death in the SNpc, as evidenced by the greater number of TH-positive neurons, compared with those that received MPTP only (Fig. 1). Although less prominent, a similar observation was made for striatal dopaminergic nerve terminals (Fig. 2). The magnitude of resistance to MPTP in mice appears to result from a balance between the dose of minocycline and the dose of MPTP (Fig. 1), with the greatest neuroprotection observed in mice that received >11.25 mg/kg minocycline twice daily and MPTP at 16 mg/kg four times in 1 d and the least neuroprotection in mice that received the regimen of minocycline at 6.1 mg/kg twice daily and MPTP at 18 mg/kg four times in 1 d. In our study, minocycline was given twice daily beginning on the day of MPTP administration and continuing through 4 d thereafter because of its long half-life (>12 hr) and because we showed that, with this MPTP regimen, nigrostriatal degeneration occurs during the first 4 d after the last injection of MPTP (Jackson-Lewis et al., 1995). Therefore, we cannot exclude that greater protection could have been achieved if minocycline had been administered more frequently or for a longer period of time. Also, because we focused our assessment of nigrostriatal neurodegeneration at 7 d after MPTP administration, we cannot exclude with certainty that minocycline had delayed rather than prevented neuronal death. However, in light of what we know about how minocycline presumably mitigates cellular damage in a variety of experimental models (Tikka and Koistinaho, 2001; Tikka et al., 2001a), the aforementioned possibility appears unlikely. In addition, we did not pretreat mice with minocycline

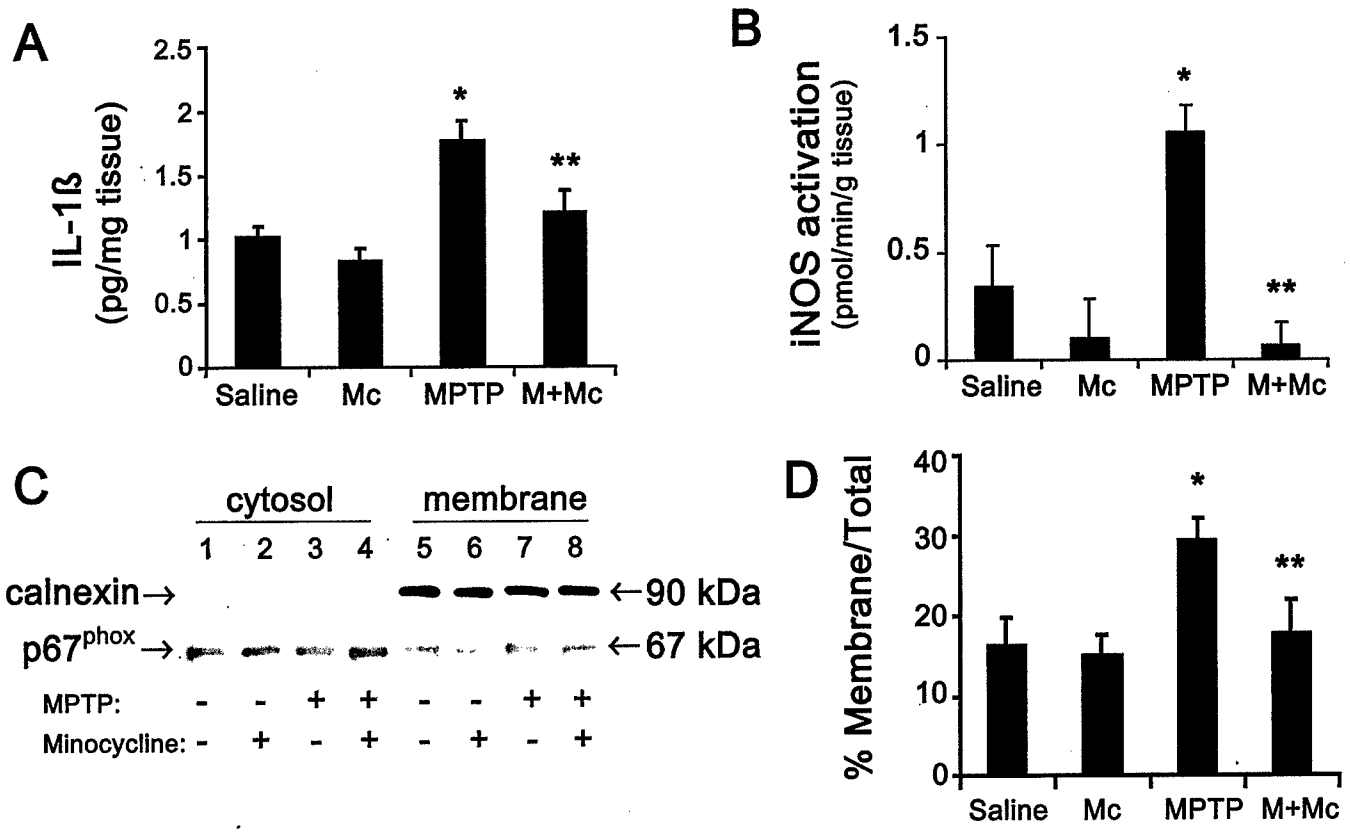

Figure 6. Effects of MPTP and minocycline on microglial-derived deleterious factors IL-1β (*A*), iNOS (*B*), and NADPH–oxidase (*C*, *D*). MPTP (18 mg/kg for 4 injections) increases ventral midbrain mature IL-1β formation, iNOS catalytic activity, and NADPH–oxidase activation, as evidenced by the translocation of its subunit $p67^{phox}$ from the cytosol to the plasma membrane, 1 d after the last injection of MPTP. Minocycline (45 mg/kg twice daily) attenuates MPTP-related effects on mature IL-1β, iNOS, and NADPH–oxidase. *Saline*, Saline-treated; *Mc*, minocycline-treated; *M*, MPTP-treated; *M+Mc*, MPTP plus minocycline-treated. $^{*}p < 0.05$, more than saline-injected or minocycline-injected control mice. $^{**}p < 0.05$, less than MPTP-injected mice but not different from both control groups. Values are means ± SEM (n = 5–8 mice per group).

because we found that administration of minocycline before MPTP injection reduces striatal MPP^+ levels by 20% (Table 2), which could complicate the interpretation of minocycline neuroprotection. Indeed, it is established that striatal contents of MPP^+ correlate linearly with magnitudes of MPTP toxicity (Giovanni et al., 1991). Thus, to avoid this potential confounding factor in our study, all mice were injected first with MPTP and then with minocycline, which we found not to affect striatal MPP^+ levels (Table 2). Along this line, it is also worth mentioning that minocycline, as used here, not only failed to alter MPP^+ levels but also failed to interfere with other key aspects of MPTP metabolism (Przedborski et al., 2000), such as entry of MPP^+ into dopaminergic neurons and inhibition of mitochondrial respiration at concentrations as high as 333 μM (Table 2).

Nitrotyrosine is a fingerprint of NO-derived modification of protein and has been documented as one of the main markers of oxidative damage mediated by MPTP (Schulz et al., 1995; Ara et al., 1998; Liberatore et al., 1999; Pennathur et al., 1999; Przedborski et al., 2001a). Consistent with our previous studies (Liberatore et al., 1999; Pennathur et al., 1999), nitrotyrosine levels increased substantially in brain regions affected by MPTP, such as ventral midbrain, but not in brain regions unaffected by MPTP, such as cerebellum (Table 1). As with the loss of SNpc neurons and striatal fibers, minocycline dramatically attenuated ventral midbrain increases in nitrotyrosine levels (Table 1). Collectively, our data demonstrate that minocycline protects against morphological as well as biochemical abnormalities that arise

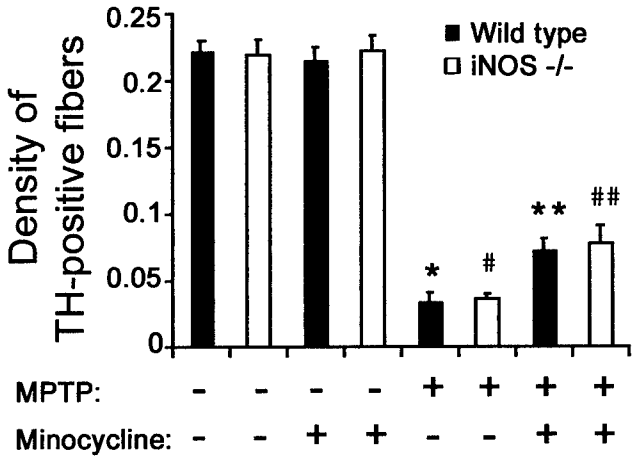

Figure 7. Minocycline attenuates MPTP-induced striatal damage by inhibiting microglia but not just by inhibiting iNOS. The optical density of striatal TH-positive fibers varied significantly among the various groups ($F_{(7,47)} = 83.576$; $p < 0.001$). *Minocycline*, Mice injected with minocycline 45 mg/kg twice daily. MPTP, Mice injected with MPTP (4 injections of 16 mg/kg). $^{*}p < 0.05$, fewer than saline-injected or minocycline-injected control mice. $\#p < 0.05$, fewer than control mice but no different than wild-type mice injected with MPTP. $^{**}p < 0.05$, fewer than control but more than MPTP-injected mice. $\#\ \#p < 0.05$, more than MPTP-injected mice but no different from wild-type mice injected with both MPTP and minocycline.

from MPTP insult. That said, we now need to consider the nature of the mechanism underlying the beneficial effects of minocycline on MPTP neurotoxicity.

Previously, we demonstrated that, aside from a dramatic loss of dopaminergic neurons, gliosis is a striking neuropathological feature in the SNpc and the striatum in the MPTP mouse model as in PD (Liberatore et al., 1999). However, activated microglia appear in the SNpc earlier than reactive astrocytes (Liberatore et al., 1999) and at a time when only minimal neuronal death occurs (Jackson-Lewis et al., 1995). This supports the contention that the microglial response to MPTP arises early enough in the neurodegenerative process to contribute to the demise of SNpc dopaminergic neurons. Consistent with this is the demonstration that direct injection of the known microglial activator lipopolysaccharide into the rat SNpc causes a strong microglial response associated with significant dopaminergic neuronal death (Castano et al., 1998; Herrera et al., 2000; Kim et al., 2000). Given these data, the key to the minocycline neuroprotective effect in the MPTP mouse model may lie in the second main finding of our study, which is that minocycline prevented MPTP-induced microglial response in both the SNpc and the striatum (Figs. 3, 4). In contrast, minocycline did not alter MPTP-related astrocytic response (Fig. 5). These results suggest that minocycline acts on microglia specifically and not on all components of gliosis. Our data also support the view that reduction of MPTP-related microglial response seen after minocycline administration is not secondary to the attenuation of neuronal loss but rather the reverse. This interpretation does not rule out, however, that at least some of the neuroprotection of minocycline against MPTP is attributable to a direct action on neurons as suggested previously (Tikka et al., 2001b).

Inhibition of microglial activation using minocycline has also been demonstrated *in vitro* (Tikka et al., 2001b) and in other experimental models of acute and chronic brain insults (Yrjanheikki et al., 1998, 1999; Tikka and Koistinaho, 2001; Tikka et al., 2001a) and results, presumably, from the blockade of p38 mitogen-activated protein kinase (Tikka et al., 2001a). It is believed that activated microglia exerts cytotoxic effects in the brain through two very different and yet complementary processes (Banati et al., 1993). First, they can act as phagocytes, which involve direct cell-to-cell contact. Second, they are capable of releasing a large variety of potentially noxious substances (Banati et al., 1993). Consistent with the notion that minocycline inhibits the ability of microglia to respond to injury, we show that minocycline not only prevents the microglial morphological response to MPTP but also the microglial production of cytotoxic mediators such as IL-1β and the induction of critical ROS- and NO-producing enzymes such as NADPH–oxidase and iNOS (Fig. 6). Although we did not test this, it is quite relevant to mention that minocycline may also prevent the induction of cyclooxygenase-2, a key enzyme in the production of potent proinflammatory prostanoids, either directly or indirectly via the blockade of IL-1β formation (Yrjanheikki et al., 1999). Little is known about the actual role of IL-1β in either MPTP or PD neurodegenerative process, except that IL-1β immunoreactivity is found in glial cells from postmortem PD SNpc samples (Hunot et al., 1999) and that blockade of interleukin converting enzyme, the known activator of IL-1β, attenuates MPTP-induced neurodegeneration in mice (Klevenyi et al., 1999). As for ROS, oxidative stress is a prominent pathogenic hypothesis in both MPTP and PD (Przedborski and Jackson-Lewis, 2000). However, many of the microglial-derived ROS, such as superoxide, cannot readily transverse cellular membranes (Halliwell and Gutteridge, 1991), making it unlikely that these extracellular reactive species gain access to dopaminergic neurons and trigger intraneuronal toxic events. Alternatively, superoxide can react with NO in the extracellular space to form the highly reactive tissue-damaging species peroxynitrite, which can cross the cell membrane and injure neurons. Therefore, microglial-derived superoxide, by contributing to peroxynitrite formation, may be significant in this model. As for NO in both MPTP and PD, the pivotal pathogenic role for microglial-derived NO is supported by the demonstration that ablation of iNOS attenuates SNpc dopaminergic neuronal death (Liberatore et al., 1999; Dehmer et al., 2000) and the production of ventral midbrain nitrotyrosine after MPTP administration (Liberatore et al., 1999). In this context, it is worth mentioning that minocycline, which protects in global brain ischemia (Yrjanheikki et al., 1998) and in a mouse model of Huntington's disease (Chen et al., 2000), appears to do so by abating iNOS expression and activity. Remarkably, iNOS ablation does protect SNpc neurons from MPTP toxicity but does not protect striatal nerve terminals and does not prevent microglial activation (Liberatore et al., 1999). This is in striking contrast to the effect of minocycline treatment, which protects both dopaminergic cell bodies and nerve fibers and inhibits the entire microglial response. This strongly suggests that microglial-associated deleterious factors other than iNOS are involved in the demise of the nigrostriatal pathway in the MPTP mouse model of PD and possibly in PD itself. Consistent with this interpretation are our data in iNOS$^{-/-}$ mice (Fig. 7), which show that minocycline protects striatal dopaminergic fibers regardless of the presence or absence of iNOS expression. Therefore, our study provides strong support to the idea that activated microglia are important contributors to the overall demise of SNpc dopaminergic neurons in the MPTP mouse model of PD and, possibility, in PD itself. It also suggests that therapeutic interventions aimed at preventing the loss of striatal dopaminergic fibers, which is essential to maintaining dopaminergic neurotransmission, must target microglial-derived factors other than iNOS.

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Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration

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Parkinson's disease (PD) is a neurodegenerative disorder of uncertain pathogenesis characterized by the loss of the nigrostriatal dopaminergic neurons, which can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Increased expression of cyclooxygenase type 2 (COX-2) and production of prostaglandin E_2 have been implicated in neurodegeneration in several pathological settings. Here we show that COX-2, the rate-limiting enzyme in prostaglandin E_2 synthesis, is up-regulated in brain dopaminergic neurons of both PD and MPTP mice. COX-2 induction occurs through a JNK/c-Jun-dependent mechanism after MPTP administration. We demonstrate that targeting COX-2 does not protect against MPTP-induced dopaminergic neurodegeneration by mitigating inflammation. Instead, we provide evidence that COX-2 inhibition prevents the formation of the oxidant species dopamine-quinone, which has been implicated in the pathogenesis of PD. This study supports a critical role for COX-2 in both the pathogenesis and selectivity of the PD neurodegenerative process. Because of the safety record of the COX-2 inhibitors, and their ability to penetrate the blood–brain barrier, these drugs may be therapies for PD.

Parkinson's disease (PD) is a common neurodegenerative disease characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (1). Its main neuropathological feature is the loss of the nigrostriatal dopamine-containing neurons, whose cell bodies are in the substantia nigra pars compacta (SNpc) and nerve terminals in the striatum (2). Except for a handful of inherited cases related to known gene defects, PD is a sporadic condition of unknown pathogenesis (1).

Epidemiological studies suggest that inflammation increases the risk of developing a neurodegenerative condition such as Alzheimer's disease (3). In keeping with this suggestion, inflammatory processes associated with increased expression of the enzyme cyclooxygenase type 2 (COX-2) and elevated levels of prostaglandin E_2 (PGE_2) have been implicated in the cascade of deleterious events leading to neurodegeneration in a variety of pathological settings (4–6). COX converts arachidonic acid to PGH_2, the precursor of PGE_2 and several other prostanoids, and exists in eukaryotic cells in two main isoforms: COX-1, which is constitutively expressed in many cell types; and COX-2, which is normally not present in most cells, but whose expression can readily be induced in inflamed tissues (7). Although both isoforms synthesize PGH_2, COX-1 is primarily involved in the production of prostanoids relevant to physiological processes, whereas COX-2 is mainly responsible for the production of prostanoids linked to pathological events (7).

In this study, we asked whether PD is associated with COX-2 up-regulation, and, if so, whether COX-2 expression contributes to the PD neurodegenerative process. We found that COX-2 expression is induced specifically within SNpc dopaminergic neurons in postmortem PD specimens and in the 1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine (MPTP) mouse model of PD during the destruction of the nigrostriatal pathway. We also show that COX-2 induction occurs through a JNK/c-Jun-dependent mechanism and that COX-2 ablation and inhibition attenuate MPTP-induced nigrostriatal dopaminergic neurodegeneration, not by curtailing inflammation, but possibly by mitigating oxidative damage. These findings provide compelling evidence that COX-2 is involved in the pathogenesis of PD and suggest a potential mechanism for the selectivity of neuronal loss in this disease.

Materials and Methods

Animals and Treatments. Wild-type mice were 8-week-old C57/BL/6 specimens (Charles River Breeding Laboratories). *Ptgs1*$^{-/-}$ mice deficient in COX-1 (B6;129P2-Ptgs1^{tm1}), *Ptgs2*$^{-/-}$ mice deficient in COX-2 (B6;129P2-Ptgs2^{tm1}), and their respective wild-type littermates were obtained from Taconic Farms. Genotyping was performed by PCR (8). For each study, 4–10 mice per group received four i.p. injections of MPTP-HCl (20 mg/kg free base; Sigma) dissolved in saline, 2 h apart in one day, and were killed at selected times ranging from 0 to 7 days after the last injection. Control mice received saline only. MPTP handling and safety measures were in accordance with our published guidelines (9). Rofecoxib (12.5–50 mg per kg per day; a gift from Merck Frosst Labs, Pointe Claire, PQ, Canada) was given to mice by gavage for 5 days before and after MPTP-injection. Control mice received vehicle only. This regimen was well tolerated and yielded 0.40 ± 0.06 ng of rofecoxib per mg of tissue (mean ± SEM for five mice) 2 h after the last gavage (measurements were kindly performed by Pauline Luk from Merck Frosst by HPLC with UV detection). Rofecoxib inhibited MPTP-induced PGE_2 production in a dose-dependent manner and did not affect striatal 1-methyl-4-phenylpyridinium (MPP^+) levels in mice (see Tables 2 and 3, which are published as supporting information on the PNAS web site, www.pnas.org). JNK pathway inhibitor CEP-11004 (1 mg/kg; gift from Cephalon, West Chester, PA) was given to mice by s.c. injections 1 day before and 6 days after MPTP-injection as described (10); CEP-11004 did not affect striatal MPP^+ levels in mice (see Table 3). Control mice received the vehicle only. This protocol was in accordance with National Institutes of Health guidelines for the use of live animals and was approved by the Institutional Animal Care and Use Committee of Columbia University.

RNA Extraction and RT-PCR. Total RNA was extracted from selected mouse brain regions as described (11). The primer sequences for COX-1, COX-2, IL-1β-converting enzyme (ICE), the 91-kDa subunit of NADPH oxidase (gp91), macrophage antigen complex-1 (MAC-1), inducible nitric oxide synthase (iNOS), and GAPDH can be found in refs. 4 and 11. All products were quantified by a phosphorimager (Bio-Rad) or a FluorChem 8800 digital image system (Alpha Innotech, San Leandro, CA).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PG, prostaglandin; COX, cyclooxygenase; SNpc, substantia nigra pars compacta; MPP^+, 1-methyl-4-phenylpyridinium; MAC-1, macrophage antigen complex-1; TH, tyrosine hydroxylase; ICE, IL-1β converting enzyme; iNOS, inducible NO synthase.

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Immunoblots. Mouse and human brain protein extracts were prepared as described (4); for phosphorylated c-Jun analysis, isolating mixture also contained 50 mM NaF and 1 mM Na_3VO_4. Western blot analyses were performed as described (4). Primary antibodies used were as follows: COX-2 (1:250; Transduction Laboratories, Lexington, KY), COX-1 (1:250; Santa Cruz Biotechnology), phosphorylated c-Jun (1:200; Cell Signaling, Beverly, MA), total c-Jun (1:200; Santa Cruz Biotechnology), or β-actin (1:10,000; Sigma). A horseradish-peroxidase-conjugated secondary antibody (1:500–1:25,000; Amersham Pharmacia) and a chemiluminescent substrate (SuperSignal Ultra; Pierce) were used for detection. Bands were quantified by using a FluorChem 8800 digital image system (Alpha Innotech).

PGE_2 Tissue Content. PGE_2 content was assessed in mouse and human tissues by a commercially available high sensitivity chemiluminescence enzyme immunoassay (EIA) kit (4) from Cayman Chemical, Ann Arbor, MI, according to the manufacturer's instructions.

COX-2, Tyrosine Hydroxylase (TH), Glial Fibrillary Acidic Protein (GFAP), and MAC-1 Immunohistochemistry. These were all performed according to our standard protocol for single or double immunostaining (11). Primary antibodies were COX-1 (1:100; Santa Cruz Biotechnology), COX-2 (1:250; gift from W. L. Smith, Michigan State University, East Lansing), TH (1:500; Chemicon), GFAP (1:500; Chemicon), and MAC-1 (1:1,000; Serotec). Immunostaining was visualized by 3,3′-diaminobenzidine with or without nickel enhancement or by fluorescein and Texas red (Vector Laboratories) and was examined by either regular light or confocal microscopy.

TH immunostaining was carried out on striatal and midbrain sections (11) and the TH- and Nissl-stained SNpc neurons were counted by stereology using the optical fractionator method described (11). The striatal density of TH immunoreactivity was determined as described (11).

Measurement of Protein-Bound 5-Cysteinyl-dopamine. Quantification of protein-bound 5-cysteinyl-dopamine was achieved by HPLC with electrochemical detection (12) using mouse brain extracts at 2 and 4 days after MPTP injections.

MPTP Metabolism. Striatal MPP^+ levels were determined by HPLC-UV detection (wavelength, 295 nm; ref. 11) 90 min after the fourth i.p. injection of 20 mg/kg MPTP. Synaptosomal uptake of [^{3}H]MPP^+ was performed as before (11) in *Ptgs2*$^{+/+}$ and *Ptgs2*$^{-/-}$ littermates.

Human Samples. Human samples were obtained from the Parkinson brain bank at Columbia University. Selected PD and controls samples were matched for age at death and interval from death to tissue processing (see *Supporting Text*, which is published as supporting information on the PNAS web site, for details).

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 factor. 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 0.05 level.

Results

MPTP Induces COX-2 Expression and Activity in Mouse Ventral Midbrain. To determine whether the expression of COX isoforms is affected during the nigrostriatal neurodegeneration, we assessed the contents of COX-1 and COX-2 mRNA and protein in ventral midbrains (the brain region that contains the SNpc) of saline- and MPTP-injected mice, at different time points. Ventral midbrain COX-1 mRNA and protein were detected in saline-treated mice and their contents were not significantly changed by MPTP administration (Fig. 1 *A*, *B*, *D*, and *E*); there was a decrease of COX-1 mRNA (but not of protein) at 2 and 4 days after MPTP administration, suggesting a transient reduction in COX-1 transcription because of the toxic insult. In contrast, ventral midbrain COX-2 mRNA and protein were almost undetectable in saline-treated mice (Fig. 1 *A*, *C*, *D*, and *F*), but were detected in MPTP-treated mice at 24 h after injections and thereafter (Fig. 1 *A*, *C*, *D*, and *F*). To determine whether MPTP-related COX-2 up-regulation paralleled an increase of its enzymatic activity, we quantified tissue contents of PGE_2. Ventral midbrain PGE_2 is detectable in saline-injected mice, and, as shown by the use of *Ptgs2*$^{-/-}$ and *Ptgs1*$^{-/-}$ mice, derives primarily from COX-1 (see Table 2). Ventral midbrain PGE_2 contents rose during MPTP neurotoxicity, coincidentally to the changes in COX-2 expression (Fig. 1*G*). Although whole-tissue PGE_2 deriving from COX-2 almost doubles after MPTP, ≈65% still originates from COX-1 (see Table 2). Unlike in ventral midbrain, levels of COX-2 mRNA, proteins, and catalytic activity in cerebellum (brain region unaffected by MPTP) and striatum were unaffected by MPTP administration (data not shown). Thus, COX-2, but not COX-1, is up-regulated in the MPTP mouse model.

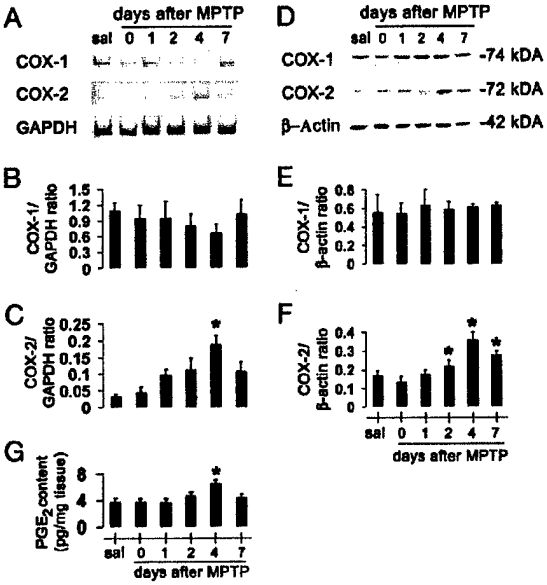

Fig. 1. Ventral midbrain COX-1 and COX-2 mRNA and protein expression after MPTP. COX-2 mRNA levels are increased by 4 days after MPTP injection (*A*) compared with controls (*C*), and almost return to basal levels by 7 days. COX-2 protein contents are minimal in saline-injected mice (sal) (*D*) but rise in a time-dependent manner after MPTP injection (*F*). COX-1 expression is not altered by MPTP (*A*, *B*, *D*, and *E*). Ventral midbrain PGE_2 levels are also increased 4 days after MPTP (*G*). Data are mean ± SEM for four to six mice per group. *, $P < 0.05$, compared with saline (Newman–Keuls post hoc test).

COX-2-Specific Induction in SNpc Dopaminergic Neurons After MPTP Administration. To elucidate the cellular origin of COX-2 up-regulation in the ventral midbrain of MPTP-treated mice, we performed immunohistochemistry. In saline controls, faint COX-2 immunoreactivity was seen in the neuropil (Fig. 2 *A* and *B*). In MPTP-treated mice, at 2 and 4 days after the last injection, ventral midbrain COX-2 immunostaining of the neuropil was increased and several COX-2-positive cells with a neuronal morphology were seen in the SNpc (Fig. 2 *C* and *D*). COX-2-positive neurons showed immunoreactivity over the cytoplasmic and nuclear areas (Fig. 2*D*), which is consistent with the known subcellular localization of this

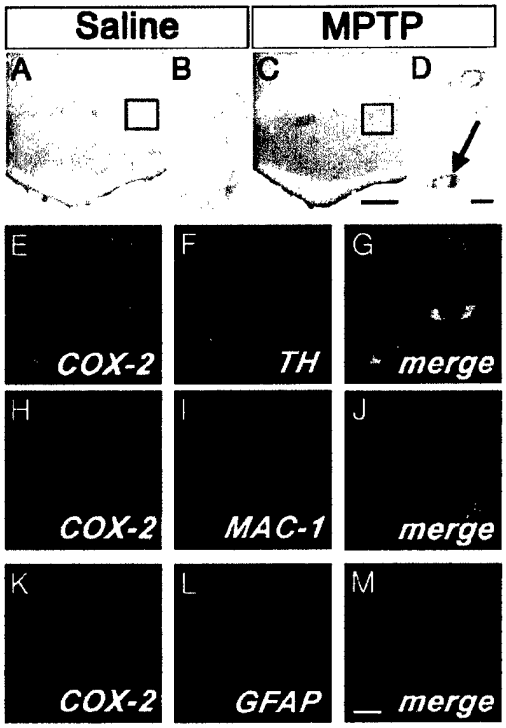

Fig. 2. Ventral midbrain illustration of COX-2 immunolocalization. No COX-2-positive cells are seen in saline-injected mice (*A* and enlarged *Inset* from *A* in *B*). Conversely, COX-2-positive cells are abundant after MPTP (*C* and enlarged *Inset* from *C* in *D*, arrow). Double immunofluorescence confirms that COX-2 (green) is highly expressed in TH-positive neurons (red; *E–G*) and not in MAC-1-positive cells (*H–J*; red) or GFAP-positive cells (*K–M*; red). [Scale bars, 250 μm (*A* and *C*), 10 μm (*B* and *D–G*), and 20 μm (*H–M*).]

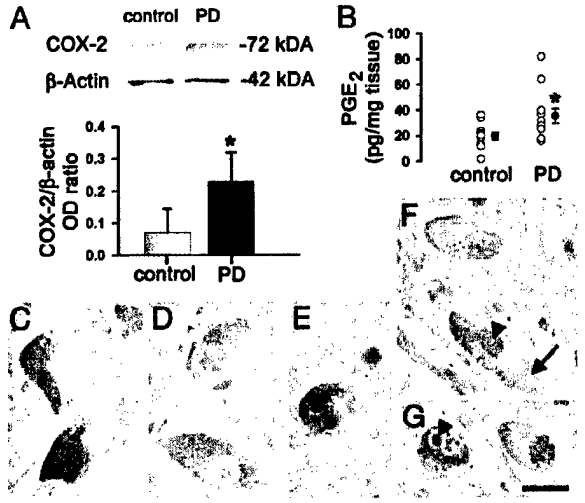

Fig. 3. Ventral midbrain COX-2 expression is minimal in normal human specimens but is increased 3-fold in PD samples (*A*). Ventral midbrain PGE_2 levels are also increased in PD (*B*). COX-2 (blue) is not detected in neuromelanized (brown) dopaminergic neurons in controls (*C* and *D*) but is well detected in PD (*E–G*). COX-2 immunostaining (*F*; *arrow*) is visible in cells with neuromelanin (*F*; arrowhead). COX-2 immunostaining is found in the core of a Lewy body (*G*; arrowhead). Data are mean ± SEM for 3–6 samples for COX-2 protein and 11 samples for PGE_2 assessment. *, $P < 0.05$, compared with normal controls (Newman–Keuls posthoc test). (Scale bar, 25 μm.)

enzyme (13). By double immunofluorescence, we found that ventral midbrain COX-2-positive cells were indeed neurons, among which almost all were dopaminergic (Fig. 2 *E–G*). COX-2 immunofluorescence did not colocalize with the microglial marker MAC-1 (Fig. 2 *H–J*), or with the astrocytic marker GFAP (Fig. 2 *K–M*). No difference in COX-2 immunoreactivity was observed in the striatum between saline- and MPTP-treated mice (data not shown). These data demonstrate that COX-2 is primarily up-regulated in ventral midbrain dopaminergic neurons during MPTP neurotoxicity.

COX-2 Up-Regulation in Postmortem Ventral Midbrain Samples from PD. To assess whether the changes in COX-2 seen after MPTP were present in PD, we assessed COX-2 protein and PGE_2 contents in postmortem SNpc samples. Consistent with the MPTP findings, PD samples had significantly higher contents of COX-2 protein and PGE_2 than normal controls (Fig. 3 *A* and *B*). As in the mice, no significant change in PGE_2 content was seen in the striatum of PD patients (data not shown). Histologically, cellular COX-2 immunoreactivity was not identified in a normal control (Fig. 3 *C* and *D*), but it was in PD midbrain sections, where it was essentially found in SNpc neuromelanized neurons (Fig. 3 *E–G*). Within these dopaminergic neurons, COX-2 immunostaining was seen in cytosol and in the typical intraneuronal proteinacious inclusions, Lewy bodies (Fig. 3*G*). The similarity of the COX-2 alterations between the MPTP mice and the PD postmortem specimens strengthens the value of using this experimental model to study the role of COX-2 in the PD neurodegenerative process.

Ablation of COX-2 Mitigates MPTP-Induced Neurodegeneration. In light of the MPTP- and PD-induced SNpc COX-2 up-regulation, we asked whether this enzyme is implicated in the nigrostriatal degeneration seen in these two pathological situations. Therefore, we compared the effects of MPTP in *Ptgs2*$^{-/-}$, *Ptgs2*$^{+/-}$, and *Ptgs2*$^{+/+}$ mice. Stereological counts of SNpc dopaminergic neurons defined by TH and Nissl staining did not differ among the three genotypes after saline injections (Fig. 4 *A* and *B* and Table 1). SNpc dopaminergic neuron numbers were reduced in all three genotypes after MPTP injections (Fig. 4 *A* and *B* and Table 1). However, in *Ptgs2*$^{-/-}$ mice, and to a lesser extent in *Ptgs2*$^{+/-}$ mice, significantly more TH- and Nissl-stained SNpc neurons survived MPTP administration than in *Ptgs2*$^{+/+}$ mice (Fig. 4*C* and Table 1). In the striatum, the density of TH-positive fibers was decreased to 16% of saline values in MPTP-treated *Ptgs2*$^{+/+}$ and to 21% in *Ptgs2*$^{+/-}$ mice, but only to 63% in *Ptgs2*$^{-/-}$ mice (Table 1). In contrast to the lack of COX-2, the lack of COX-1 did not decrease MPTP neurotoxicity: *Ptgs1*$^{-/-}$ mice [saline = 8,640 ± 725, MPTP = 4,247 ± 554 (mean ± SEM for three to eight mice per group)] and *Ptgs1*$^{+/+}$ littermates (saline = 8,577 ± 334, MPTP = 5,274 ± 147; $P > 0.05$, between MPTP-treated groups, Newman–Keuls posthoc test). Thus, COX-2, but not COX-1, participates in the MPTP neurotoxic process affecting dopaminergic cell bodies in the SNpc and nerve fibers in the striatum.

MPTP-Induced Toxicity Requires COX-2 Catalytic Activity. In the absence of catalytic activity, COX-2 can still exert deleterious effects in transfected cells (14). To test whether a similar situation occurs *in vivo* in the demise of dopaminergic neurons mediated by MPTP, nigrostriatal degeneration was assessed in regular mice injected with this neurotoxin and treated with the selective COX-2 inhibitor rofecoxib. The selected regimens of rofecoxib did not cause any distress in the animals (see *Materials and Methods* for details) or any alteration in MPTP metabolism (see below), and afforded meaningful brain accumulation (see *Materials and Methods* for details). At both 25 and 50 mg/kg, rofecoxib completely blocked ventral midbrain COX-2-derived PGE_2 production (see Table 2). In mice injected with MPTP that received either 25 or 50 mg/kg rofecoxib, ≈74% and 88%,

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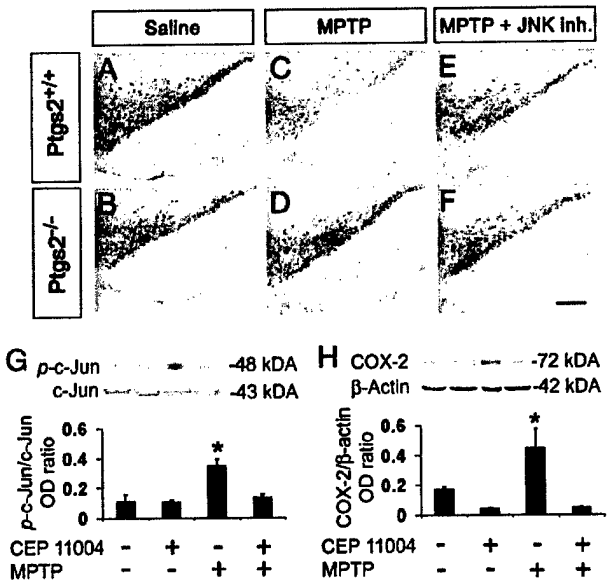

Fig. 4. Effect of COX-2 ablation and JNK pathway inhibition on MPTP-induced neuronal loss. TH-positive neuronal counts are shown in Table 1 and appear comparable between saline-injected *Ptgs2*$^{-/-}$ and *Ptgs2*$^{+/+}$ mice (*A* and *B* and Table 1). SNpc TH-positive neurons are more resistant to MPTP in *Ptgs2*$^{-/-}$ (*D*) than in *Ptgs2*$^{+/+}$ (C) mice, 7 days after MPTP injection. CEP-11004 protects *Ptgs2*$^{+/+}$ mice against MPTP neurotoxicity (*E*). Treatment of *Ptgs2*$^{-/-}$ mice with CEP-11004 does not enhance protection against MPTP (*F* and Table 1). (*G*) Ventral midbrain MPTP-induced c-Jun phosphorylation (*p*-c-Jun) inhibition by 1 mg/kg CEP-11004. (*H*) Ventral midbrain MPTP-induced COX-2 up-regulation is also inhibited by 1 mg/kg CEP-11004. Data are mean ± SEM for three to six mice per group. *, $P < 0.05$, compared with the other three groups (Newman–Keuls posthoc test). (Scale bar, 250 μm.)

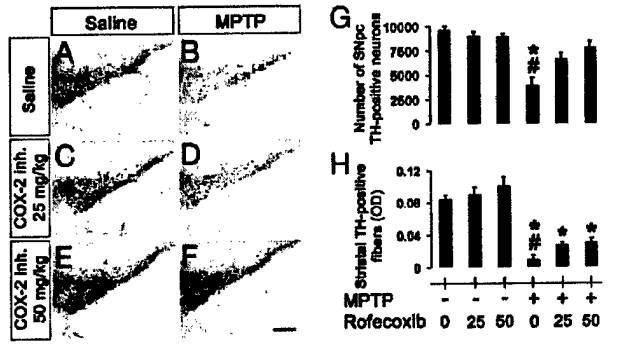

Fig. 5. TH-positive neurons and striatal fibers are more resistant to MPTP in mice treated with rofecoxib (25 or 50 mg/kg p.o.; *D* and *F*) than in mice receiving vehicle (*B*), 7 days after MPTP injection (SNpc neuronal counts are shown in *G* and striatal fiber optical density is shown in *H*). Rofecoxib by itself has no effect on TH-positive neurons (*A*, *C*, and *E*). Data are mean ± SEM for three to six mice per group. *, $P < 0.05$, compared with saline-treated controls; #, $P < 0.05$, compared with rofecoxib-treated MPTP animals (Newman–Keuls posthoc test). (Scale bar, 250 μm.)

respectively, of SNpc TH-positive neurons survived, compared with 41% in mice injected with MPTP only (Fig. 5 *C–G*). Similarly, both doses of rofecoxib attenuated the loss of TH-positive fibers caused by MPTP (Fig. 5*H*) in a dose-dependent manner, although this beneficial effect was less profound than was seen with COX-2 ablation (Table 1). These findings demonstrate how crucial the enzymatic function of COX-2 is to its neurotoxic effects on at least SNpc dopaminergic neurons.

JNK Activation Controls COX-2 Induction During MPTP-Induced Death. Stress-activated protein kinase JNK can regulate COX-2 transcription in mammalian cells (15). We therefore investigated whether MPTP-induced COX-2 up-regulation is a JNK-dependent event. After MPTP administration to mice there was a robust ventral midbrain activation of JNK, as evidenced by c-Jun phosphorylation (Fig. 4*G*) and, as shown above, a marked up-regulation of COX-2 (Fig. 4*H*). Conversely, in mice in which JNK activation was blocked by 1 mg/kg CEP-11004, MPTP caused almost no c-Jun phosphorylation and no COX-2 up-regulation (Fig. 4 *G* and *H*), thus demonstrating the critical role of the JNK/c-Jun pathway in MPTP-mediated COX-2 induction.

Administration of CEP-11004 at 1 mg/kg decreased MPTP-induced SNpc dopaminergic neuronal death, but failed to attenuate striatal dopaminergic fiber loss in *Ptgs2*$^{+/+}$ mice (Fig. 4 *C* and *E* and Table 1). The magnitude of neuroprotection against MPTP provided by the lack of COX-2 did not differ between CEP-11004-treated and untreated *Ptgs2*$^{-/-}$ mice (Fig. 4 *D* and *F* and Table 1). These data show that although both blockade of JNK and lack of COX-2 attenuate MPTP-induced SNpc dopaminergic neuronal death, the combination of the two strategies does not enhance neuroprotection.

COX-2 Ablation and Inhibition Do Not Impair MPTP Metabolism. The main determining factors of MPTP neurotoxic potency are its conversion in the brain to MPP^+ followed by MPP^+ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (16). To ascertain that resistance to the neurotoxic effects of MPTP provided by COX-2 ablation or inhibition was not because of alterations in any of these three key MPTP neurotoxic steps, we measured striatal levels of MPP^+ 90 min after the last injection of MPTP, striatal uptake of [^{3}H]MPP^+ into synaptosomes, and striatal MPP^+-induced lactate production, a reliable marker of mitochondrial inhibition (17). Striatal levels of MPP^+ were not lower in MPTP-injected *Ptgs2*$^{-/-}$ mice compared with *Ptgs2*$^{+/+}$ mice, regardless of whether or not mice received the JNK pathway inhibitor (see Table 3). Striatal levels of MPP^+ did not differ between MPTP-injected regular mice that either received or did not receive rofecoxib (see Table 3). The absence of the COX-2 gene or the presence of rofecoxib up to 32 μM did not affect MPP^+-induced lactate production (lactate in μM/100 mg of protein: *Ptgs2*$^{+/+}$ = 56.1 ± 1.9, *Ptgs2*$^{-/-}$ = 58.6 ± 5.2; regular mice/vehicle = 60.8 ± 3.8, regular mice/rofecoxib = 53.8 ± 7.3;

Table 1. Effect of COX-2 ablation and JNK pathway inhibition on MPTP toxicity

	SNpc: no. of TH-positive neurons			Striatum: TH-positive fibers, OD × 100		
Treatment	*Ptgs2*$^{+/+}$	*Ptgs2*$^{+/-}$	*Ptgs2*$^{-/-}$	*Ptgs2*$^{+/+}$	*Ptgs2*$^{+/-}$	*Ptgs2*$^{-/-}$
Saline	9,153 ± 328	9,104 ± 643	9,200 ± 643	11.9 ± 2.7	12.2 ± 1.9	11.5 ± 1.9
MPTP	5,228 ± 283*†‡	6,296 ± 356*‡	7,600 ± 610	1.9 ± 1.0*‡	2.6 ± 0.6*‡	7.3 ± 0.4
MPTP/CEP-11004	6,933 ± 501	—	8,420 ± 799	2.4 ± 0.5*‡	—	7.4 ± 0.3

Values are mean ± SEM for four to eight mice per group. *, $P < 0.05$ compared with the other groups of saline-treated mice; †, $P < 0.05$ compared with MPTP-injected *Ptgs2*$^{+/+}$ mice treated with the JNK pathway inhibitor CEP-11004; ‡, $P < 0.05$ compared with all three groups of *Ptgs2*$^{-/-}$ mice.

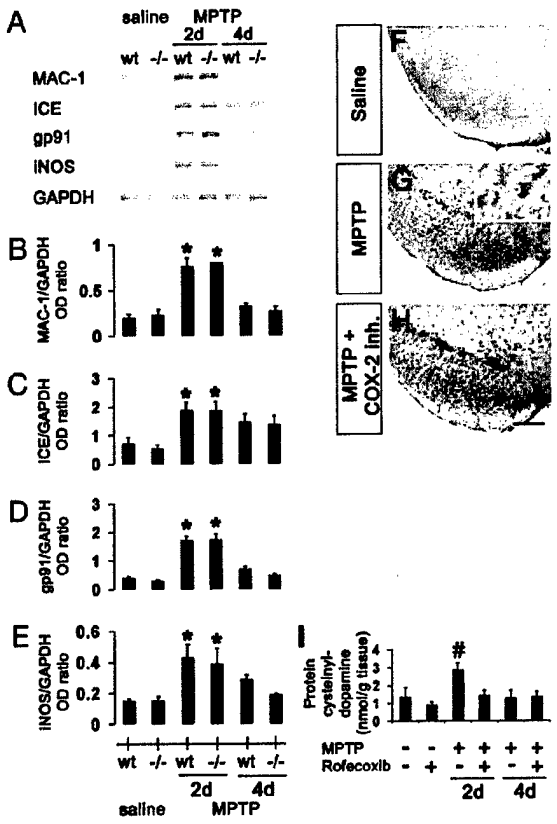

Fig. 6. Expression of inflammatory and oxidative stress markers after MPTP. Two days after MPTP injection, mRNA expression of MAC-1 (*A* and *B*), ICE (*A* and *C*), gp91 (*A* and *D*), and iNOS (*A* and *E*) are increased in the ventral midbrain and none is attenuated by COX-2 ablation. MAC-1 immunoreactivity is minimal in saline-injected mice in ventral midbrain (*F*), but is increased after MPTP injection (*G*; *Inset* shows MPTP-induced microglial activation at higher magnification). (*H*) COX-2 inhibition does not attenuate MPTP-induced microglial activation. (*I*) MPTP increases ventral midbrain protein-bound cysteinyl-dopamine, which is blocked by rofecoxib. Data are mean ± SEM for four to six mice per group. *, $P < 0.05$, compared with saline treated groups; #, $P < 0.05$, compared with the other five groups (Newman–Keuls posthoc test). (Scale bar, 250 μm.)

mean ± SEM for six mice per group). Striatal uptake of [^{3}H]MPP$^+$ was not impaired in *Ptgs2*$^{-/-}$ mice, compared with *Ptgs2*$^{+/+}$ mice, with an IC_{50} of 226.3 ± 21 nM for *Ptgs2*$^{-/-}$ mice and 195.3 ± 6.38 nM for their wild-type littermates (mean ± SEM for three mice per group). These findings suggest that COX-2-mediated neurotoxicity during MPTP-induced neuronal death operates either in parallel or downstream to MPTP's key metabolic steps.

COX-2 Modulation Does Not Alleviate MPTP-Associated Microglial Activation. Given the proinflammatory role of prostanoids such as PGE_2, we investigated the potential involvement of SNpc dopaminergic neuron production of prostaglandins in MPTP-associated microglial activation. As shown before (11, 18), there is a robust microglial activation in mice after MPTP administration. This activation was evidenced by increased contents of MAC-1, iNOS, gp91, and ICE mRNAs in ventral midbrains (Fig. 6 *A–E*), as well as by increased numbers of MAC-1-positive cells in both SNpc (Fig. 6*G*) and striatum (data not shown). Whereas both COX-2 abrogation and inhibition attenuated MPTP-mediated death, neither prevented the microglial response described above (RT-PCR: Fig. 6 *A–E*, data not shown for COX-2 inhibition; immunostaining for MAC-1: Fig. 6 *F–H*; data not shown for COX-2 ablation). Thus, COX-2 plays a negligible role in the microglial activation and the production of microglial-derived noxious factors after MPTP intoxication.

COX-2 Mediates Oxidative Stress During MPTP-Induced Neurodegeneration. Aside from production of extracellular prostanoids, COX-2 can also damage intracellular protein-bound sulfhydryl groups through the oxidation of catechols such as dopamine (19). To investigate whether such a mechanism is in play here, we quantified ventral midbrain content of protein 5-cysteinyl-dopamine, a stable modification engendered by the COX-related oxidation of dopamine (19). In saline-injected mice, baseline levels of protein 5-cysteinyl-dopamine were slightly lower in mice treated than those not treated with rofecoxib (Fig. 6*I*). In MPTP-injected mice that did not receive rofecoxib, protein 5-cysteinyl-dopamine levels were >2-fold higher than in their saline-injected counterparts (Fig. 6*I*). In contrast, in MPTP-injected mice that did receive rofecoxib, there was no significant increase in protein 5-cysteinyl-dopamine levels compared with their saline controls (Fig. 6*I*).

Discussion

This study shows an up-regulation of COX-2 in the brain regions that house nigrostriatal dopaminergic neurons in both MPTP mice and human PD samples. Increased COX-2 expression was associated with increased PGE_2 tissue content, thus indicating that the increased COX-2 is catalytically active. However, we found that ventral midbrain PGE_2 reflects mainly COX-1 activity in both normal and MPTP-injured mice. Although affected brain regions in MPTP and PD are cellularly heterogeneous, conspicuous COX-2 immunoreactivity was essentially found in SNpc dopaminergic neurons from MPTP-treated mice and postmortem PD samples. This finding raises the possibility that COX-2 up-regulation could amplify the neurodegenerative process specifically in SNpc dopaminergic neurons, thus rendering these neurons more prone than any other neurons to succumb to MPTP toxicity or PD injury.

Consistent with the involvement of COX-2 in MPTP and PD neurodegenerative processes, approximately twice as many SNpc dopaminergic neurons and striatal dopamine fibers survived in *Ptgs2*$^{-/-}$ mice compared with their wild-type littermates after MPTP administration. These results agree with the previous demonstrations that COX-2 modulation mitigates MPTP-mediated SNpc dopaminergic neurotoxicity in mice (20, 21). Because COX-2 can also exert deleterious effects unrelated to its catalytic activity (14), it must be noted that lack of COX-2 protein and inhibition of COX-2 by rofecoxib produced comparable protection of SNpc dopaminergic neurons against MPTP; striatal dopaminergic fibers were better protected by COX-2 ablation than by inhibition. It can thus be concluded that the deleterious effect of COX-2, at least on SNpc dopamine neurons in the MPTP model, and probably in PD, relies on COX-2 catalytic activity. Unlike ablation of COX-2, ablation of COX-1 failed to produce any protection against MPTP, thus indicating that induction of COX-2 expression, but not COX-1 or COX-1 gene products (e.g., COX-3; ref. 22), is instrumental in MPTP neurotoxicity.

Our data confirm the activation of the JNK/c-Jun signaling pathway after MPTP administration (23) and demonstrate that the blockade of this pathway by CEP-11004 at a concentration that inhibits c-Jun phosphorylation also inhibits COX-2 induction. In mice lacking both JNK-2 and JNK-3 genes, we found that MPTP fails to cause any phosphorylation of c-Jun or induction of COX-2 (S.H., M.V., P.T., R.J. Davis, S.P., E.C. Hirsch, P. Rakic, and R. A. Flavell, unpublished data). These results support a critical role for the JNK/c-Jun signaling pathway in the regulation of COX-2 expression in SNpc dopaminergic neurons after MPTP administration. However, COX-2 ablation attenuated MPTP-induced SNpc dopaminergic neuronal and striatal dopaminergic fiber loss, whereas JNK pathway inhibition protected only against SNpc neuronal death. This finding suggests that, in the absence of any COX-2 induction, residual COX-2 proteins in CEP-11004-treated

mice suffice to damage at least striatal dopaminergic fibers, which are more sensitive to MPTP than to SNpc dopaminergic neurons. We also show that the combination of JNK blockade and COX-2 ablation did not confer neuroprotection against MPTP beyond that produced by COX-2 ablation alone. It can thus be concluded that among the host of genes regulated by JNK, COX-2 may be the mediator of JNK's deleterious effects on SNpc dopaminergic neurons in the MPTP model of PD.

COX-2 toxicity is presumably mediated by its production of inflammatory prostanoids. Accordingly, neurons expressing COX-2 would cause their own demise through a harmful interplay with glial cells: COX-2-positive neurons release PGE_2, which promotes the production of microglial-derived mediators, which, in turn, help in killing neurons. Although we have previously demonstrated that activated microglia and derived factors do amplify MPTP-induced neurodegeneration (11), the present study shows that COX-2 modulation alters neither the morphological nor the functional correlates of microglial activation after MPTP administration. Therefore, neuronal COX-2 cytotoxicity in this model of PD does not appear to be linked to the inflammatory response. This view is consistent with our finding that most of the ventral midbrain PGE_2 originates not from COX-2, but from COX-1.

Alternatively, neuronal COX-2 overexpression may kill neurons in a cell-autonomous manner (5, 6, 24). Relevant to the leading pathogenic hypothesis for PD (25) is the fact that COX-2 cell-autonomous toxicity may arise from the formation of reactive oxygen species generated during COX peroxidase catalysis of PGG_2 conversion to PGH_2 (26). On donation of electrons to COX, cosubstrates such as dopamine become oxidized to dopamine-quinone (19), which is highly reactive with glutathione and protein amino acids such as cysteine, tyrosine, and lysine. Supporting the occurrence of such an oxidative process after MPTP injection is the marked increase in ventral midbrain protein cysteinyl-dopamine content, a fingerprint of protein cysteinyl attack by dopamine-quinone (19), in MPTP-intoxicated mice. We also demonstrated the COX-2 dependency of this toxic event by showing that COX-2 inhibition prevented the rise in protein cysteinyldopamine seen after MPTP injections. The deleterious consequences of dopamine-quinone can include depletion of vital antioxidants such as glutathione, inactivation of critical enzymes such as TH (27), and accumulation of α-synuclein protofibrils, a proposed key event in PD pathogenesis (28). Given these findings, it is thus undeniable that COX-2 up-regulation in SNpc dopaminergic neurons can unleash an array of oxidative assaults, which ultimately may play a decisive role in determining the fate of these neurons in the MPTP model and in PD itself.

Collectively, our data provide evidence for COX-2 up-regulation in MPTP and PD and support a significant role for COX-2 in both the mechanism and the specificity of MPTP- and PD-induced SNpc dopaminergic neuronal death. The present study suggests that inhibition of COX-2 may be a valuable target for the development of new therapies for PD aimed at slowing the progression of the neurodegenerative process.

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D-β-Hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease

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Parkinson disease (PD) is a neurodegenerative disorder characterized by a loss of the nigrostriatal dopaminergic neurons accompanied by a deficit in mitochondrial respiration. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes dopaminergic neurodegeneration and a mitochondrial deficit reminiscent of PD. Here we show that the infusion of the ketone body D-β-hydroxybutyrate (DβHB) in mice confers partial protection against dopaminergic neurodegeneration and motor deficits induced by MPTP. These effects appear to be mediated by a complex II–dependent mechanism that leads to improved mitochondrial respiration and ATP production. Because of the safety record of ketone bodies in the treatment of epilepsy and their ability to penetrate the blood-brain barrier, DβHB may be a novel neuroprotective therapy for PD.

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Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer disease (1). PD is clinically characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (2), and its main neuropathological feature is the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons (3).

While PD is a sporadic condition of uncertain etiology (2), several lines of evidence suggest that a defect in oxidative phosphorylation contributes to its pathogenesis. For instance, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that blocks complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain (4), recapitulates in humans the hallmarks of PD (5). Furthermore, reduction in complex I activity has been reported in PD tissues (reviewed in ref. 6). This defect is not confined only to the brain (7), since low complex I activity has also been found in platelets from PD patients (8, 9) and in *cybrid* cells engineered to contain mitochondria derived from platelets of patients suffering from PD (10).

D-β-Hydroxybutyrate (DβHB) is a ketone body produced by hepatocytes and, to a lesser extent, by astrocytes (11). It is an alternative source of energy in the brain when glucose supply is depleted such as during starvation (12). In vitro DβHB prevents neuronal damage seen following glucose deprivation (13) and mitochondrial poison exposure (14). Herein, we show that DβHB infusion protects SNpc dopaminergic neurons against MPTP in a dose-dependent and stereospecific manner and prevents the development of PD-like motor abnormalities in mice. We also provide in vivo and in vitro evidence that DβHB protects not by alleviating MPTP-related complex I inhibition, but by enhancing oxidative phosphorylation via a mechanism dependent on mitochondrial complex II (succinate-ubiquinone oxidoreductase).

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Nonstandard abbreviations used: Parkinson disease (PD); substantia nigra pars compacta (SNpc); 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); D-β-hydroxybutyrate (DβHB); L-B-hydroxybutyrate (LβHB); 3-nitropropionic acid (3-NP); tyrosine hydroxylase (TH); 1-methyl-4-phenylpyridinium (MPP^+); carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); transmembrane potential ($\Delta\psi_m$); arbitrary fluorescence unit (AFU); dihydroxyphenylacetic acid (DOPAC); homovanillic acid (HVA); reactive oxygen species (ROS); tricarboxylic acid (TCA).

Methods

Animals and treatment. All animals were 8- to 10-week-old male C57BL mice (Charles River Laboratories, Wilmington, Massachusetts, USA). Mice were divided into four groups: vehicle (i.e., saline), DβHB, L-hydroxybutyrate (LβHB), and DβHB plus 3-nitropropionic acid (3-NP). Vehicle, DβHB (1.6, 0.8, or 0.4 mmol/kg/d in saline, pH 7.4; Sigma-Aldrich, St. Louis, Missouri, USA), and LβHB (1.6 mmol/kg/d in saline, pH 7.4; Sigma-Aldrich) were administered subcutaneously (1 μl/h) using Alzet mini-osmotic pumps (DURECT Corp., Cupertino, California, USA). 3-NP (Sigma-Aldrich;

15 mg/kg, in 0.1 M PBS adjusted to pH 7.4) was given intraperitoneally 2 hours before the implantation on the first day and then once a day until the animals were sacrificed. This dosage of 3-NP was selected to inhibit complex II but not to induce cell loss. After surgery, animals were allowed to rest for 1 day. Each mouse was then randomly assigned to receive four intraperitoneal injections of either MPTP (18 mg/kg of free base in saline; Sigma-Aldrich) or saline at 2-hour intervals.

Tyrosine hydroxylase immunostaining and quantitative morphology. Seven days after the last MPTP injection, mice were killed and their brains were processed for immunohistochemical studies. Sections (30 μm) were incubated with a polyclonal anti-tyrosine hydroxylase (TH; 1,000 dilution; Calbiochem-Novabiochem Corp., San Diego, California, USA) for 48 hours at 4°C. Biotinylated secondary antibodies followed by avidin-biotin complex were used. Immunoreactivity was visualized by incubation in 3,3′-diaminobenzidine/glucose/glucose oxidase. Total numbers of TH-positive neurons in SNpc were counted stereologically using the optical fractionator method (15). Striatal OD of TH immunostaining, determined by the Scion Image program (Scion Corp., Frederick, Maryland, USA), was used as an index of striatal density of TH innervation (16). The concentration of anti-TH antibody and 3,3′-diaminobenzidine (DAB) and the duration of incubation of striatal sections in DAB were optimized to fall within the linear range of the plot of the immunostaining intensities and the scanned ODs.

Measurement of DβHB and succinate levels. At different time points after the implantation of the osmotic pumps, blood was collected from tails, and brains were quickly removed, freeze-clamped under liquid nitrogen, and stored at -80°C. Frozen tissues were treated with perchloric acid and neutralized with sodium hydroxide as previously described (17). Both DβHB and succinate were measured spectrophotometrically at 340 nm using commercial kits from Sigma-Aldrich and from Roche Molecular Biochemicals (Indianapolis, Indiana, USA) respectively, following the manufacturers' instructions.

Measurement of striatal 1-methyl-4-phenylpyridinium levels. Mice infused with either saline or DβHB (1.6 mmol/kg/d) were injected with MPTP (18 mg/kg) as described above and killed 90 minutes after the fourth injection. HPLC with UV detection (295 nm) was used to measure striatal 1-methyl-4-phenylpyridinium (MPP+) levels as previously described (18) with the following modifications: a reverse-phase Altima C18 column (Alltech Associates Inc., Deerfield, Illinois, USA) and a mobile phase consisting of 89% 50 mM KH_2PO_4 and 11% acetonitrile were used. Data represent mean ± SEM of five mice per group.

Synaptosomal uptake of MPP+. Striata were dissected out from naive mice and processed for uptake experiments as described previously (19) with a few modifications. Briefly, striata were homogenized in 0.32 M sucrose and centrifuged at 700 *g*, 4°C, for 10 minutes. The supernatant was removed and centrifuged at 27,000 *g* for 30 minutes. The resulting synaptosomal pellet was suspended at 1.2 mg/ml (original wet weight) in Krebs-Ringer phosphate buffer (pH 7.4). The uptake reaction was initiated by addition of 0.6 mg of synaptosomes to tubes containing [^{3}H]MPP+ (~4 nM, ~800,000 degradations per minute, specific activity 31.6 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, Missouri, USA) in the absence or presence of DβHB (up to 5 mM) at 37°C for 6 minutes. Nonspecific uptake was assessed in the presence of 10 μM mazindol. Data represent mean ± SEM of three mice per group.

Isolation of brain mitochondria. Brains from C57BL mice were homogenized in isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, and 2 mg/ml fat-free BSA) using a motorized Dounce homogenizer with eight up-and-down strokes. The homogenate was centrifuged at 1,000 *g* for 10 minutes, and the resulting supernatant was layered onto 5 ml of 7.5% Ficoll medium on top of 5 ml of 10% Ficoll medium and centrifuged at 79,000 *g* for 30 minutes (the Ficoll medium contained 0.3 M sucrose, 50 μM EGTA, and 10 mM HEPES). The mitochondrial pellet was resuspended in isolation buffer. Protein concentrations were determined by the bicinchoninic assay (Pierce Chemical Co., Rockford, Illinois, USA) method with BSA as a standard protein.

Mitochondrial accumulation of MPP+. Brain mitochondria were isolated and resuspended in buffer as described previously (20) but with a few modifications. The uptake reaction was initiated by addition of 0.6 mg of mitochondria to tubes containing 5 μM [^{3}H]MPP+ and 45 μM MPP+ in the absence or presence of DβHB (up to 5 mM) at 25°C for 3 minutes. Nonspecific uptake was assessed in the presence of 5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Data represent mean ± SEM of four or five mice per group.

Polarography. Brain mitochondria were suspended in respiration buffer consisting of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 5 mM HEPES, 5 mM K_2HPO_4, and freshly added 1 mg/ml defatted BSA at 30°C, and oxygen-consumption rates were measured in a closed-chamber cuvette with a mini-stirring bar using a Clark-type electrode (Hansatech Instruments Ltd., Norfolk, United Kingdom). For each reading, 300 μg protein was used in a final 1-ml respiration buffer, and all mitochondria preparations had an average respiratory control ratio of at least 5 when 10 mM glutamate and 5 mM malate were used as NADH-linked substrates.

ATP measurements. Samples were prepared under conditions identical to those of polarographical study. Mitochondria suspended in respiration buffer were incubated in the presence or absence of different substrates or inhibitors using the same incubation times as those of polarographical study. Where 3-NP was used, it was added from the beginning with MPP+ (5 minutes) or rotenone (2.5 minutes) to mitochondria before the addition of DβHB. When the reaction was stopped, mitochondrial suspension from the cuvette was lysed in an equal volume of lysis buffer from the ATP biolumi-

nescence assay kit (Roche Molecular Biochemicals), and the content of ATP was measured according to the manufacturer's instructions. Light emitted from luciferase-mediated reaction was captured in a tube luminometer and calculated from a log-log plot of the standard curve of known ATP concentrations.

Measurements of mitochondrial H_2O_2 production. Samples were prepared under conditions identical to those of polarographical study. Mitochondria suspended in respiration buffer were incubated in the presence or absence of different substrates or inhibitors using the same incubation times as those of polarographical study. Phenazine methosulfate (0.1 mM) was used to oxidize NADH (21). Hydrogen peroxide, converted from superoxide by manganese-superoxide dismutase, was measured using 5 μM Amplex red (Molecular Probes, Eugene, Oregon, USA) and 5 U/ml HRP. Fluorescence was detected by a Perkin-Elmer (Boston, Massachusetts, USA) LS55 spectrofluorometer with an excitation wavelength of 550 nm (slit 1.5 nm) and an emission wavelength of 585 nm (slit 3 nm). H_2O_2 production was calculated from a standard curve generated from known concentrations of H_2O_2.

Measurements of mitochondrial transmembrane potential. Safranine, a cationic fluorescence dye accumulated and quenched inside energized mitochondria (22, 23) was used to measure transmembrane potential ($\Delta\psi_m$). Mitochondria were incubated with 10 mM glutamate, 5 mM malate, and 5 μM safranine (Sigma-Aldrich) in respiration buffer 5 minutes before 5 mM DβHB was added, and $\Delta\psi_m$ was monitored for an additional 5 minutes. FCCP (5 μM) was used as a positive control to collapse $\Delta\psi_m$. Fluorescence was detected by a Perkin-Elmer LS55 spectrofluorometer with an excitation wavelength of 495 nm (slit 3 nm) and an emission wavelength of 586 nm (slit 5 nm). Data are reported in arbitrary fluorescence units (AFUs).

Complex I activity. Largely based on protocols described by Birch-Machin and Turnbull (24), brain mitochondria were lysed by freeze-thawing in hypotonic buffer (25 mM KH_2PO_4 [pH 7.2], 5 mM $MgCl_2$) three times. To initiate the reaction, 50 μg mitochondria were added to the assay buffer (hypotonic buffer containing 65 μM ubiquinone$_1$, 130 μM NADH, 2 μg/ml antimycin A, and 2.5 mg/ml defatted BSA) in the absence or presence of different concentrations of rotenone (2.5–15 μM) or MPP^+ (10–30 mM). The oxidation of NADH by complex I was monitored at 340 nm spectrophotometrically for 3 minutes at 30°C prior to the addition of rotenone (2 μg/ml), after which the activity was measured for an additional 3 minutes. The difference in rate before and after the addition of rotenone (2 μg/ml) was used to calculate complex I activity.

Complex II histochemistry. Animals were injected intraperitoneally with either saline or 3-NP (15 mg/kg) once daily for 8 days, the same regimen used in the animals that received DβHB. As described previously (25), animals were perfused with PBS containing 10% glycerol. Brains were rapidly removed, frozen in dry ice–cooled isopentane, and stored at -80°C. Brains were sectioned at 20 μm throughout the entire nigra and striatum. Sections were mounted onto glass microscope slides, and complex II activity was revealed by incubation of sections at 37°C for 20 minutes in 50 mM phosphate buffer (pH 7.6) containing 50 mM succinate as a substrate and 0.3 mM Nitroblue tetrazolium (NBT) as an electron acceptor.

Immunoblots. Total tissue proteins from ventral midbrains of MPTP- and saline-treated mice were isolated as described previously (26), and 20 μg proteins were separated on 12% SDS-PAGE. Membranes were blotted with polyclonal anti-β-hydroxybutyrate dehydrogenase (1:100; a generous gift from Andrew Marks, Columbia University, New York, New York, USA) and monoclonal anti-β-actin (1:5,000) overnight at 4°C. Secondary antibodies conjugated with HRP were used. Bands of interest were analyzed and quantified using FluorChem 8800 (Alpha Innotech Corp., San Leandro, California, USA).

Rotarod performance. The Economex system (Columbus Instruments, Columbus, Ohio, USA), consisting of four rotating rods of 3 cm diameter in separated compartments, enables four mice to be recorded simultaneously. Seven days after MPTP or saline injections, implanted pumps containing 1.6 mmol/kg/d DβHB were removed, and mice (4–13 animals per group) were allowed to recover from surgery and dehydration for an additional 7 days. On the testing day, animals were first pretrained three times (1 hour apart) using an accelerating mode. After these training sessions, the time on the rod, with a maximum recording time of 240 seconds, was recorded for successive rotational speeds (15, 18, 21, 24, 27, 30, 32, 36, and 40 rpm), and the overall rod performance (ORP) for each mouse was calculated by the trapezoidal method as the area under the curve in the plot of time on the rod versus rotation speed (27). To assess the responsiveness of the MPTP-related motor deficit to dopaminergic stimulation, mice were injected intraperitoneally with L-3,4-dihydroxyphenylalanine (L-DOPA) methyl ester/benserazide (100/25 mg/kg), and Rotarod performance was assessed 45 minutes later.

Measurement of dopamine and its metabolite levels in striatal and ventral midbrain tissues. Animals from the Rotarod study were sacrificed, and their striata and ventral midbrains were dissected out and stored at -80°C until analysis. On the day of the assay, striatal and ventral midbrain tissues were sonicated in 50 and 10 volumes (wt/vol), respectively, of 0.1 M perchloric acid containing 50 ng/ml dihydrobenzylamine as internal standard. After centrifugation at 15,000 *g* for 15 minutes at 4°C, 20 μl of supernatant was injected onto a C18 reverse-phase HR-80 catecholamine column (ESA Inc., Bedford, Massachusetts, USA). The mobile phase consisted of 94% 50 mM sodium phosphate/0.2 mM EDTA/1.2 mM heptanesulfonic acid (pH 3.2) solution and 6% methanol. The flow rate was 1.5 ml/min. Peaks were detected by an ESA 8 Channel CoulArray system. Data were collected and processed using the CoulArray data analysis program (version 1.12).

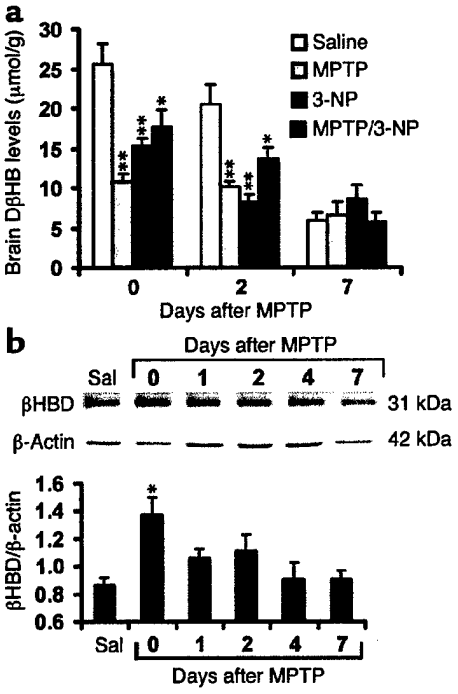

Figure 1
Brain levels of DβHB and β-hydroxybutyrate dehydrogenase (βHBD) under different treatments. (**a**) One day after implantation of pumps containing DβHB, animals were injected intraperitoneally with saline (Sal), MPTP, or 3-NP as described in Methods, and brain levels of DβHB were measured at 0 days (90 minutes after the fourth injection), 2 days, and 7 days thereafter. The utilization of DβHB was increased when cells were under metabolic stress induced by these toxins. $n = 4$–6; $^*P < 0.05$ and $^{**}P < 0.01$ compared with the respective control saline groups. (**b**) Western blot analysis of ventral midbrains from MPTP-intoxicated mice shows upregulation of this enzyme as early as day 0. $n = 4$–5 per group; $^*P < 0.05$ compared with the control saline group. β-Actin is used to normalize βHBD values.

Statistical analysis. All values are expressed as mean ± SEM. Differences between means were analyzed using one-way ANOVA followed by Newman-Keuls post-hoc testing for pairwise comparison. The null hypothesis was rejected when P was greater than 0.05.

Results

MPTP upregulates DβHB-metabolizing enzyme and increases utilization of DβHB in the brain. To assure sustained high tissue levels of DβHB during the experiment, this short-half-life (28) compound was infused subcutaneously at a dose of 1.6 mmol/kg/d for the entire 7 days. This regimen seemed well tolerated and yielded a stable plasma level of approximately 0.9 mM throughout the 7-day period. Likewise, brain DβHB levels in mice intoxicated with MPTP did not significantly change throughout the experiment (Figure 1a). Brain DβHB levels in mice that received saline instead of receiving MPTP, 3-NP, or both were significantly higher, at least at the beginning of the experiment (Figure 1a).

Circulating DβHB readily crosses the blood-brain barrier and enters mitochondria, where it is metabolized by β-hydroxybutyrate dehydrogenase to acetoacetate; the latter is converted to acetyl-CoA, which feeds into the Krebs cycle (29). In saline-injected control mice, β-hydroxybutyrate dehydrogenase protein content in ventral midbrain (the brain region that contains the SNpc) was detectable (Figure 1b). In MPTP-injected mice, β-hydroxybutyrate dehydrogenase protein content in ventral midbrain rose rapidly and remained elevated for 2 days after the last injection of MPTP (Figure 1b). These data suggest that MPTP-related cellular stress is associated with a β-hydroxybutyrate dehydrogenase upregulation, which in turn may facilitate utilization of DβHB in the brain.

DβHB attenuates MPTP-induced dopaminergic neurodegeneration. One day after implantation of pumps containing either vehicle or DβHB, mice were injected with MPTP. Seven days later, 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 mice infused with either vehicle or DβHB, numbers of TH-positive neurons in the SNpc were identical (Table 1; Figure 2, a and b), as were TH ODs in the striatum (Table 1; Figure 2, i and j). In MPTP-injected mice infused with vehicle, there was an approximately 70% loss of SNpc TH-positive neurons and an approximately 90% reduction of striatal TH ODs (Table 1; Figure 2, e and m) compared with saline-injected controls (Table 1; Figure 2, a and i). In contrast, in MPTP-injected mice

Table 1
TH- and Nissl-positive neurons in SNpc and striatal TH density

	MPTP	3-NP	Nigral TH	Nigral Nissl	Striatal TH OD (×100)
Vehicle	–	–	9,770 ± 694	15,525 ± 930	21.78 ± 1.90
DβHB (1.6 mmol/kg/d)	–	–	9,293 ± 590	14,880 ± 416	23.76 ± 2.10
LβHB (1.6 mmol/kg/d)	–	–	9,040 ± 705	12,987 ± 1,274	20.47 ± 1.43
Vehicle	–	+	8,933 ± 1,040	12,387 ± 1,169	23.11 ± 4.43
Vehicle	+	–	3,233 ± 280	6,445 ± 380	1.61 ± 0.16
Vehicle	+	+	2,600 ± 654	5,860 ± 850	1.76 ± 0.10
DβHB (0.4 mmol/kg/d)	+	–	3,168 ± 625	5,392 ± 847	1.80 ± 0.12
DβHB (0.8 mmol/kg/d)	+	–	3,720 ± 185	7,693 ± 659	2.00 ± 0.39
DβHB (1.6 mmol/kg/d)	+	–	6,300 ± 506[A]	9,597 ± 601	3.73 ± 0.10[B]
LβHB (1.6 mmol/kg/d)	+	–	2,780 ± 236	7,525 ± 360	1.10 ± 0.33
DβHB (1.6 mmol/kg/d)	+	+	1,947 ± 389	4,627 ± 701	1.73 ± 0.27

Animals with pumps containing either vehicle (saline) or different isoforms of β-hydroxybutyrate were injected intraperitoneally with MPTP, 3-NP, or saline (not shown). Data represent mean ± SEM of six to nine mice per group. [A]$P < 0.01$ and [B]$P < 0.05$ compared with the saline-MPTP group.

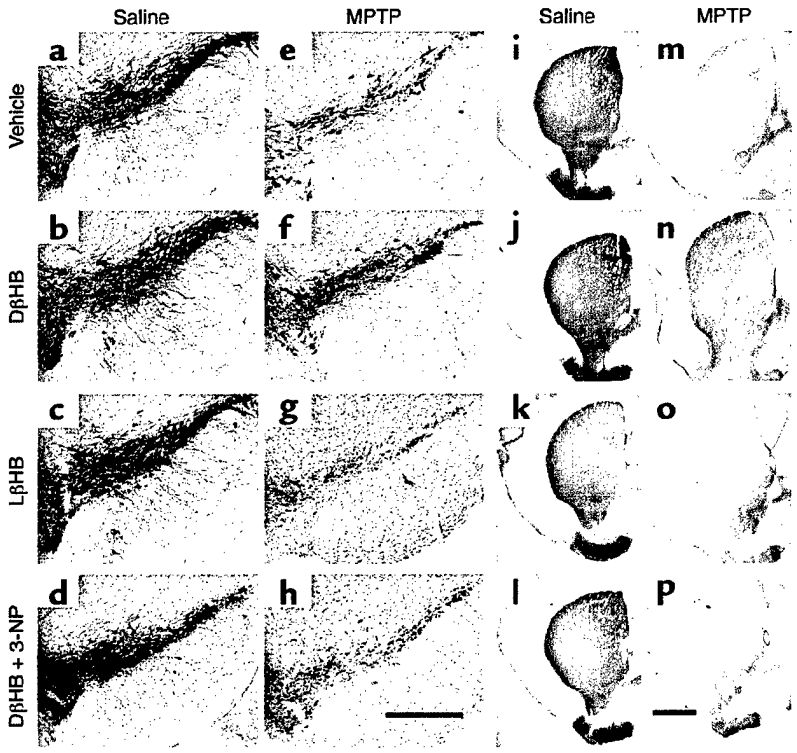

Figure 2
Protective effect of DβHB against MPTP-induced neurodegeneration. (**a–h**) TH-positive neurons in SNpc, and (**i–p**) TH-positive terminals in striatum. Animals were infused subcutaneously with vehicle (saline; **a**, **e**, **i**, and **m**), DβHB (1.6 mmol/kg/d; **b**, **d**, **f**, **h**, **j**, **l**, **n**, and **p**), or LβHB (1.6 mmol/kg/d; **c**, **g**, **k**, and **o**) 1 day before receiving intraperitoneal injections of either saline (**a–d** and **i–l**) or MPTP (18 mg/kg; **e–h** and **m–p**). There is an extensive loss of TH-positive neurons (**e**) and terminals (**m**) in MPTP-injected animals. This loss is attenuated by DβHB (**f** and **n**) but not by its inactive isomer LβHB (**g** and **o**). The complex II inhibitor 3-NP was given intraperitoneally (15 mg/kg) daily for the entire period of DβHB infusion. In the presence of 3-NP, DβHB does not confer neuroprotection. Scale bars: 500 μm (**a–h**) and 1 mm (**i–p**). Please refer to Table 1 for quantification of neurons and terminals in each animal group.

infused with DβHB, less reduction in SNpc TH-positive neurons and striatal TH ODs was observed (Table 1; Figure 2, f and n). To control for the specificity of DβHB neuroprotection, another set of MPTP-injected mice received infusion of the inactive isomer LβHB. In these mice, the loss of dopaminergic neurons was as severe as in mice infused with vehicle (Table 1; Figure 2, g and o). Thus, DβHB, but not its inactive isomer, can attenuate neurotoxic effects of MPTP on dopaminergic cell bodies in the SNpc and nerve fibers in the striatum.

DβHB attenuates the loss of dopamine and the motor deficit induced by MPTP. To examine whether DβHB protects not only against structural damage but also against functional deficits caused by MPTP, we assessed levels of dopamine and two of its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in ventral midbrain and striatum, as well as locomotor activity, in these animals. In MPTP-injected mice that did not receive DβHB, there was a reduction in dopamine and its metabolites (Table 2) in both ventral midbrain and striatum. Behaviorally, the length of time that these MPTP-injected mice remained on the rotating rods was significantly shorter than that of the saline-injected controls (Figure 3). The motor deficit observed in MPTP-treated mice was alleviated by the administration of L-DOPA/benserazide (data not shown), indicating that this motor deficit results from a loss of dopamine. In MPTP-injected mice that did receive DβHB, the levels of dopamine and its metabolites were all significantly higher than those in MPTP-injected mice that did not receive DβHB (Table 2). Of note, the attenuation of MPTP-induced dopamine loss by DβHB was smaller than the attenuation of MPTP-induced SNpc neuronal death by DβHB. Similarly, MPTP-injected mice that received DβHB performed much better on the rotating rods than MPTP-injected mice that did not receive DβHB (Figure 3). Saline-injected mice that received DβHB had similar levels of dopamine and metabolites (Table 2) and simi-

Table 2
Levels of dopamine and its metabolites in ventral midbrain and striatal tissues

	Ventral midbrain levels (ng/mg tissue)			Striatal levels (ng/mg tissue)		
	DA	DOPAC	HVA	DA	DOPAC	HVA
Vehicle	0.32 ± 0.01	0.098 ± 0.003	1.07 ± 0.02	15.81 ± 0.69	0.91 ± 0.06	1.41 ± 0.03
DβHB	0.33 ± 0.02	0.104 ± 0.010	1.09 ± 0.11	16.92 ± 0.53	1.02 ± 0.01	1.40 ± 0.11
Vehicle/MPTP	0.17 ± 0.01	0.046 ± 0.003	0.50 ± 0.03	0.86 ± 0.21	0.10 ± 0.02	0.36 ± 0.05
DβHB/MPTP	0.23 ± 0.01[A]	0.070 ± 0.005[A]	0.71 ± 0.05[A]	2.41 ± 0.45[B]	0.24 ± 0.03[B]	0.64 ± 0.04[A]

Animals from the Rotarod study were killed, and their brains were removed and measured by HPLC for the levels of dopamine and its metabolites. Data represent mean ± SEM of 4–13 mice per group. [A]$P < 0.01$; [B]$P < 0.05$ compared with the MPTP-treated group without DβHB.

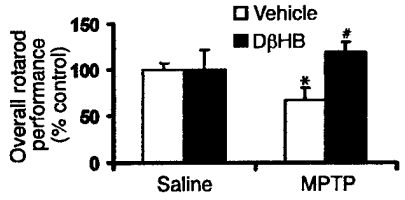

Figure 3
Protective effect of DβHB against motor deficit in MPTP-treated mice. Animals were infused subcutaneously with either vehicle (saline) or DβHB (1.6 mmol/kg/d) 1 day before receiving intraperitoneal injections of either saline or MPTP (18 mg/kg). Pumps were removed at day 7, and animals were allowed to recover from surgery and dehydration for an additional 7 days before their Rotarod performance was assessed. Motor deficit is observed in the MPTP-treated animals, but DβHB significantly improves this impairment. DβHB does not affect base-line motor function in saline-injected mice. $n = 4$–13; $^*P < 0.05$ compared with the saline-vehicle group; $^{\#}P < 0.05$ compared with the MPTP-vehicle group.

lar motor performance (Figure 3) to those of saline-injected mice that did not receive DβHB.

DβHB does not affect MPTP activation. MPTP is a protoxin whose effect correlates with the striatal content of its active metabolite MPP^+ (30). Striatal levels of MPP^+ 90 minutes after the last injection of MPTP did not differ between mice that received DβHB (30.9 ± 1.6 μg/g tissue) or vehicle [26.8 ± 1.3 μg/g tissue; Student's t test with 6 degrees of freedom ($t(6) = 1.98$; $P = 0.1$]. MPTP-induced dopaminergic neurotoxicity relies on the entry of MPP^+ into dopaminergic neurons via dopamine transporters (31). DβHB did not impair the uptake of [^{3}H]MPP^+ by striatal synaptosomes at concentrations up to 5 mM, which is more than five times the plasma concentration found in DβHB-infused animals (vehicle, 100% ± 2.3% of control; DβHB, 99.1% ± 1.8% of control; $t(6) = 0.3$; $P = 0.8$). Inside dopaminergic neurons, MPP^+ is concentrated within mitochondria by a mechanism that depends on mitochondrial $\Delta\psi_m$ (20). At 5 mM, DβHB did not alter the uptake of [^{3}H]MPP^+ by purified brain mitochondria (vehicle, 100% ± 4.1% of control; DβHB, 93.2% ± 0.5% of control; $t(6) = 1.7$; $P = 0.1$). Thus, it is unlikely that the neuroprotective effect of DβHB in the MPTP model of PD results from alterations in the key MPTP toxicokinetic steps described above.

DβHB increases mitochondrial oxygen consumption. DβHB has been used as a mitochondrial substrate (32, 33). We thus asked whether DβHB could support oxidative phosphorylation in brain mitochondria, and, if so, whether it may rescue mitochondrial respiration depressed by MPP^+-mediated complex I blockade (34). Consistent with DβHB being a mitochondrial substrate, we found that it increased oxygen consumption in a dose-dependent manner (Figure 4, a and b). The effects of DβHB in supporting mitochondrial respiration are stereospecific, since the inactive isomer LβHB failed to improve oxidative phosphorylation (Figure 4c). We also found that DβHB ameliorated oxygen consumption impaired by different concentrations of MPP^+ (Figure 4a) and of another complex I inhibitor, rotenone (Figure 4b). At 25 μM MPP^+ and 25 nM rotenone, which we found to inhibit about 25% of the oxygen consumption in glutamate- and malate-supported mitochondria, DβHB restored completely the oxygen consumption depressed by these inhibitors (Figure 4, a and b). At 100 μM MPP^+ and 100 nM rotenone inhibits more than 90% of the oxygen consumption in glutamate- and malate-supported mitochondrial respiration (data not shown). At these concentrations, DβHB restored completely the oxygen consumption inhibited by MPP^+, but only partially that inhibited by rotenone (Figure 4, a and b).

DβHB does not uncouple mitochondria. To assure that the increase in rate of oxygen consumption induced by DβHB is not an artifact of uncoupled mitochondria, we measured $\Delta\psi_m$. As expected, the uncoupler FCCP at 5 μM collapsed the $\Delta\psi_m$ in isolated mitochondria (FCCP, 419 ± 23 AFUs; no FCCP, 69 ± 2 AFUs). Conversely,

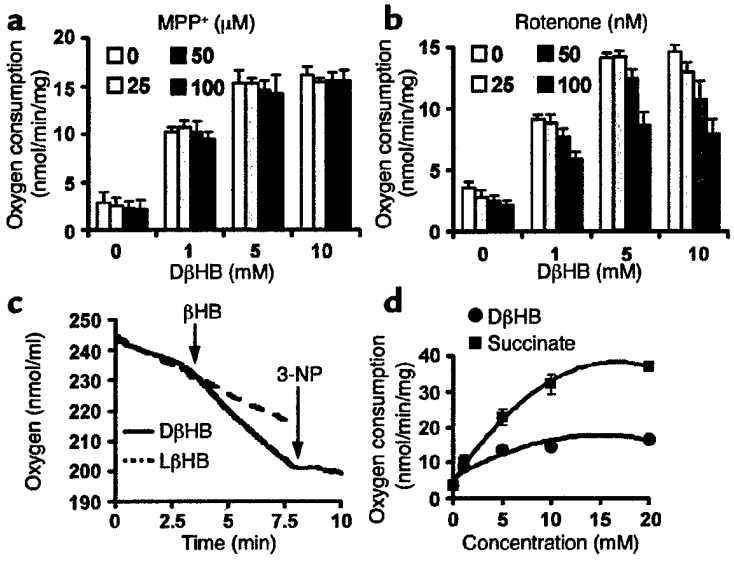

Figure 4
DβHB increases oxygen consumption in purified brain mitochondria. Mitochondria (300 μg) were incubated in the absence or presence of MPP^+ (5 minutes; **a**) or rotenone (2.5 minutes; **b**) at 30°C, and then 5 mM DβHB was added to induce oxygen consumption. DβHB attenuated inhibition of mitochondrial respiration induced by MPP^+ (**a**) or rotenone (**b**) at indicated concentrations, which blocked about 25–90% of oxygen consumption when glutamate and malate were used as NADH-linked substrates (data not shown). (**c**) The improvement of oxygen consumption by DβHB is stereospecific and is blocked by 10 mM 3-NP, a complex II inhibitor. (**d**) DβHB increases oxygen consumption in a dose-dependent and saturable fashion as seen with succinate, a complex II substrate, although not as efficiently as succinate does on an equimolar basis. $n = 3$–4.

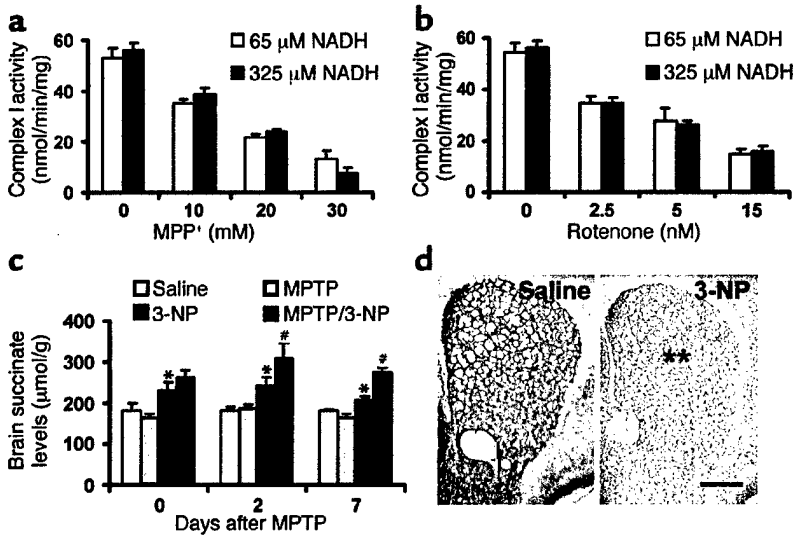

Figure 5

Dose-response study of NADH in complex I activity (**a** and **b**) and brain levels of succinate (**c**). In mitochondria lysed by freeze-thawing, when the inhibition of complex I activity was titrated with different concentrations of MPP^+ (**a**) or rotenone (**b**), different amounts of NADH did not produce different responses in complex I activity (n = 4 per group). (**c**) Levels of succinate were measured in the brains of animals treated with 18 mg/kg/d MPTP or 15 mg/kg 3-NP, or both. Levels of succinate in the group that received DβHB (1.6 mmol/kg/d) are significantly increased in the presence of 3-NP. n = 3–10 per group; $^*P < 0.05$ compared with the control saline group; $^*P < 0.05$ compared with the 3-NP group. (**d**) Histochemical analysis in striatal sections shows that when animals were treated with 3-NP (right panel) at this concentration for 8 days, there was approximately 40% reduction in complex II activity in the striatum compared with that in the group treated with saline (left panel). n = 5 per group; $^{**}P < 0.01$. Scale bar: 500 μm.

DβHB at concentrations as high as 5 mM had no effect on mitochondrial $\Delta\psi_m$ (DβHB, 68.58 ± 3.07 AFUs; no DβHB, 65.21 ± 3.03 AFUs; n = 5 per group; $P > 0.05$). We also found that the increase in oxygen-consumption rate produced by DβHB could be blocked by antimycin A, a complex III inhibitor (base line, 4.49 ± 0.62 nmol/min/mg; DβHB, 14.14 ± 0.43 nmol/min/mg; DβHB + antimycin A, 5.99 ± 0.95 nmol/min/mg; n = 3 per group; $P > 0.05$ comparing base line with the DβHB + antimycin A group). These experiments indicate that DβHB does not uncouple mitochondria at concentrations that increased oxygen consumption.

Effects of DβHB on mitochondrial respiration seem driven by complex II. One product generated from the metabolism of DβHB is NADH, which provides the driving force for the mitochondrial respiration through complex I. Can an increase in availability of NADH compensate for the loss of oxygen consumption due to complex I inhibition? To test this possibility, freeze-thawed disrupted brain mitochondria were incubated with MPP^+, or rotenone, and NADH. Concentrations of MPP^+ and rotenone were selected to produce complex I inhibition ranging from about 40% to 100%, and supplementation of NADH ranged from 0.5 to 2.5 times the normal concentration used in the assay (Figure 5, a and b). These changes in NADH supplementation did not modify the degree of complex I inhibition (Figure 5, a and b). This indicates that DβHB-derived NADH cannot explain the improvement seen in mitochondrial respiration produced by DβHB.

Based on its metabolic pathway, DβHB can also generate succinate, which is capable of stimulating the rate of oxygen consumption in isolated brain mitochondria through complex II. In keeping with this metabolic pathway, we found that both DβHB and succinate did improve oxygen consumption in a dose-dependent and saturable manner, although DβHB was not as potent as succinate (Figure 4d). This is not unexpected, since DβHB has to go through several metabolic steps to generate succinate. In addition, we found that the beneficial effects of DβHB on mitochondrial respiration in the presence of MPP^+ or rotenone were completely abolished by two different complex II inhibitors, 3-NP at 10 mM (Figure 4c) and malonate at 10 mM (data not shown). Together, these data are consistent with the idea that DβHB increases mitochondrial respiration in the face of complex I inhibition by a complex II–dependent mechanism.

DβHB neuroprotection is abrogated by mitochondrial complex II inhibition in vivo. To determine whether our in vitro data are relevant to DβHB neuroprotection seen in vivo, we first measured succinate levels in the brains of DβHB-infused mice. Upon inhibition of complex II, DβHB infusion indeed increased levels of succinate in the brain (Figure 5c). Next, MPTP-injected mice infused with DβHB were injected with 3-NP. This irreversible complex II inhibitor was administered daily for the entire period of DβHB infusion at a dosage of 15 mg/kg/d. As illustrated in Figure 5d, this regimen of 3-NP inhibited approximately 40% of complex II activi-

Table 3

ATP levels in purified brain mitochondria

ATP levels (nmol/mg mitochondrial protein)	
Base line (no substrate)	5.37 ± 0.30
DβHB (5 mM)	76.16 ± 6.11[A]
DβHB plus MPP^+ (100 μM)	90.49 ± 9.73[A]
DβHB plus rotenone (100 nM)	25.96 ± 5.22[B]
DβHB plus MPP^+ plus 3-NP (10 mM)	0.62 ± 0.21
DβHB plus rotenone plus 3-NP	0.73 ± 0.23
LβHB	3.85 ± 0.24

Mitochondrial samples were prepared as in the polarographical studies, and ATP levels were measured using a luciferase kit. Data represent mean ± SEM of four mice per group. $^AP < 0.01$ and $^BP < 0.05$ compared with the base-line endogenous ATP level.

Table 4
H_2O_2 measurements in purified brain mitochondria

Treatment	Mitochondrial H_2O_2 production (pmol/min/mg protein)
DβHB (5 mM)	73.83 ± 8.04
Rotenone (100 nM)	132.39 ± 19.68
DβHB plus rotenone	506.00 ± 40.47[A]
DβHB plus rotenone plus 3-NP (10 mM)	522.76 ± 62.23[A]
DβHB plus rotenone plus PM (0.1 mM)	160.50 ± 20.62
LβHB (5 mM) plus rotenone	105.91 ± 7.45
MPP^+ (500 μM)	55.24 ± 12.98
DβHB plus MPP^+	94.92 ± 6.79[B]
DβHB plus MPP^+ plus PM	73.76 ± 6.38
LβHB plus MPP^+	54.28 ± 4.93

Mitochondrial samples similar to those in the polarographical studies were prepared, and the fluorescence dye Amplex red was used to measure H_2O_2 converted from superoxide. Data represent mean ± SEM of four mice per group. [A]$P < 0.01$ compared with the rotenone-alone group; [B]$P < 0.05$ compared with the MPP^+-alone group. PM, phenazine methosulfate.

ty in the striatum without causing cell death in either the SNpc (Table 1) or the striatum, as evidenced by TH or Nissl staining (Table 1; data not shown for striatal Nissl staining). As before, DβHB protected against MPTP neurotoxicity in mice that did not receive 3-NP. However, DβHB failed to reduce MPTP-induced dopaminergic neurodegeneration in mice that did receive 3-NP (Table 1; Figure 2, h and p). Supporting the effectiveness of the 3-NP regimen in blocking complex II is our demonstration that succinate levels in the brain were higher in mice that received 3-NP than in those that did not (Figure 5c). Thus, these results are consistent with the hypothesis that complex II is a pivotal mediator in DβHB's neuroprotective effects.

DβHB does not have antioxidant effects but increases ATP production. Inhibition of complex I by MPP^+ and rotenone generates reactive oxygen species (ROS), raising the possibility that the beneficial effects of DβHB are mediated by an antioxidant action, as previously suggested (14). In isolated mitochondria, DβHB did not reduce but stimulated ROS production in the presence of rotenone or MPP^+ (see Table 4). To elucidate the basis of DβHB-related ROS production, 3-NP was added to the incubation mixture (see Table 4). This complex II inhibitor was unable to block the DβHB-related ROS production, thus ruling out the possibility of a reversed flux of electrons from complex II to complex I as the ROS generator (22, 23). Instead, we suspected that the DβHB-related ROS resulted from additional NADH generated by DβHB metabolism. To test this alternative possibility, phenazine methosulfate, a compound that oxidizes NADH (21), was included in the incubation mixture. Consistent with this possibility, phenazine methosulfate abolished ROS production (see Table 4). These data argue against DβHB having antioxidant properties, at least in this in vitro setting.

Inhibition of complex I by MPP^+ and rotenone also impairs ATP production, raising the possibility that the beneficial effects of DβHB are mediated by attenuation of ATP depletion. We thus measured ATP production in isolated brain mitochondria under conditions similar to those of polarographical study. As shown in Table 3, DβHB increased ATP production from a base line of 5.37 ± 0.30 nmol/mg protein to 76.16 ± 6.11 nmol/mg protein. The increase of ATP production was not detected with the inactive isomer LβHB (3.85 ± 0.24 nmol/mg protein). In agreement with the oxygen-consumption data, DβHB prevented the loss of ATP production caused by 100 μM MPP^+ or 100 nM rotenone (Table 3). Yet, upon addition of 3-NP, DβHB-related ATP production was abolished (Table 3). Together, these data are consistent with the contention that the effects of DβHB seen in the polarographical studies correspond to an increase in oxidative phosphorylation.

Discussion

The present study shows that the ketone body DβHB, a crucial alternative source of glucose for brain energy, confers protection against the structural and functional deleterious effects of the parkinsonian toxin MPTP; these include degeneration of SNpc dopaminergic neurons and striatal dopaminergic fibers, loss of striatal dopamine, and PD-like motor deficit. The beneficial effects of DβHB were achieved by its subcutaneous infusion using osmotic mini-osmotic pumps, which, without apparent distress, allowed its reliable continuous delivery to the brain. While DβHB levels in the brain were stable in DβHB-infused mice exposed to MPTP, in mice injected with saline they were higher at the beginning and then dropped during the experimental period of 7 days. Although the basis for these differences remains to be elucidated, it is possible that the utilization of DβHB in the brain increases rapidly following exposure to mitochondrial poisons such as MPTP and augments progressively in normal brain as part of a metabolic adaptation to sustained high DβHB concentrations.

Utilization of DβHB in the brain is contingent on its conversion to acetoacetate by β-hydroxybutyrate dehydrogenase, which is scarce in the adult brain, especially in the basal ganglia (35). The activity of β-hydroxybutyrate dehydrogenase correlates with its protein content (36), and, following MPTP administration, it is upregulated in the ventral midbrain. MPTP-induced β-hydroxybutyrate dehydrogenase upregulation precedes peak dopaminergic neuronal death in this model (37). It can thus be envisioned that β-hydroxybutyrate dehydrogenase activity increases early enough to allow effective utilization of DβHB by the compromised dopaminergic neurons.

A critical step in activation of MPTP is its conversion into MPP^+ by monoamine oxidase (38). The possibility that DβHB infusion confers protection by interfering with monoamine oxidase activity can be ruled out given the fact that brain levels of MPP^+ were similar between mice that received and those that did not receive DβHB. Also arguing against the possibility that DβHB confers protection by impairing MPTP activation is the fact that DβHB attenuates dopaminergic neuronal death in primary ventral midbrain cultures exposed to MPP^+ (14). DβHB also did not interfere with other key aspects of

MPTP metabolism (39), such as entry of MPP^+ into dopaminergic neurons and mitochondria at concentrations as high as 5 mM. Together these data indicate that DβHB protects not by a pre-complex I mechanism but rather by mitigating the deleterious effects of complex I inhibition on the survival of dopaminergic neurons.

In isolated brain mitochondria, DβHB improves oxygen consumption in the presence of the complex I poisons MPP^+ and rotenone. The DβHB effect is dose dependent and stereospecific. The metabolism of DβHB leads to an elevated mitochondrial [NADH]/[NAD^+] ratio due to NADH generated from the conversion of DβHB to acetoacetate and also from the tricarboxylic acid (TCA) cycle, whose turnover is increased by high levels of acetyl-CoA produced by acetoacetate. NADH is used by complex I to drive mitochondrial respiration. DβHB may increase oxygen consumption by fueling mitochondria with NADH. However, in the presence of complex I inhibition by MPP^+ or rotenone, NADH oxidation is impaired and, as shown in this study, an increase in NADH content is unable to alleviate complex I blockade.

In addition to generating NADH, increased TCA turnover, in theory, should also lead to increases in production of other TCA intermediates such as succinate. Here, we show that DβHB infusion does increase brain succinate content. While succinate is a TCA cycle substrate, its oxidation by succinate dehydrogenase is coupled to a transfer of electrons to ubiquinone of the mitochondrial respiratory chain, and thus succinate is routinely used to support oxygen consumption in the presence of complex I blockade. We demonstrate that inhibition of complex II (a) abrogates DβHB-mediated increases in oxygen consumption in isolated mitochondria and (b) abolishes DβHB-mediated protective effects on SNpc dopaminergic neurons and striatal dopaminergic fibers after MPTP administration. Thus, these data strongly support our hypothesis that the beneficial effect of DβHB in the MPTP model of PD involves a complex II–dependent mechanism.

It has been proposed that the ability of DβHB to decrease MPP^+ neurotoxicity in primary ventral midbrain cultures is related to the oxidation of the coenzyme Q couple, which should, by decreasing the semiquinone, decrease ROS production (14). Contrary to this prediction, we found, at least in isolated mitochondria, that rather than decreasing ROS production induced by MPP^+ or rotenone, DβHB enhanced it even further. These findings cast doubt that DβHB protects the nigrostriatal pathway through an antioxidant mechanism. How can DβHB increase ROS? Succinate is the most effective ROS-generating substrate in intact brain mitochondria (22, 23), by stimulating a reversed flux of electrons from complex II to complex I (22, 23). However, rotenone blocks this ROS signal (22, 23); thus, in the context of the present study, in which complex I is inhibited, this mechanism may not be operative. Instead, our data suggest that DβHB-derived NADH, by feeding complex I, increases the accumulation of electrons upstream to the blockade, thereby stimulating ROS production.

Mitochondrial respiration is tightly linked to ATP synthesis (40). It may thus be speculated that DβHB, by restoring oxygen consumption in MPTP-intoxicated animals, may increase ATP cellular stores. Ablation and inhibition of poly(ADP-ribose) polymerase-1 (41, 42) and creatine supplements (43) mitigate MPTP-induced death of dopaminergic neurons in the SNpc by buffering ATP depletion. These studies underscore the importance of ATP deficit in the MPTP neurodegenerative process. In normal rodents, dopaminergic structures represent less than 1.5% of the cellular elements in the striatum (44) and hardly more in the ventral midbrain. This renders precarious any detection of ATP changes in brain tissues of MPTP-intoxicated mice (45). To avoid this problem, we studied the effects of DβHB on ATP production in isolated brain mitochondria. By this approach, we were able to demonstrate that DβHB does increase ATP levels in both the absence and the presence of complex I inhibitors. Consistent with the oxygen-consumption data, we also found that the stimulation of ATP production by DβHB likely relies on complex II, as inhibitors of this electron transport chain enzyme eliminated the effect. Data generated in isolated mitochondria may only approximate the more complex situation found in vivo. Despite this caveat, we believe that the most parsimonious explanation for DβHB-induced neuroprotection in the MPTP model of PD is that energy crisis is attenuated by an enhancement of oxidative phosphorylation. It is thus tempting to conclude that, under the current DβHB regimen, the benefit due to the improved ATP production overcomes the possible detriment due to the increased ROS formation in this PD model.

The present study demonstrates that modulation of body DβHB levels may be a straightforward neuroprotective strategy for the treatment of neurodegenerative diseases such as PD. Relevant to this view is the demonstration that mice subjected to dietary restriction (e.g., alternate-day fasting) exhibit higher serum DβHB concentrations and are more resistant to kainic acid–induced hippocampus damage (46) and to MPTP-induced SNpc damage (47). At this point, however, the long-term effects of the chronic use of DβHB on the cell metabolism and, especially, on the mitochondrial function are not known. DβHB has been administered orally for several months to two 6-month-old infants with hyperinsulinemic hypoglycemia (48). Despite the high dosage (up to 32 g/d), these patients seem to tolerate quite well. In addition, the ketogenic diets, which result in high levels of DβHB, have been used for more than 70 years in humans as a treatment for refractory epilepsy and have proven safe and well tolerated.

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Engineered modeling and the secrets of Parkinson's disease

Miquel Vila, Du Chu Wu and Serge Przedborski

The development of new methods for manipulating the animal genome by transgenic and gene-targeting technologies provides a unique means of studying the most intimate aspects of countless human diseases, including Parkinson's disease (PD). In this review, the contribution of such engineered models to our current understanding of the pathophysiology, etiology and pathogenesis of PD will be discussed.

Parkinson's disease (PD) is a common neurodegenerative disorder, the cardinal clinical features of which include tremor, stiffness, slowness of movement and postural instability[1]. It is estimated that, in the USA alone, more than one million individuals are currently affected by this disabling disease[1]. However, because the incidence of PD rises with age[1], it is expected that this number will increase significantly in the future because of the aging character of society. Despite this bleak perspective, several recent discoveries have unquestionably brought closer the day when the secrets of PD will be unlocked. A rapid survey of PD research not only shows the impressive pace at which advances have been made, but also that the bulk of published studies can be divided into three groups: those that investigate the pathophysiology of PD (that is, neurochemical perturbations), those that search for the etiology of PD (the cause), and those that explore the pathogenesis of PD (the mechanisms of neuronal death). This review investigates these three approaches to PD research through the appraisal of engineered models, which are, in our opinion, the spearhead of most of the recent breakthroughs accomplished in this field (Table 1). The goal of this review is to present an overview of the many recent advances in PD and not an in-depth discussion of selected topics. For further information on any of the presented subjects, the reader is encouraged to peruse the original articles referenced in this review.

Pathophysiology of PD

The main, but not sole, neurochemical alteration of PD is the deficit in brain dopamine, which is believed to be the primary culprit in the development of the motoric and non-motoric manifestations of PD (Ref. 1). This view is supported by the finding that administration of the dopamine precursor L-DOPA, which replenishes the brain with dopamine, alleviates most of the signs of PD. However, although L-DOPA is essential for the fine control of motor function, it is not necessary for the normal development of the brain circuitry within which dopamine plays such a pivotal role. Indeed, mutant mice deficient in tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, produce almost no dopamine and yet harbor a normal cytoarchitecture within the basal ganglia and the different midbrain dopaminergic neuronal groups[2]. Remarkably, pigmented $TH^{-/-}$ mice have more dopamine levels in the brain than their albino counterparts, presumably owing to the conversion of tyrosine to L-DOPA by the melanin-synthesizing enzyme tyrosinase[3]. If a similar phenomenon exists in humans then PD patients with pigmented skin or those living in sunny regions would have higher residual dopamine in the brain and thus fare clinically better than others.

Transgenic and homologous recombination technology has been extensively used to manipulate different factors in dopamine metabolism. Most of these studies have investigated the contribution of dopamine on spontaneous and drug-induced motor and non-motor behaviors. From these studies, it appears that ablation of either of the two key enzymes responsible for dopamine catabolism, namely monoamine oxidase (MAO) and catechol-O-methyl-transferase, fails to alter brain dopamine levels or locomotor activity, although all of the mutant mice studied were found to exhibit increased aggressive behavior[4,5]. Similarly, mice heterozygous for vesicular monoamine transport-2 (VMAT-2), which is the brain

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Table 1. Selected engineered models used in the study of Parkinson's disease[a]

Category	Refs
Pathophysiology	
Tyrosine hydroxylase$^{-/-}$ mice	2,3
Monoamino oxidase$^{-/-}$ mice	4
Catechol-O-methyl-transferase$^{-/-}$ mice	5
Vesicular monoamine transporter$^{+/-}$ mice	6
Vesicular monoamine transporter transfected cells	7
Dopamine transporter$^{-/-}$ mice	8
Dopamine receptor$^{-/-}$ mice	9
Etiology	
α-Synuclein$^{-/-}$ mice	12
α-Synuclein transgenic (wild-type, A53T, A30P)	13–18
Pathogenesis	
Oxidative stress	
Cu–Zn-superoxide dismutase$^{-/-}$ mice	26
Manganese-superoxide dismutase$^{+/-}$ mice	28
Glutathione peroxidase$^{-/-}$ mice	26
Monoamino-oxidase B transgenic	29
Trophic factor support	
GDNF$^{-/-}$ mice	30
BDNF$^{-/-}$ mice	31
TGFα$^{-/-}$ mice	34
Mitochondria	
Adenine nucleotide translocator$^{-/-}$ mice	35
Complex I 'cybrids'	36

[a]Abbreviations: BDNF, brain-derived neurotrophic factor; GDNF, glial-derived neurotrophic factor; TGF, transforming growth factor.

carrier that enables translocation of cytosolic monoamines into synaptic vesicles, also show minimal baseline abnormalities[6]. However, as predicted, VMAT-2$^{+/-}$ mice exhibit diminished extracellular striatal dopamine levels, as well as reduced K^+- and amphetamine-evoked dopamine release[6]. Conversely, VMAT-2 overexpression in small synaptic vesicles of transfected dopaminergic neurons shows increased quantal size and frequency of dopamine release consistent with the recruitment of synaptic vesicles, which do not normally release dopamine[7]. Together, these studies demonstrate that VMAT-2 is a critical regulator of the rate of transmitter accumulation and synaptic strength in the monoamine system. Mutant mice deficient in dopamine transporter (DAT) are spontaneously much more metabolically perturbed in that, by virtue of lacking the ability to recapture released dopamine, they exhibit a dramatic increase in striatal extracellular dopamine levels and are already grossly hyperactive at baseline[8]. This abnormal motor behavior is probably related to a hyperstimulation of the postsynaptic dopaminergic receptors in response to the higher extracellular content of dopamine. If this interpretation is correct, then it also explains why mutant mice deficient in at least dopamine D2 receptors have an impaired capacity for responding to extracellular dopamine and why, as a consequence, are so hypoactive[9]. As expected, this poor locomotor activity could not be improved by the administration of dopamine agonist[9]. In agreement with our current knowledge of the distribution and function of the minor dopamine receptors, manipulation of D3-, D4- and D5-receptor expression demonstrates that they all seem to exert opposing motoric effects to those of the D2 receptor[9].

The aforementioned studies indicate that many tools are already available to target key components of the dopamine system, including its synthesis and degradation, its intracellular compartmentalization, its release, and its postsynaptic neurotransmission. So far, published data demonstrate that, unless conspicuous changes in striatal dopamine or in dopamine postsynaptic neurotransmission occurs, no gross motor abnormalities arise in these engineered animals. This conclusion is consistent with the finding that parkinsonism emerges in humans only after severe alterations in dopaminergic pathways[1].

Etiology of PD

The cellular basis of PD is a dramatic loss of dopaminergic neurons, primarily in the substantia nigra pars compacta (SNpc)[1]. The cause of PD neurodegeneration remains unknown. However, the discovery that rare familial forms of PD are linked to genetic mutations has raised the prospect that, through the study of these unique pedigrees, some hints into the etiology of PD can be obtained. So far, five disease-causing loci and mutations in three genes, as well as several allelic associations, have been linked to PD[1]. Mutant proteins derived from causal mutations include: (1) α-synuclein, which is linked to an autosomal dominant form of PD; (2) parkin, which is linked to an autosomal recessive form of early-onset PD; and (3) ubiquitin carboxy-terminus hydrolase L1 (UCH-L1), which is linked to an autosomal dominant form of typical PD. Other mutations have been identified in association with clinical syndromes that comprise parkinsonism, such as point mutations in the Tau gene[10],

Table 2. Transgenic α-synuclein animals[a]

Species	Form of α-synuclein	Promoter	Loss of SNpc DA cells	Striatal DA deficit	Inclusions	Motor deficits	Refs
Mouse	Wild-type	PDGF	No	Yes	Nuclear and cytoplasmic, no fibrillar aggregates, in neocortex, hippocampus and, 'occasionally' in SN	Yes	13
Mouse	Wild-type and mutant (A53T)	Thy-1	No	No	Lewy-like pathology, especially in motor neurons	Yes	14
Mouse	Mutant (A30P)	Thy-1 and TH	No	No	Somal and neuritic accumulation of mutant α-synuclein	No	15
Mouse	Wild-type and mutant (A53T, A30P)	TH	No	No	No	No	16
Mouse	Wild-type and mutant (A30P)	Thy-1	No	No	Abnormal accumulation of α-synuclein in cell bodies and neurites	No	17
Drosophila	Wild-type and mutant (A53T, A30P)	GAL4	Yes	Yes	Yes	Yes	18

[a]Abbreviations: DA, dopamine; PDGF, platelet-derived growth factor; SN, substantia nigra; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase

which in humans causes a form of frontotemporal dementia with parkinsonism.

α-Synuclein and PD

To date, two PD-causing missense mutations in α-synuclein (A53T and A30P) have been identified[1]. Cytotoxicity of mutant α-synuclein is probably related to the fact that both identified point mutations might enhance the propensity of this small presynaptic protein to interact with other proteins and aggregate[11] to form Lewy-body-like intraneuronal inclusions, a pathologic hallmark of PD (Ref. 1). The lack of α-synuclein results in neither the parkinsonian phenotype nor alterations in dopaminergic pathways in mice[12]. Still, these mutant animals exhibit increased dopamine release following paired stimuli, a mild reduction in striatal dopamine content and an attenuation of dopamine-dependent locomotor response to amphetamine, suggesting that α-synuclein negatively regulates dopamine neurotransmission. More complicated is the situation of transgenic animals overexpressing either wild-type or mutant α-synuclein, which have generated inconsistent results (Table 2). For instance, overexpression of wild-type α-synuclein driven by platelet-derived growth factor promoter is associated with the accumulation of α-synuclein- and ubiquitin-immunoreactive inclusions reminiscent of Lewy bodies in the neocortex, hippocampus and occasionally in the substantia nigra[13]. Despite an absence of nigral dopamine-mediated neuronal loss, aged transgenic animals show a reduction in striatal TH protein content and enzymatic activity that ascribes to impaired motor performance. In a second line of transgenic mice overexpressing either the wild-type or mutant allele, α-synuclein-containing inclusions with some neuronal death were found in the spinal cord but not in the SNpc, where the promoter used is not expressed[14]. And, in three additional lines of transgenic animals in which expression of wild-type or mutant α-synuclein is driven by either a neuronal glycoprotein Thy-1 promoter or a TH promoter that enables transgene expression in SNpc, no nigrostriatal pathology was found at all[15–17]. Unlike the mouse, expression of wild-type or mutant α-synuclein in fruit flies reproduces most of the features of PD, including a selective age-dependent loss of dorsomedial dopaminergic neurons, Lewy-body-like α-synuclein-positive inclusions and a progressive age-dependent locomotor dysfunction[18]. Because α-synuclein can be damaged by oxidative stress[19,20], it is worth mentioning that fruit flies have a more intense oxidative metabolism than mice. Thus, it is possible that the apparent discrepancy between the inconsistent and subtle abnormalities found in transgenic α-synuclein mice and the robust alterations found in transgenic α-synuclein *Drosophila* is related to a different degree of post-translational modification of α-synuclein inflicted by oxidative stress.

Ubiquitin metabolism-linked mutations and familial parkinsonism

As indicated previously, another PD-causing mutation (I93M) is found in the gene encoding UCH-L1, a key

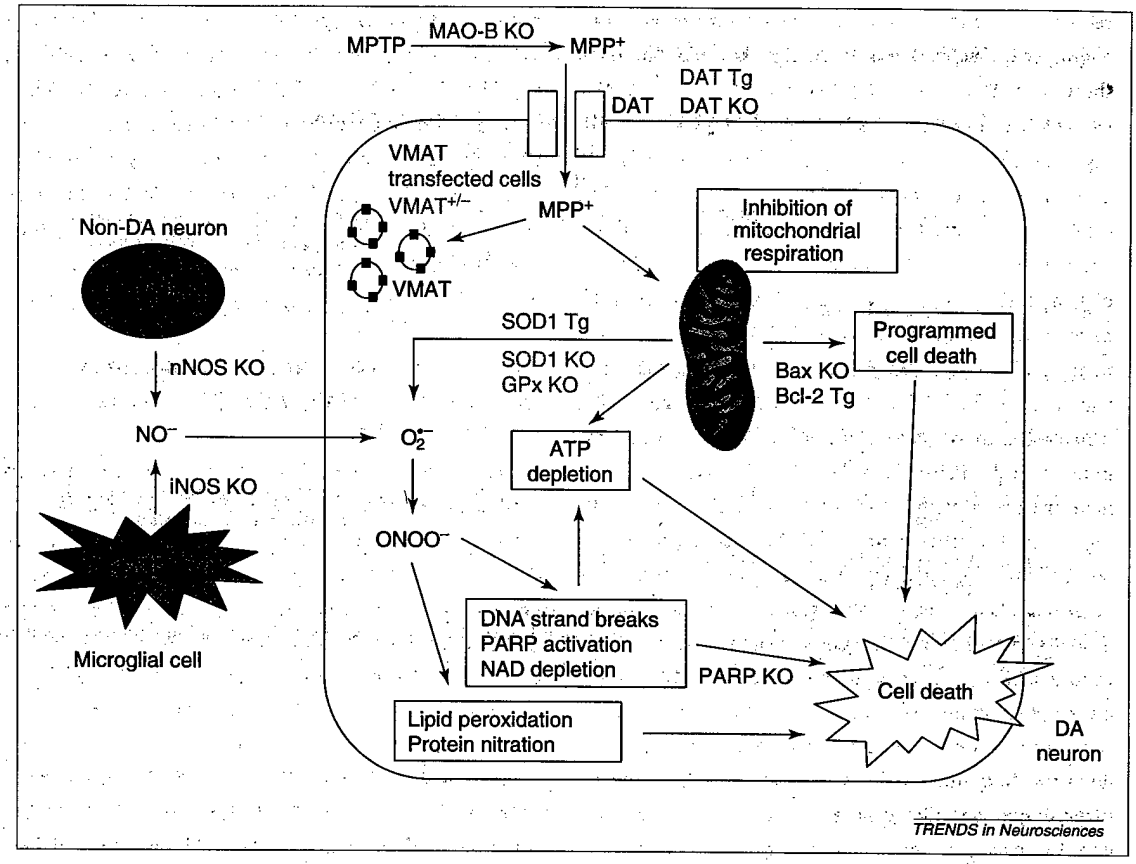

Figure 1. Engineered manipulations of MPTP molecular mechanism

Engineered animals (knockouts, KO, or transgenics, Tg) enable targeting of specific molecular factors hypothesized to be instrumental in the demise of dopaminergic neurons induced by the parkinsonian neurotoxin MPTP. Intervention aimed at blocking neurotoxic effects of MPTP is shown in red. Intervention aimed at enhancing the neurotoxic effects of MPTP is shown in green. Further information regarding the use of engineered animals in the MPTP model can be found in Ref. 24. Abbreviations: DAT, dopamine transporter; GPx, glutathione peroxidase; iNOS, inducible nitric oxide synthase; MAO-B, monoamino oxidase B; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; nNOS, neuronal nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; SOD1, copper–zinc superoxide dismutase; VMAT, vesicular monoamine transporter.

enzyme in the ubiquitin pathway[21]. This is an exciting finding in light of the fact that ubiquitin is highly expressed in Lewy bodies and that parkin, which, upon mutation, causes a juvenile form of PD, possesses a ubiquitin-related activity[22]. Although, so far, no engineered animals exist for either UCH-L1 or parkin mutations, it has been established that the gracile axonal dystrophy (*gad*) mutant mouse carries a spontaneous autosomal recessive mutation resulting in an in-frame deletion of *UCH-L1*[23]. These mutant mice do not show any clinical or pathological similarity to PD patients harboring UCH-L1 mutation, but they represent the first mammalian model of neurodegeneration with a defect in the ubiquitin system.

Pathogenesis of PD

Following the initiation of the disease by an etiological factor, mounting evidence indicates that a cascade of deleterious events is set in motion, which will ultimately be responsible for the demise of dopaminergic neurons. Over the years, several pathogenic hypotheses have been proposed and, with the development of engineered animals, these are now testable. Investigations geared toward studying the pathogenesis of PD can be divided into two broad categories: (1) those in which engineered animals are used directly to test hypothesized pathogenic mechanisms; and (2) those in which engineered animals are used to modulate the susceptibility of dopaminergic neurons to a given insult, such as the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[24] or rotenone[25].

Engineered animals used to test PD pathogenic mechanisms directly

The most popular pathogenic hypotheses for PD include oxidative stress (related to dopamine metabolism or other mechanisms), inappropriate trophic support, reactivation of apoptosis, excitotoxicity, and mitochondrial dysfunction. In keeping with these dominant themes, it is

relevant to mention that mice lacking various key scavenging enzymes for reactive oxygen species (ROS) do not show overt SNpc neurodegeneration[24,26–28]. By contrast, transgenic mice overexpressing the dopamine-metabolizing enzyme MAO-B show no actual neuronal death but do exhibit a striking atrophy of SNpc neurons[29], probably caused by increased ROS production during dopamine deamination. Even more dramatic is the effect of the lack of trophic factors. First, mutant mice deficient in glial-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF) do not survive beyond postnatal day 21 (Refs 30 and 31). GDNF$^{-/-}$ neonates suffer from major abnormalities in both peripheral and central noradrenergic neurons, although midbrain dopamine neurons appear intact[30]. This observation is quite surprising given the importance of GDNF in dopaminergic neuron survival[32] and the intense developmental SNpc neuronal death seen in the mouse[33], a phenomenon thought to be highly sensitive to trophic support. More in line with this latter argument is the fact that mutant mice deficient in transforming growth factor-α have a significantly lower number of SNpc dopaminergic neurons than their wild-type littermates[34]; but here, surprisingly, the other midbrain dopaminergic neuronal populations appear unaffected by the lack of this trophic factor[34].

In connection with the proposed role of mitochondria as the main source of ROS responsible for the oxidative attack in PD, it has been demonstrated that oxidative phosphorylation can be inhibited by ablating the adenine nucleotide translocator (Ant-1), thus resulting in widespread oxidative damage in these mice[35]. However, contrary to this hypothesis, the brunt of Ant-1 deficit was found in muscles and not in the SNpc (or in the brain as a whole)[35]. Furthermore, in cells, transgenicity (cytoplasmic hybrids or 'cybrids') has been used to evaluate the consequences of the defect in complex I in PD (Ref. 36). In this study, cybrid cells with reduced complex I activity exhibit a variety of major functional alterations, which all have potential pathogenic significance and could easily, either separately or in combination, kill SNpc dopaminergic neurons. Finally, there are several mouse models with SNpc alterations that occur for unclear reasons. These include: (1) transgenic mice expressing a mutant form of the enzyme superoxide dismutase-1 (SOD1), which not only show a loss of spinal cord motor neurons but also a reduction in the number of SNpc dopaminergic neurons[37]; (2) mice lacking the orphan nuclear receptor Nurr-1, which fail to generate midbrain dopaminergic neurons and are hypoactive[38]; (3) mutant mice lacking engrailed genes En-1 and En-2, showing that these genes control the survival of midbrain dopaminergic neurons in a dose-dependent manner and regulate the expression of α-synuclein[39]; (4) mice knockout for the estrogen receptor-β, which exhibit several morphological brain abnormalities and a pronounced neuronal degeneration with aging, particularly in the SNpc[40]; and (5) mice deficient in the ATM gene (known to be involved in DNA repair), and which also develop severe degeneration of SNpc dopaminergic neurons[41].

Engineered animals used to modulate the susceptibility of dopaminergic neurons to a given insult

To date, several potent neurotoxins have been used to duplicate most of the biochemical and neuropathological hallmarks of PD. Worth noting, however, is the fact that the chronic infusion of the mitochondrial poison rotenone into rats seems to be the only toxin-induced animal model of PD that is unequivocal in showing Lewy-body-like intraneuronal inclusions[25]. So far, however, rotenone-induced SNpc toxicity has only been documented in rats[25] and, to our knowledge, has yet to be used in engineered animal models, which precludes its discussion here. The situation is quite different for MPTP, which has been used in transgenic and knockout mice[24] and which has contributed significantly to our current understanding of the pathogenesis of PD. After the first wave of fruitful studies in the 1980s led to the characterization of the key steps in MPTP metabolism, this neurotoxin had a second wave of interest with the advent of engineered animals[24]. This powerful combination has provided a well-recognized and validated tool to not only injure, specifically, dopaminergic neurons but also to target specific molecular factors hypothesized to be instrumental in the demise of these neurons (Fig. 1). The benefit of this dual approach has definitively confirmed the nature of those factors that determine the neurotoxic potency of MPTP (Ref. 24). For instance, it has been shown that mutant mice deficient in MAO-B fail to transform the pro-toxin MPTP into its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) and consequently are refractory to the deleterious effect of this toxin[42]. Mutant mice deficient in DAT are also resistant to MPTP[43], thus confirming the mandatory role of DAT in MPP^+ entry into dopaminergic neurons and the ensuing cytotoxicity. As for VMAT-2, which enables MPP^+ sequestration into synaptic vesicles, engineered mice have shown that the lower the VMAT-2 expression, the greater the MPTP-induced dopaminergic toxicity[44]. This shows how crucial cytosolic MPP^+ is to the MPTP neurotoxic process.

This combined approach has also shed light on the molecular mechanisms that could be of importance to

unraveling the pathogenesis of PD (Fig. 1). It is well established that once MPP^+ is in the dopaminergic neuron, it is actively concentrated into the mitochondria where, through its binding to complex I, it interrupts the flow of electrons, which leads to a deficit in ATP formation and to increased production of ROS, especially superoxide radical[24]. The importance of the latter in the neurotoxic process of MPTP is supported by the finding that transgenic mice with increased activity of SOD1, the key protective enzyme against superoxide, are more resistant to MPTP than their non-transgenic littermates, whereas mutant mice deficient in SOD1 or in glutathione peroxidase are more sensitive[24,26]. However, it is known that superoxide, which is poorly reactive, is unlikely to be the sole mediator of oxidative damage inflicted on dopaminergic neurons following MPTP administration. To circumvent this issue, it has been proposed that superoxide reacts with nitric oxide (NO), a small molecule present in great abundance in the brain, to produce the highly reactive tissue-damaging species, peroxynitrite. Relevant to the hypothesized involvement of peroxynitrite in MPTP-induced neuronal death are the observations that mutant mice deficient in either neuronal or inducible NO synthase are partially protected against MPTP (Refs 24 and 27) by depleting NO in the brain. Another consequence of MPTP attack or intoxication is the activation of the apoptotic cascade, an active form of cell death, presumably implicated in the overall neurodegenerative process in PD. So far, supportive evidence for this pathogenic mechanism is provided by the finding that ablation of the pro-apoptotic protein Bax in mutant mice[45] or, conversely, overexpression of the anti-apoptotic protein Bcl-2 in transgenic mice, attenuates dopaminergic neuronal death caused by MPTP (Ref. 24).

Concluding remarks

In this review, we have tried to compile the most recent and significant reports dealing with the complex issue of how to better understand and treat PD. Using the angle of engineered models, we have shown how eclectic and important the advances accomplished in the field of PD research are. From this, it clearly emerges that the contribution of transgenic and knockout animals has been, and will continue to be, tremendous as far as the study of the pathogenesis of PD is concerned. To date, comparable strides have not been achieved in the study of the etiology of PD but, with several PD-linked gene mutations still to be tested using this technology, it is our opinion that brighter days are ahead and major breakthroughs, which could change the landscape of PD, are just around the corner.

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REVIEW ARTICLE

The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety

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Parkinson's disease (PD) is a common disabling neurodegenerative disorder the cardinal clinical features of which include tremor, rigidity and slowness of movement (Fahn and Przedborski 2000). These symptoms are attributed mainly to a profound reduction of dopamine in the striatum due to a dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski 2000). Thus far, both the cause and the mechanisms of PD remain unknown. Over the years, investigators have used experimental models of PD produced by several compounds such as reserpine, 6-hydroxydopamine, methamphetamine, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to provide insights into the mechanisms responsible for the demise of dopaminergic neurons in PD. To this end, MPTP has emerged unquestionably as a popular tool for inducing a model of PD in a variety of animal species including monkeys, rodents, cats, and pigs (Kopin and Markey 1988). The sensitivity to MPTP and therefore its ability to induce parkinsonism closely follows the phylogenetic tree where the species most closely related to humans are the most vulnerable to this neurotoxin. Due to the significant neurotoxicity of MPTP, it is important that researchers appreciate the potential hazards of this toxin. Given this, the purpose of this review is to inform the researcher of the hazardous nature of MPTP and to provide guidance for its safe handling and use.

MPTP models of PD

MPTP is a by-product 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 and Irwin 1986). Recognition of MPTP as a neurotoxin occured 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, including tremor, rigidity, slowness of movement, postural instability, and even freezing; in non-human primates, a resting tremor characteristic of PD has only been demonstrated convincingly in the African green monkey (Tetrud *et al.* 1986). The responses, as well as the complications, to traditional antiparkinsonian therapies are virtually identical to those seen in PD. 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;

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Abbreviations used: MAO-B, monoamine oxidase B; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSDS, material safety data sheet, PD, Parkinson's disease; PPE, personal protective equipment; SNpc, substantia nigra pars compacta.

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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 studies and thus, the comparison between PD and the MPTP model is largely limited to primates (Forno *et al.* 1993). Neuropathological data show that MPTP administration causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD (Agid *et al.* 1987), yet there is a resemblance that goes beyond the loss of SNpc dopaminergic neurons. Like PD, MPTP causes greater loss of dopaminergic neurons in SNpc than in ventral tegmental area (Seniuk *et al.* 1990; Muthane *et al.* 1994) and, at least in monkeys treated with low doses of MPTP but not in humans, 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 SNpc, pigmented nuclei such as 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).

Modes of administration

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/or 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 SNpc damage with the least number of undesirable consequences 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).

Monkeys

The most commonly used regimens in monkeys are the multiple intraperitoneal or intramuscular injections and the intracarotid infusion of MPTP (Petzinger and 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 levodopa is required to enable the animal to eat and drink adequately (Petzinger and Langston 1998). On the other hand, the unilateral intracarotid infusion is technically more difficult, but causes symptoms mainly on one side (Bankiewicz *et al.* 1986; Przedborski *et al.* 1991), which enables the monkey to maintain normal nutrition and hydration without the use levodopa.

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 and 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, it is the chronic model that is, presumably, the most suitable for the testing of neuroprotective strategies.

Mice

In addition to monkeys, many other mammalian species are also susceptible to MPTP (Kopin and Markey 1988; Heikkila *et al.* 1989; Przedborski *et al.* 2000). Mice have become the most commonly used species for both technical and financial reasons. 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, presenting a far greater hazardous situation. Second, in contrast to the situation in monkeys, mice treated with MPTP do not develop parkinsonism. Third, the magnitude of nigrostriatal damage depends on the dose and dosing schedule (Sonsalla and Heikkila 1986).

Rats

The use of MPTP in rats presents an interesting situation (Kopin and 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; Giovanni *et al.* 1994b). Conversely, rats injected with much higher doses of MPTP do exhibit significant dopaminergic neurodegeneration (Giovanni *et al.* 1994a; Giovanni *et al.* 1994b) 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 often in MPTP studies (Storey *et al.* 1992; Giovanni *et al.* 1994a; Giovanni

et al. 1994b; Staal and 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 MPTP's toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (Storey *et al.* 1992; Giovanni *et al.* 1994a; Giovanni *et al.* 1994b; Staal and Sonsalla 2000; Staal *et al.* 2000).

Intervening factors

Several factors influence the reproducibility of the lesion in monkeys, rats, and mice. However, to our knowledge, the extensive and systematic assessment of these factors has only been done in mice, and can be found in the following references (Heikkila *et al.* 1989; Giovanni *et al.* 1991; Giovanni *et al.* 1994a; Giovanni *et al.* 1994b; Miller *et al.* 1998; Hamre *et al.* 1999; Staal and Sonsalla 2000), the highlights of which can be summarized as follows: different strains of mice (and even within a given strain obtained from different vendors) can exhibit strikingly distinct sensitivity to MPTP. This differential sensitivity acts in an autosomal dominant fashion (Hamre *et al.* 1999). Gender, age, and body weight are also factors that modulate MPTP sensitivity as well as reproducibility of the lesion, in that female mice are less sensitive and exhibit more variability in the extent of damage than males, as do mice younger than 8 weeks and lighter than 25 g. From our experience, optimal reproducibility in MPTP neurotoxicity is obtained using male C57 BL/6 mice 8–10 weeks of age and 25–30 g in weight. Also of importance is that, following MPTP administration, some mice will die within the first 48 h postinjection; note that C57 BL/6 mice from different vendors exhibit dramatically different magnitudes of acute lethality, ranging from 5% to 90%. This common issue is unlikely related to a toxic effect in the central nervous system but rather toxicity to the peripheral nervous and cardiovascular systems. Although, to our knowledge, this possibility has never been formally studied, we believe that, following acute MPTP administration, mice develop fatal alterations in heart rate and blood pressure. Moreover, MPTP intoxication causes a transient drop in body temperature, which not only can modulate the extent of dopaminergic damage (Moy *et al.* 1998), but can also contribute to acute lethality. Death rate can be reduced by maintaining the body temperature of the injected mice using a temperature-controlled warming pad (do not use a lamp, which can kill mice by overheating them as there is no control of the temperature).

Metabolism of MPTP

MPTP has a complex multistep metabolism (Tipton and Singer 1993; Przedborski *et al.* 2000). It is highly lipophilic, and freely and rapidly crosses the blood–brain barrier. Within a minute after MPTP injection, levels of the toxin are detectable in the brain (Markey *et al.* 1984). Once in the brain, MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) in non-dopaminergic cells. Then $MPDP^+$ is oxidized to the active MPTP metabolite, MPP^+, which is then released into the extracellular space, where it is taken up by the dopamine transporter and is concentrated within dopaminergic neurons, where it exerts its toxic effects. The essential role of these different metabolic steps in MPTP-induced neurotoxicity and the fact that MPP^+ is the actual culprit are demonstrated by the following observations: (1) pretreatment with MAO-B inhibitors such as deprenyl prevents MPTP biotransformation to MPP^+ and blocks dopaminergic toxicity (Heikkila *et al.* 1984; Markey *et al.* 1984); (2) pretreatment with dopamine uptake inhibitors (e.g. mazindol) prevents MPP^+ entry into dopaminergic neurons and also blocks dopaminergic toxicity (Javitch *et al.* 1985), at least in mice; and (3) striatal MPP^+ content correlates linearly with dopaminergic toxicity in mice (Giovanni *et al.* 1991).

Body distribution and environmental contamination

Knowing where MPTP and its toxic metabolite, MPP^+, accumulate both inside and outside of the body of the injected animal following MPTP administration is germane to the formulation of any set of standard practices for the safe use of MPTP.

Following MPTP administration to both mice and monkeys, only the interior surfaces of the cage, the surfaces that the animals and/or their excreta could physically touch, including food and drinking bottle, are contaminated with MPTP and its metabolites (Yang *et al.* 1988). Conversely, no evidence of contamination is found outside of the cage or on the outside surrounding surfaces (Yang *et al.* 1988). At two days postinjection, 70% of the total injected dose of MPTP and its metabolites is recovered from the inside cage-wash, urine and feces, of which about 15% in mice and 2% in monkeys is unmetabolized MPTP, while the rest is due to MPTP metabolites, such as MPP^+. Moreover, it appears that the excretion of unmetabolized MPTP occurs mainly during the first day postinjection, while mainly MPTP metabolites are excreted up to 3 days postinjection (Yang *et al.* 1988). There is no evidence either in mice or in monkeys that MPTP and its metabolites are still being excreted after 3 days post MPTP administration. Although high concentrations of MPTP are found in the bile, the main route of MPTP excretion is the urine (Johannessen *et al.* 1986). MPTP in urine will likely be ionized and not volatile, and be well absorbed by the animal bedding. Also, less than 0.01% of the total injected dose of MPTP is detected as volatile MPTP, which probably originates from the animals exhaling MPTP or from vapors from contaminated urine (Yang *et al.* 1988).

One day after an injection of radiolabeled MPTP to mice, most of the radioactivity is localized in the brain and the adrenal gland, while all other organs contain 50–75% lower amounts of radioactivity (Johannessen *et al.* 1986). Analysis of the radioactive species recovered from different organs and body fluids such as bile, urine, blood, and CSF demonstrates variable amounts of unmetabolized MPTP soon after injection, but by 12–24 h postinjection, essentially all of the radioactivity corresponds to MPP^+ (Markey *et al.* 1984; Johannessen *et al.* 1986).

From the above, it appears that the potential risks of exposure to MPTP are through direct contact with the animal, the animal cage inner surfaces, and its bedding material. There is minimal risk from exposure due to airborne or vapor-borne forms of MPTP. Although safety procedures, as outlined below, must always be followed, the period of maximal risk of MPTP contamination is from the moment of injection to the time that MPTP or its metabolites are no longer found in the excreta of treated animals; as a precautionary measure, we recommend extending the period of high-risk from 3 days to 5 days post-MPTP injection.

Personal protection

Prior to discussing MPTP preparation, injection and animal experimentation, it is necessary to discuss the issues of the recommended facility and personal protective equipment (PPE). As a rule, only investigators and/or staff members who are trained in handling hazardous agents and who are familiar with MPTP safety procedures and practices should prepare and administer MPTP, and monitor the animals during the high-risk period (i.e. 5 days post-MPTP injection). Of note, any staff member who undertakes these tasks should give informed consent and not be coerced into taking on MPTP-related duties. Moreover, it is strongly recommended that all aspects of the MPTP experiment, including storage and solution preparation, take place in a dedicated procedure room (for small animals) or area within the animal room (for large animals), and not in a regular laboratory. For personal safety, when using MPTP, researchers are required to wear the PPE described below, during the preparation of the MPTP solution, the injection period, and 5 days postinjection. Thereafter, regular laboratory attire as required to handle animals is sufficient.

It is important to emphasize that in laboratories committed to MPTP research, one cannot exclude that exposure to even trace amounts of MPTP over many years of the same investigator and/or staff member may have negative consequences. This is one more reason why a heightened standard of protection must be implemented for any individual involved in MPTP experiments.

Dedicated procedure room and area within animal room

All MPTP experiments including preparation of solutions must be performed in a procedure or animal room under negative-pressure because aerosols from MPTP and its metabolites can be generated from bedding, excreta and animal fur. All animals should be acclimated to the room for 4–7 days prior to any MPTP experiment to allow for monoamine stabilization before MPTP injection since monoamine level alterations may affect intragroup lesion reproducibility. The procedure or animal room should have a 12-h light-dark cycle, a bench with a working area, a sink, and be temperature-controlled. For small animals like mice, it should also be equipped with an animal rack to hold all of the cages and a fume hood. All furniture should be of stainless steel or of any material, except wood, that is acid-resistant and washable. All working surfaces including the fume hood and animal racks should be covered with materials that are absorbent on the face-up side and non-absorbent on the face-down side. The entire floor of the procedure room or working area in the animal room for large animals should be covered with plastic-backed absorbent sheets. A warning sign clearly stating 'Danger! MPTP Neurotoxin Use Area – Entry Restricted' must be posted on the outside of the procedure or animal room door. The room must be locked at all times and the animal care staff informed of the ongoing use of MPTP and its dangers. They must also be informed that this room is off limits unless allowed to enter by the responsible investigator.

This procedure room or designated procedure area should be completely equipped with all of the necessary supplies for the MPTP experiments. It should also contain a sharps disposal container clearly labeled as hazardous waste, a container lined with a hazardous waste disposal bag for solid waste (diapers, gloves, animal shavings, etc.), gloves, absorbent pads, paper towels, markers, weighing scales for animals and MPTP, sterile saline, syringes with needles, 1% bleach (sodium hypochlorite) solution in water, a strong biodegradable detergent, personal protective equipment (see below), and deprenyl (selegiline), an MAO-B inhibitor, for accidental exposure to MPTP. It is imperative that the material safety data sheet (MSDS) for MPTP, which is supplied by the manufacturer, be kept in the room. Thus, once in the room or area, there should be no need to exit during the injection period.

Personal protection equipment

PPE must be worn during all procedures involving MPTP, including during the 5 days post-MPTP injection. The PPE is far more important when injecting mice than monkeys as mice require significantly higher doses of MPTP. The PPE consists of a one-piece garment with an

attached hood, elasticized wrists and attached boots made of a lightweight, chemically and biologically inert, non-absorbent material that is tear-resistant and provides protection from airborne particles. This garment should be easy enough to get into and economical enough to throw away after one wearing. For example, coveralls made of Tyvek fabric with elasticized wrists and boots and an attached hood (Kapplar, Guntersville, AL, USA) can be used. A full-face respirator with removable HEPA filter cartridges that is fit-tested to the individual is preferred for facial and respiratory protection. Alternatively, a half-face air-purifying respirator with removable HEPA cartridges that is approved by the National Institute of Occupational Safety and Health (NIOSH)/Mine Safety Health Administration (MSHA) for respiratory protection against dusts that is fit-tested to the individual using the respirator can also be used. The respirator is re-usable and should be thoroughly wiped with 1% bleach solution then washed with detergent after each use; wipes must be disposed of in the hazardous waste container. Splash-proof goggles and double-layered nitrile under latex gloves complete the PPE attire. All items comprising the PPE attire can be obtained from a large general laboratory supply company. The office of environmental health and safety in any Institution where MPTP is to be used must be consulted for guidance in obtaining PPE attire for use with MPTP.

Housing

For small animals such as mice, disposable cages and accessories are strongly recommended as they permit incineration of waste without bedding changes. Covering cages with filter bonnets is recommended to significantly reduce both room contamination and cross contamination of other animals. Small animal cages should be placed on the animal rack in the procedure room prior to and during the five-day period post-MPTP injection. All injections must be performed in the fume hood in the procedure room.

For large animals such as monkeys, enclosed cages should be used. The base of the cage and the drop pan must be lined with plastic-backed absorbent pads.

MPTP storage and handling

MPTP can be purchased from several commercial sources. Unless specifically required, do not use MPTP as the free base, but only as the hydrochloride or other non-volatile salt conjugate. MPTP storage and handling must be restricted to the procedure room or designated area within the animal room. Minimize the use of large volumes, concentrated solutions, and handling of MPTP powder and never transport MPTP solutions or opened vials of MPTP outside of the dedicated room. MPTP may be purchased in small quantities of 10 mg or 100 mg in glass septum bottles. Vials of MPTP must be kept closed until used and stored at room temperature in a container within a vacuum-sealed desiccated container. This second container should be kept in a locked cabinet with a permanently affixed 'MPTP – Neurotoxin' label. This cabinet must be secured to a non-removable surface in the procedure room or area.

Only investigators appropriately trained in the handling of MPTP should perform manipulations involving the powder. Use of glass containers will reduce handling problems that result from the electrostatic properties of plastic. It is strongly recommended that a balance dedicated to weigh MPTP powder be kept in the procedure room. Prior to weighing MPTP powder, cover the weighing area with pads dampened with 1% bleach solution to reduce the risk of airborne MPTP powder particles. To minimize the risk of MPTP powder spills, the weighing procedure described by Pitts *et al.* (1986) is a safe method: tare a small container (e.g. small scintillation glass vial with a screw cap); take the tared container and place an approximated amount of MPTP in it, close and wipe container with 1% bleach solution; weigh container; then add solvent to give desired concentration; again wipe container and all other items with 1% bleach solution; dispose of all wastes in a hazardous waste container. Alternatively, if a given experiment requires a total daily dose of less than 10 mg or 100 mg of MPTP, then it is safer not to open the vial and weigh the powder but to add the desired volume of solvent/vehicle directly to the sealed 10 mg or 100 mg vial. It must be understood that this MPTP solution has to be used in one day and the remainder discarded since MPTP in solution oxidizes at room temperature; prior to discarding the used MPTP sealed vial, inject a volume of 1% bleach solution equivalent to the volume of MPTP solution remaining into the vial, then discard the vial as biohazardous liquid waste. We previously found that storing MPTP solution at −80°C retards its oxidation as MPTP solution appears stable up to 2 months (personal observation). However, unless one has a dedicated −80°C freezer for MPTP storage, other issues such as laboratory safety will arise and that even without mentioning the negative impact of thawing and freezing of MPTP solution on its neurotoxic potency.

Animals should be injected only with sterile solutions of MPTP prepared by either filtration through a disposable 0.22 μm filter unit or by dissolving the compound in sterile saline or water. Do not autoclave MPTP solutions, as this will vaporize the compound and may lead to exposure from inhalation.

Injection of MPTP

As mentioned above, a number of different injection regimens have been used to produce the desired MPTP lesions. These are based on a number of factors, including experimental design, degree of desired lesion, and species

used. As indicated, mice, which typically require greater amounts of MPTP to produce lesions, can be injected either subcutaneously or intraperitoneally, single or multiple injection, and with a wide range of concentrations. Whatever the regimen used, it is recommended that all MPTP injections to mice be performed in a fume hood. Vials from which MPTP is drawn should have a septum or be covered with parfilm to eliminate potential aerosols and spills and to avoid drops on the needle end. Change gloves frequently during the course of and at the end of the injection schedule. This will prevent any contamination of the PPE and decrease the possibility of overt contamination of equipment.

On the day of or on the evening before the experiment, all animals are weighed and coded. About a half-hour before starting the injection schedule, sterile MPTP solution should be prepared to the desired working concentration. During animal injection, care must be taken to avoid self-inoculation; special attention to animal restraint will significantly reduce this risk. For injection, place the mouse cage in the fume hood and when injecting, hold the animal so that any urine spray will fall into the cage and not on the surrounding areas, since mice, when held, tend to expel urine which can contain significant amounts of MPTP (Yang *et al.* 1988). Make sure the mouse is not held so tightly as to cause backflow of the injected MPTP from the peritoneum. Larger animals such as squirrel monkeys must be placed in restrainers for injection. It is not practical to inject large animals in a fume hood. Inspect injection site for leakage or spilled solution and wipe with a small pad dampened with 1% bleach solution. When discarding syringes, do not clip, recap or remove needles from syringes; fill the syringe with 1% bleach solution and then place the syringe with attached needle in a sharps container to be disposed of as biohazardous waste. At the end of the injection schedule, the remaining MPTP solution must be destroyed with an equivalent volume of 1% bleach solution as described above.

Cage changing

The greatest potential for exposure to MPTP and its metabolites is from contaminated bedding and caging immediately following MPTP injection and during the period that MPTP or its metabolites are likely to be in the excreta of treated animals. Therefore, when handling cages and their contents, it is important that the PPE be worn.

Used disposable mouse cages containing contaminated bedding should be dampened with 1% bleach solution and then be carefully placed into a plastic biohazard bag, tied off, and sent for incineration. When using re-usable cages, bedding should also be dampened with 1% bleach solution, then carefully placed in the biohazard bag, packaged and disposed of as biohazardous waste. Immediately after emptying re-usable cages, soak cages and accessories with 1% bleach solution for 10 min, rinse, then wash with detergent and rinse thoroughly with water. Mouse cages may then be sent to central cage washing facilities. The absorbent material that covered the rack surfaces should be sprayed with 1% bleach solution, allow to soak for 10 min and then disposed of as hazardous waste. For large animal cages, spray plastic-backed absorbent pads that line the cage bottoms and drop pans with 1% bleach solution, allow to soak for 10 min, then remove pads and place them in the biohazardous waste container; replace used linings with fresh pads. This needs to be done on a daily basis. Wash cages and accessories thoroughly with 1% bleach solution, rinse, then wash with detergent and rinse thoroughly with water. The procedure described above assumes that MPTP-injected animals remain in the same cage for 5 days postinjection and change out should occur only after the 5 days postinjection period. In the case of prolonged MPTP exposure protocols (i.e. weeks to months), while the procedure room or area will remain off-limits throughout the treatment period (plus the five days postinjection period), for mice, change only cage bottoms once a week following the procedure described above and, for monkeys, it is advisable to move monkeys to clean cages every other week and to handle the dirty cages as described above.

Counter tops in the procedure room or area should be cleaned with 1% bleach solution. Floor coverings should be carefully removed and disposed of as hazardous waste. Routine animal care can be re-instituted five days post last MPTP injection and once the procedure room or area has been cleaned by the responsible investigator and/or staff member.

Animal tissues

Potential risk of exposure to MPTP or MPP^+ may occur when animals are killed for tissue collection up to 5 days following MPTP administration. During this period, mice should be killed in the fume hood and the appropriate PPE worn by the researcher during blood and tissue harvesting procedures. All working surfaces are lined with plastic-backed absorbent pads, which should be changed if stained with body fluids. Since decapitation is the primary method of killing for small animals in MPTP studies, care should be taken to prevent blood spatters, and urine and feces should be contained. Brain tissues are best dissected on an inverted glass Petri dish covered with water-dampened filter paper and placed on regular ice. All instruments, including the Petri dish used for dissection, should be soaked in 1% bleach solution for 10 min, rinsed, then washed with detergent and rinsed with water. Collected tissues should always be handled with double gloves, and brain remnants and the remaining carcass, which may contain MPTP and

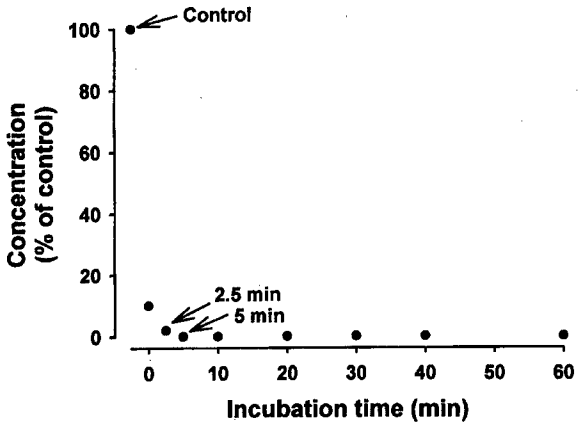

Fig. 1 Time-dependent effect of 1% bleach solution on MPTP. A 5-mg/mL of MPTP-HCl solution in saline was incubated at room temperature for different lengths of time with 1 volume (v/v) of 1% bleach (sodium hypochlorite) solution in water. After the indicated time of incubation, an aliquot of the mixture was injected into an HPLC-UV system and MPTP levels were quantified as described (Przedborski *et al.* 1996).

metabolites (Yang *et al.* 1988), must be discarded following biohazardous waste practices for animal waste.

For the perfusion of small animals, a grid overlaying a collection pan works best. Thus, blood and perfusion solution will be collected in the pan and can then be poured into a bottle or can be discarded as biohazardous waste. As per proper biohazardous waste disposal, the outside of the waste container must be wiped with 1% bleach solution.

For the perfusion of large animals, plastic tubing should be attached to the drain of the dissection table and a liquid biohazard waste container. This will catch any perfusion solution and prevent contamination of the water system. The collected perfusate will be discarded as biohazardous waste. After the perfusion procedure, the table must be washed with 1% bleach solution, rinsed, then washed with detergent and rinsed with water.

Decontamination, cleaning, and disposal

Often, one may see that 0.1 M HCl is used for cleaning up following MPTP experiments. However, we have HPLC evidence showing that HCl, up to 2 M and after incubation for more than 1 h at room temperature, does not destroy MPTP at all. Conversely, a 5% potassium permanganate solution in water completely destroys MPTP almost immediately. However, since potassium permanganate is such a powerful oxidant, it can produce hazardous exothermic reactions with several compounds like detergents and must be neutralized with ascorbic acid prior to being discarded as non-toxic waste. We have also found that bleach is as efficient as potassium permanganate in destroying MPTP, yet more friendly to use as it does not cause dangerous reactions with detergents and does not require specific treatment prior to discarding. Bleach is commercially available as a 5–10% stock solution. It can be readily diluted to the desired concentration with water and kept at room temperature indefinitely. Using a 1% bleach solution in water, which corresponds to twice the Environmental Protection Agency (EPA) recommended concentration for disinfection, we found that the action of bleach on 5 mg/mL of MPTP–HCl in saline is rapid in that after 5 min, at room temperature, there is no longer any detectable MPTP (Fig. 1). The 'almost' instantaneous destruction of MPTP by the bleach solution, as illustrated in Fig. 1, is not a surprising finding since the bleach-mediated reaction corresponds not to an enzymatic reaction but to a straight biochemical oxidation. In addition, we found that 10 min incubation of 5 mg/mL MPTP–HCl with different concentration of bleach solutions, ranging from 0.5 to 2.5%, had similar effects on MPTP. Therefore, our recommendation for MPTP decontamination is 10 min of soaking in 1% bleach solution. In contrast to their effects on MPTP, neither 2.5% bleach solution nor 5% potassium-permanganate destroyed MPP^+, even after an overnight incubation. This is not surprising, as MPP^+ is notoriously stable and resists destruction even after exposure to extremely harsh chemical and physical treatments. High doses of MPP^+ administered systemically (i.e. 25 mg/kg intraperitoneal) to mice produce oxidative damage to the lung, but fail to affect the nervous system (Johannessen *et al.* 1985). This is consistent with our observation that the intraperitoneal or subcutaneous injection of different doses of radiolabeled and non-radiolabeled MPP^+ to mice failed to show any accumulation of radioactivity in the striatum or to produce any damage to the dopaminergic systems of the brain (unpublished observation). Nevertheless, the direct injection of MPP^+ into the striatum does produce dopaminergic neurotoxicity (Giovanni *et al.* 1994b). These data indicate that the work-related hazards of MPP^+ involve peripheral organs such as the lungs and then only if high amounts reach the blood stream or the respiratory tract. Therefore, MPP^+ is far less hazardous than its parent compound and thus the real safety goal is the destruction of MPTP.

Only investigators appropriately trained in the handling of MPTP should clean up spills. Prior to any decontamination procedure, determine the maximum quantity of MPTP involved in the spill and the location of the spill.

If the room is properly maintained as stated above, linings and underpads will catch any spills. In case a liquid spill does occur, wearing the PPE, the researcher should immediately spray the linings and underpads with 1% bleach solution, allow to soak for 10 min, then remove, and place these in hazardous waste disposal bags. In the event that pads and linings have not caught all of the spill, absorb MPTP spill with absorbent plastic-backed pads to prevent

MPTP solution from contaminating gloves and discard as hazardous waste. The dry area is then soaked with 1% bleach solution, rinsed with water, then washed several times with detergent, rinsed with water, and dried with pads. Discard these materials in hazardous waste bags as well. Recover work area and inform the environmental health and safety office that an MPTP spill has occured and what measures were used to remove that spill.

To clean up MPTP powder spills, cover with a disposable towel dampened with 1% bleach solution, then pick up all materials and put into a hazardous waste container. Then, soak the area with 1% bleach solution, rinse with water, then wash several times with detergent, rinse with water, and dry with pads. Discard these materials in hazardous waste bags. Recover area, then inform the environmental health and safety office that a MPTP powder spill has occured and what measures were taken to contain and clean up the powder spill.

If clothes become contaminated with MPTP, immediately remove clothing and shower. After obtaining fresh clothing, report directly to a medical service. A very careful evaluation of any potential MPTP exposure is critical (see medical emergency and surveillance). Persons assisting exposed individuals should wear the PPE attire.

Plan experiments to avoid generating large quantities of contaminated glass or metal; these materials are difficult to incinerate, and large quantities can create waste disposal problems. Contaminated glass and metal can be decontaminated using 1% bleach solution followed by detergent washes and rinses. Decontaminate all equipment with wipes dampened with 1% bleach solution before repair work is performed, before transferring equipment to other operations, and before discarding. Pay special attention to internal parts of equipment that may have become contaminated.

Prevention, medical emergency and surveillance

To date, there has been no report in the literature of the inadvertent exposure of a researcher to MPTP while conducting MPTP experiments. A single report of a research chemist who suffered a fatal exposure to large amounts of MPTP during its synthesis has been documented and represents the only inadvertent human exposure to MPTP (Langston and Ballard 1983). However, despite the safe track record of MPTP use, precautionary emergency procedures must be employed to avoid potential injury from acute exposure to the toxin (such as a needle prick).

As indicated above, MAO-B inhibitors prevent the conversion of MPTP to its toxic metabolite, MPP^+ thereby preventing neurotoxicity. For example, pretreatment of animals with deprenyl, a potent irreversible MAO-B inhibitor prevents MPTP-induced neurotoxicity (Cohen *et al.* 1984; Mytilineou and Cohen 1985; Fuller *et al.* 1988). On the other hand, except for a single report (Tatton 1993), there is no evidence that MAO-B inhibition by deprenyl or by other compounds, following exposure to MPTP provides any neuroprotection. However, in case of accidental exposure to MPTP, in an attempt to block the conversion of any remaining MPTP to MPP^+ it is recommended that deprenyl be administered immediately. As far as we know, there is no established deprenyl regimen for accidental exposure to MPTP. Since the goal here is to prevent the conversion of MPTP by inhibiting MAO-B, as rapidly and profoundly as possible, we suggest an initial large dose of deprenyl (e.g. four 5 mg tablets) be taken orally at once. Although it may be prudent to continue deprenyl medication (e.g. 5 mg twice a day) for some time, it is unknown whether this is justified. Short-term surveillance is necessary for the appearance of hypotension from the deprenyl or the development of acute parkinsonian symptoms from the MPTP exposure. In addition, following the administration of a large dose of deprenyl, individuals must be cautious in consuming tyramine-containing foods (i.e. cheese) and in taking medications containing pharmacologically active amines. Prior to beginning any MPTP investigation, deprenyl must be available for emergency use and must be kept in a closed container at all times in the procedure room or area for immediate use, if necessary. Furthermore, it is advisable that individuals who are planning to embark upon a series of MPTP experiments consider a treatment of 5 mg twice a day of deprenyl prior to (e.g. 3–5 days before) and during the experiments. This may be especially indicated for a person first learning the protocol or if there is an increased risk of contact with MPTP. This should be done only after consulting one's personal physician.

Conclusion

To date, MPTP remains the best experimental model of PD. To this end, it is extensively used in various animal species and especially in mice. However, even as a research tool, MPTP is an extremely hazardous compound, which can be injected, ingested, inhaled, and/or absorbed. Because of its demonstrated toxicity to humans, the use of MPTP among researchers is a serious concern. Over the years, a better understanding of the physicochemical properties of this toxin, its metabolism, and its body distribution has enabled investigators to develop practices and procedures for the safe use of this compound. These include improved procedures for preparing MPTP solutions and for its injection into animals, proper protective equipment, reducing potential exposure from animal excreta, proper decontamination and disposal procedures, and medical treatment and surveillance in case of accidental exposure. Despite the fact that we have tried to cover the most common situations and topics related to MPTP use, this review cannot cover all possible aspects of the safe use of

this hazardous compound. Accordingly, there can be no substitute for common sense and proper laboratory practices in the use of dangerous compounds such as MPTP. It is hoped, however, that this review has built upon the guidelines presented by others in the past and, in conjunction with our recent knowledge of MPTP, will lead to the effective and safe use of the MPTP animal model of PD.

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The role of glial cells in Parkinson's disease

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Parkinson's disease is a common neurodegenerative disorder characterized by the progressive loss of the dopaminergic neurons in the substantia nigra pars compacta. The loss of these neurons is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes. This glial response may be the source of trophic factors and can protect against reactive oxygen species and glutamate. Aside from these beneficial effects, the glial response can mediate a variety of deleterious events related to the production of reactive species, and pro-inflammatory prostaglandin and cytokines. This article reviews the potential protective and deleterious effects of glial cells in the substantia nigra pars compacta of Parkinson's disease. Curr Opin Neurol 14:483–489. © 2001 Lippincott Williams & Wilkins.

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Abbreviations

GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acid protein
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
MPP+	1-methyl-4-phenylperydinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NOS	nitric oxide synthase
SNpc	substantia nigra pars compacta
TNF-α	tumor necrosis factor-α

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Introduction

Parkinson's disease is a common neurodegenerative disorder characterized mainly by resting tremor, slowness of movement, rigidity, and postural instability [1•] and associated with a dramatic loss of dopamine-containing neurons in the substantia nigra pars compacta (SNpc) [2]. The number of Parkinson's disease patients has been estimated at ~1 000 000 in North America, with ~50 000 newly affected individuals each year [1•]. Thus far, the most effective treatment for Parkinson's disease remains the administration of a precursor of dopamine, L-dopa, which, by replenishing the brain in dopamine alleviates almost all Parkinson's disease symptoms. However, the chronic administration of L-dopa often causes motor and psychiatric side effects, which may be as debilitating as Parkinson's disease itself [3], and there is no supportive evidence that L-dopa therapy impedes the neurodegenerative process in Parkinson's disease. Therefore, without undermining the importance of L-dopa therapy in Parkinson's disease, there is an urgent need to acquire a deeper understanding of both etiologic (i.e., causes) and pathogenic (i.e., mechanisms of cell death) factors implicated in Parkinson's disease, not only to prevent the disease, but also to develop therapeutic strategies aimed at halting its progression. To elucidate such factors, and consequently to develop new therapies, the neuropathology of Parkinson's disease has been revisited in search of abnormalities that could shed light on these putative culprits. In keeping with this goal, it is worth mentioning that aside from the dramatic loss of dopamine neurons, the SNpc is also the site of a glial reaction in both Parkinson's disease and experimental models of Parkinson's disease [4–7]. Gliosis is a recognized prominent neuropathological feature of many diseases of the brain, whose sole and unique function has been thought, for many years, to be the removal of cellular debris. Since then, mounting evidence indicates that the role played by gliosis in pathological situations may not be restricted to its 'housekeeping' function but may also include actions which significantly and actively contribute to the demise of neurons, especially in neurodegenerative diseases like Parkinson's disease. Interestingly, several lines of evidence demonstrate that gliosis may behave in a 'yin and yang' fashion because, depending upon the situation, it may mediate either beneficial or harmful events. In this review, we will summarize the observations regarding gliosis in Parkinson's disease and in experimental models of Parkinson's disease as well as outline recent findings regarding the potential role of

gliosis in the overall neurodegenerative process that occurs in Parkinson's disease.

As a preamble to our review, it is important to remind the reader that glia is composed of macroglia, including astrocytes and oligodendrocytes, and microglia. As mentioned by Wilkin and Knott [8], so far, oligodendrocytes, which are involved in the process of myelination, have not been implicated in Parkinson's disease, whereas both astrocytes and microglial cells have. Accordingly, the focus of this section will be on astrocytes and microglial cells. Astrocytes are crucial, in the normal, undamaged adult brain, to the homeostatic control of the neuronal extracellular environment [8]. Conversely, little is known about microglial functions in the normal brain. Following an injury to the brain, both astrocytes and microglial cells undergo various phenotypic changes that enable them to respond to and to play a role in the pathological processes [9,10]. For instance, microglial activation is characterized by proliferation, increased or de-novo expression of marker molecules, such as major histocompatibility complex antigens, migration, and eventually changes into a macrophage-like appearance [11].

Glial reaction in Parkinson's disease

In normal brains, neither resting astrocytes nor microglial cells are evenly distributed [12,13]. For instance, density of microglial cells is remarkably higher in the substantia nigra compared to other midbrain areas and brain regions such as hippocampus [14]. This observation, together with the finding that substantia nigra neurons are much more susceptible to activated microglial-mediated injury [14], lend support to the idea that gliosis may play an especially meaningful role in Parkinson's disease.

The nigrostriatal pathway is the most affected dopaminergic system in Parkinson's disease. The neurons that form this pathway have their cell bodies in the SNpc and their nerve terminals in the striatum. Of particular relevance to this review is the finding that the loss of dopaminergic neurons in post-mortem parkinsonian brains is associated with a significant glial reaction [4,5,15,16]. Interestingly, however, while the damage to dopaminergic elements is consistently more severe in the striatum than in the SNpc, the response of glial cells is consistently more robust in the SNpc than in the striatum [5]. This discrepancy can be explained by the fact that dopaminergic structures are in dominance in the SNpc whereas they are in a minority in the striatum (e.g., dopamine synapses represent $<15\%$ of the entire pool of synapses in the striatum). Aside from this topographical difference, the magnitude of the astrocytic and microglial responses in parkinsonian brains are also very different. The SNpc of many but not all post-mortem Parkinson's disease patients exhibit, at best, a mild increase in the number of astrocytes and in the immunoreactivity for glial fibrillary acid protein (GFAP) [4,16]. Despite these changes, full-blown reactive astrocytes have been observed only in a few instances [4,16]. Of note, the density of GFAP-positive astrocytes appears to be inversely related to the magnitude of dopaminergic neuronal loss across the different main dopaminergic areas of the brain in Parkinson's disease post-mortem samples [12], suggesting that dopaminergic neurons within areas poorly populated with astrocytes are more prone to degenerate. Conversely, among the astrocytic pathologic features seen in Parkinson's disease, what does correlate positively with the severity of SNpc dopaminergic neuronal loss is the count of α-synuclein positive-inclusions within SNpc astrocytes [17]; whether these inclusions have any pathogenic significance remains unknown. Unlike the astrocytic response, the activation of microglial cells in Parkinson's disease is consistently dramatic [5,15,16]. Microscopically, this microglial response in the SNpc culminates in those sub-regions most affected by the neurodegenerative process [5,15,16]. Moreover, activated microglial cells are predominantly found in close proximity to free neuromelanin in the neuropil and to remaining neurons, onto which they sometimes agglomerate to produce an image of neuronophagia [5]. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated individuals, post-mortem examination reveals a marked glial reaction in the SNpc whose magnitude seems to parallel that of dopaminergic neuronal loss [18••]. In all three autopsy cases, both reactive astrocytes and activated microglial cells as well as images of neuronophagia are abundantly seen in the SNpc [18••].

The aforementioned studies indicate that the glial response in the SNpc is fairly similar between humans with Parkinson's disease and those intoxicated by MPTP, although a more significant astrocytic reaction is seen in the latter [18••]. From a neuropathological standpoint, microglial activation and especially neuronophagia is indicative of an active, ongoing process of cell death. While this contention is consistent with the fact that Parkinson's disease is a progressive condition, it challenges the notion that MPTP produces a 'hit-and-run' kind of damage and rather suggests that a single acute insult in the SNpc could set in motion a self-sustaining cascade of events with long-lasting deleterious effects. It remains that neither human post-mortem Parkinson's disease studies nor MPTP cases provide information about the temporal relationship between the loss of dopaminergic neurons and the glial reaction in the SNpc. 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 [6,19,20••]. These findings suggest that, in the MPTP mouse model [21•], the astrocytic reaction is secondary to the death of neurons and not the reverse. This is supported by the demonstration that blockade of 1-methyl-4-phenylperydinium (MPP^+, the active metabolite of MPTP [21•]) uptake into dopaminergic neurons not only completely prevents SNpc dopaminergic neuronal death but also GFAP upregulation [22]. Remarkably, activation of microglial cells, which is also quite strong in the MPTP mouse model [6,19,20••,23], occurs much earlier than that of astrocytes and, more importantly, reaches a maximum before the peak of dopaminergic neurodegeneration [20••]. In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc 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 Parkinson's disease. In the following sections, we will examine through which beneficial or detrimental mechanisms the glial response in Parkinson's disease can possibly play out in the neurodegenerative process.

The protective effect of glial cells in Parkinson's disease

As mentioned above, glial response to injury may in fact have beneficial effects which, in the case of Parkinson's disease, could attenuate neurodegeneration. Among the different mechanisms by which glial-derived neuroprotection could be mediated, the first that comes to mind involves the production of trophic factors.

To date, it is well recognized that many mature and, even more so, immature tissues and cell types, including glial cells, possess trophic properties that are essential for the survival of dopaminergic neurons. Relevant to this is the observation that striatal oligodendrocyte-type 2 astrocytes greatly improve the survival and phenotype expression of mesencephalic dopaminergic neurons in culture, while simultaneously decreasing the apoptotic demise of these neurons [24]. Although the actual identity of this glial-related trophic factor remains to be established, several others have already been well characterized. Among those, glial-derived neurotrophic factor (GDNF), which can be released by activated microglia, seems to be the most potent factor in supporting SNpc dopaminergic neurons during their period of natural developmental death in postnatal ventral midbrain cultures [25]. It is also worth emphasizing that GDNF induces dopaminergic nerve fiber sprouting in the injured rodent striatum [26], and that this effect is markedly decreased when GDNF expression is inhibited by intrastriatal infusion of antisense oligonucleotides [27]. Furthermore, GDNF, delivered either by infusion of the recombinant protein or by viral vectors, has been shown to markedly attenuate dopaminergic neuronal death and to significantly boost dopaminergic function within injured neurons in both MPTP-treated monkeys and mice [28,29••,30]. Unfortunately, in humans with Parkinson's disease, much less enthusiastic results have been obtained thus far, in that repetitive intraventricular injections of recombinant GDNF to one advanced parkinsonian patient was poorly tolerated and failed to halt the progression of the disease [31].

Glial cells may also protect dopaminergic neurons against degeneration by scavenging toxic compounds released by the dying neurons. For instance, dopamine can produce reactive oxygen species through different routes [32•]. Along this line, glial cells may protect remaining neurons against the resulting oxidative stress by metabolizing dopamine via monoamine oxidase-B and catechol-*O*-methyltransferase present in astrocytes, and by detoxifying reactive oxygen species through the enzyme glutathione peroxidase, which is detected almost exclusively in glial cells [33•]. Glia, which can avidly take up extracellular glutamate, may mitigate the presumed harmful effects of the subthalamic excitotoxic input to the substantia nigra [34]. Taken together, the data reviewed here support the contention that glial cells could have neuroprotective roles in Parkinson's disease. Whether any of those, however, actually dampen the neurodegenerative process in parkinsonian patients remains to be demonstrated.

The deleterious role of glial cells in Parkinson's disease

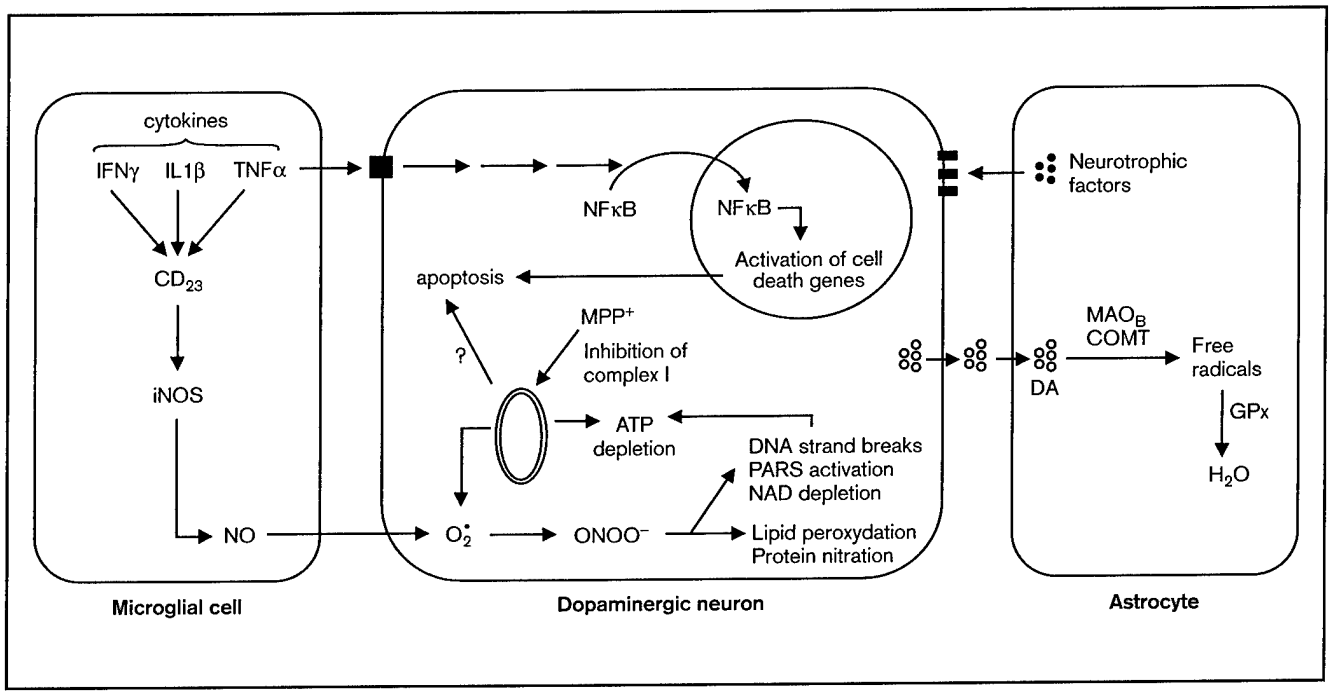
As we will see now, there are also many compelling findings which support the contention that glial cells could be harmful in Parkinson's disease. In this context, the spotlight appears to be more on activated microglial cells and less on reactive astrocytes. The importance of activated microglial cells in the neurodegenerative process is underscored by the following demonstrations in rats [35]: (1) the stereotaxic injection of bacterial endotoxin lipopolysaccharide into the SNpc causes a strong activation of microglia throughout the substantia nigra, followed by a marked degeneration of dopaminergic neurons; and (2) the pharmacological inhibition of microglial activation prevents lipopolysaccharide-induced SNpc neuronal death.

Activated microglial cells can produce a variety of noxious compounds including reactive oxygen species, reactive nitrogen species, pro-inflammatory prostaglandins, and cytokines. Among the array of reactive species, lately, the lion's share of attention has been given to reactive nitrogen species due to the prevailing idea that nitric oxide-mediated nitrating stress could be pivotal in

the pathogenesis of Parkinson's disease [36,37,38•–40•]. So far, however, none of the characterized isoforms of nitric oxide synthase (NOS) has been identified in SNpc dopaminergic neurons; hence, nitric oxide involved in the nitrating stress of Parkinson's disease most likely originates from other neurons and/or glial cells, as we hypothesized previously [36]. It is thus particularly relevant to mention that numerous glial cells in the SNpc of both Parkinson's disease patients [41•] and MPTP-treated mice [20••,23], but not of controls, express high levels of inducible NOS (iNOS). This NOS isoform, upon its induction, produces high amounts of nitric oxide for a prolonged period of time [42], as well as superoxide radicals [43], two reactive species which can either directly or indirectly promote neuronal death.

Prostaglandins and their synthesizing enzymes, such as cyclooxygenase type 2, constitute a second group of potential culprits. Indeed, cyclooxygenase type 2 has emerged as an important determinant of cytotoxicity associated with inflammation [44,45•]. In the normal brain, cyclooxygenase type 2 is significantly expressed only in specific subsets of forebrain neurons that are primarily glutamatergic in nature [46], which suggests a role for cyclooxygenase type 2 in the postsynaptic signaling of excitatory neurons. However, under pathological conditions, especially those associated with a glial response, cyclooxygenase type 2 expression in the brain can increase significantly, as does the level of its products (e.g., prostaglandin E_2), which are responsible for many of the cytotoxic effects of inflammation. Interestingly, cyclooxygenase type 2 promoter shares many features with iNOS promoter [42] and, thus, these two enzymes are often co-expressed in disease states associated with gliosis. Therefore, it is not surprising to find cyclooxygenase type 2 and iNOS expressed in SNpc glial cells of post-mortem Parkinson's disease samples [47]; prostaglandin E_2 content is also elevated in SNpc from Parkinson's disease patients [48]. Of relevance to the potential role of prostaglandin in the pathogenesis of Parkinson's disease is the demonstration that the pharmacological inhibition of both cyclooxygenase types 1 and 2 attenuates MPTP toxicity in mice [49].

Figure 1. Potential involvement of glial cells in the pathogenesis of Parkinson's disease



Activated microglial cells may contribute to dopaminergic neurodegeneration by releasing cytotoxic compounds such as cytokines. Cytokines may exert a direct effect on dopaminergic neurons by activating transduction pathways that lead to apoptosis or, alternatively, by inducing the expression of iNOS within glial cells and the subsequent formation of nitric oxide (NO). NO is membrane permeable and can diffuse to neighboring dopaminergic neurons. If the neighboring cell has elevated levels of superoxide ($O_2^{\bullet}$), there is an increased probability that superoxide will react with NO to form peroxynitrite ($ONOO^-$), which can damage lipids, proteins and DNA. Damaged DNA stimulates Poly(ADP-ribose) synthase (PARS) activity, which further contributes to the ATP depletion induced by the MPP^+-mediated inhibition of the mitochondrial complex I. Other glial cells, such as astrocytes, may have a neuroprotective effect on dopaminergic neurons by producing neurotrophic factors, such as GDNF, or by metabolizing dopamine (DA) by monoamino oxidase-B (MAO_B) or catechol-O-methyltransferase (COMT), then eliminating free radicals using glutathione peroxidase (GPx).

A third group of glial-derived compounds that can inflict damage in Parkinson's disease is the pro-inflammatory cytokines. Several among these, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are increased in both SNpc tissues and cerebrospinal fluids of Parkinson's disease patients [50–52] although some of the reported alterations may be related to the chronic use of the anti-Parkinson's disease therapy L-dopa [53]. It remains that, at autopsy, convincing immunostaining for TNF-α, IL-1β, and interferon-γ is observed in SNpc glial cells from Parkinson's disease patients [54••]. These cytokines may act, at least, at two levels in Parkinson's disease. First, while they are produced by glial cells, they can stimulate other glial cells not yet activated, thereby amplifying and propagating the glial response and consequently the glial-related injury to neurons. Relevant to this scenario are the following demonstrations [54••]: glial-derived TNF-α, IL-1β, and interferon-γ activate other microglial cells which start to express the macrophage cell surface antigen FcεR11; now, activation of FcεR11 on these newly activated microglial cells induces iNOS expression and the subsequent production of nitric oxide which, in turn, can amplify the production of cytokines within glial cells (e.g., TNF-α) and can diffuse to neighboring neurons. Second, glial-derived cytokines may also act directly on dopaminergic neurons by binding specific cell surface cytokine receptors (e.g., TNF-α receptor). Once activated, these cytokine receptors trigger intracellular death-related signaling pathways whose molecular correlates include translocation of the transcription nuclear factor-κ-B from the cytoplasm to the nucleus and activation of the apoptotic machinery. In connection with this, Parkinson's disease patients exhibit a 70-fold increase in the proportion of dopaminergic neurons with nuclear factor-κ-B immunoreactivity in their nuclei compared to control subjects [55]. In relation to apoptosis, Bax, a potent pro-apoptotic protein, is upregulated after MPTP administration and its ablation prevents the loss of SNpc dopaminergic neurons in this experimental model [56]; and caspase-3, a key effector of apoptosis, is activated in post-mortem Parkinson's disease samples [57].

Conclusion

We have tried to succinctly review the issue of glial response in Parkinson's disease and how this cellular component of Parkinson's disease neuropathology, which has been neglected far too long, can play out in the overall neurodegenerative process (Fig. 1). Accordingly, key findings and, as often as possible, recent studies were included in our discussion to provide an up-to-date look at this question. Although we have tried to provide the reader with a balanced view of this issue, it is our opinion that, given the available evidence to date, data supporting a detrimental role of the glial response in Parkinson's disease outweigh those supporting a beneficial role. We also believe that, should the glial response in Parkinson's disease indeed be implicated in the neurodegenerative process, it is unlikely that any aspect of the glial response initiates the death of SNpc dopaminergic neurons, but quite possibly propagates the neurodegenerative process. This view, if confirmed, may thus have far-reaching therapeutic implications as targeting a specific aspect of the glial-related cascade of deleterious events may prove successful in slowing or even halting further neurodegeneration in Parkinson's disease [58].

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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The Last Decade in Parkinson's Disease Research

Basic Sciences

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Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal features include tremor, slowness of movement, stiffness, and poor balance (1). Most, if not all, of these disabling symptoms are secondary to a profound reduction in striatal dopamine content, caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and of their projecting nerve fibers in the striatum (2,3). Although several approved drugs do alleviate PD symptoms, their chronic use is often associated with debilitating side effects (4), and none seems to dampen the progression of the disease. Moreover, the development of effective preventive or protective therapies is impeded by our limited knowledge of the cause (i.e., etiology) and mechanisms (i.e., pathogenesis) by which dopaminergic neurons die in PD.

Although neither the etiology nor the pathogenesis of PD has yet been elucidated, this last decade has witnessed an explosion of invaluable research, which unquestionably has provided critical insights into our current understanding of this illness. Accordingly, in this chapter, we give an overview of what we believe are the key findings of the past 10 years in this area. We also try to place each of these findings within the context of what appears to be, at least to date, the direction in which the field of PD research seems to be evolving. One caveat of our approach resides in the fact that the goal of this chapter is to provide a "flavor" for the field of PD research rather than a comprehensive review. Therefore, the reader must be aware that only selected aspects of the research performed in PD are reviewed and discussed. Along this line, the reader should also know that this chapter does not, except incidentally, review the large core of research dealing with "symptomatic therapies," whether the approach is pharmacological or surgical, which are discussed elsewhere in this book.

ETIOLOGY OF PD

If the goal is to prevent PD and to diagnose it before any actual neurodegeneration occurs, then we must unravel the etiology of this disorder. For many years, the two main hypotheses for the etiology of PD that have prevailed have been the toxic and the genetic hypotheses. As we will see, there is supportive evidence for both hypotheses, and neither one is exclusive of the other.

Toxic Hypothesis

According to this hypothesis, it is proposed that a deleterious compound may be present in our environment, even in low amounts, and

that over time it may accumulate in our organism and ultimately reach a threshold level that will cause it to unleash its damaging properties against the dopaminergic system. A significant support for an "exogenous or environmental toxin" has been provided by the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause a parkinsonian syndrome in humans almost identical to PD (5). Also relevant is the fact that 1-methyl-4-phenylpyridimium (MPP^+), MPTP's active metabolite, has been extensively used as an herbicide in many countries around the world, as has paraquat, which has a striking structural similarity to MPP^+. Furthermore, a rural environment has often been found to be associated with an elevated risk of PD (6). In keeping with this, factors such as herbicides, pesticides, and well water have all been incriminated in the occurrence of PD (6). To date, it remains, however, that despite all of these supportive observations, no actual compound, related to the MPTP family or not, has been unequivocally linked to the development of PD. Alternatively, it has also been proposed that perhaps the putative parkinsonian toxin is not exogenous but rather is produced by our own metabolism, giving rise to the "endogenous toxin" hypothesis of PD (7). According to this model, a noxious compound would be produced in response to either a defective or a variant metabolic pathway. In keeping with this view is the suggestion that patients harboring specific polymorphisms in the gene encoding for the cytochrome P450 may be at greater risk of developing young-onset PD (8). Furthermore, several isoquinoline derivatives, which can kill dopaminergic neurons, have been recovered from the brains of PD patients and thus are regarded by several experts as potential endogenous parkinsonian neurotoxins (9). Among these, tetrahydroisoquinoline (TIQ), 1-benzyl-TIQ, and *(R)*-1,2-dimethyl-5,6-dihydroxy-TIQ have the most potent neurotoxic effects (9).

Genetic Hypothesis

During the last decade, there has been a clear waning and waxing of enthusiasm for the role of genetics in the etiology of PD. However, during the past few years there has been an upsurge of interest in the genetics of PD, triggered by several breakthroughs obtained in familial forms of parkinsonism. For instance, point mutations in the α-synuclein gene, located on chromosome 4, have been found to cause an autosomal dominant parkinsonian syndrome (10,11). The two missense mutations identified thus far result in a single amino acid substitution in α-synuclein protein, that is, an alanine being replaced by a hydrophobic residue threonine at position 53 and by proline at position 30. Since the discovery of these mutations, data have been accumulated suggesting that both mutations may alter α-synuclein's normal intracellular distribution, enhance α-synuclein's propensity to interact with other intracellular proteins, and increase α-synuclein's disposition to aggregate and consequently to form intraneuronal inclusions (12–16). However, thus far, efforts to identify α-synuclein mutations in sporadic PD have failed (17–19). On the other hand, in sporadic PD, α-synuclein has been demonstrated to be a major component of the intraneuronal inclusions Lewy bodies, which are a pathologic hallmark of the disease (20,21). In addition, we have recently demonstrated that α-synuclein is up-regulated in dopaminergic neurons of the SNc after MPTP administration to mice (22). However, the normal function of this protein and its implications for the pathogenesis of PD remain to be determined.

The gene for an autosomal recessive form of juvenile parkinsonism has also recently been identified and encoded for a protein called parkin (23). More recently, a susceptibility locus for PD has been mapped to chromosome 2p13 (24), and a point mutation in the gene encoding for a key enzyme of the ubiquitin pathway has been identified in a family with parkinsonism (25). In light of all of these discoveries, we can conclude that there is increasing evidence that genetic factors might play a role in PD. However, these studies also show quite clearly that only a small number of the multigenerational fami-

lies used in the cited investigations have confirmed pathology of PD, and most of the affected family members exhibit atypical features, such as an onset at younger age, rapid course, and often dementia. Moreover, the mode of inheritance of the parkinsonism within these families is highly variable, ranging from autosomal dominant to autosomal recessive and even the possibility of being maternally transmitted (26), raising the potentiality of a genetic defect within the mitochondrial genome. Because, to date, none of the identified mutations found in familial PD has been identified in sporadic PD, it is likely that these genetic alterations may account, at most, for a very small fraction of PD patients. Nevertheless, these findings remain extremely exciting, as they raise the prospect that by elucidating the actual mechanisms by which these genetic defects produce the demise of dopaminergic neurons in familial forms of PD, they may well shed light into the etiology and pathogenesis of sporadic PD.

In our opinion, one of the most damaging arguments against a pivotal genetic component in the etiology of PD is provided by the lack of significant concordance in monozygotic twins with classical PD (27). It remains plausible that genetics may play a critical role, not as a unique etiologic factor but rather within a multifactorial cascade such as through an interaction between genetic and toxic mechanisms. This view is supported by the demonstration that individuals carrying a specific mitochondrial mutation will develop deafness only on exposure to aminoglucoside (28). Conversely, it is also relevant to point out that, among the cohort of individuals who were intoxicated with MPTP, only a fraction developed parkinsonism (Dr. J. W. Langston, *personal communication*). These two examples emphasize the potential importance of the interaction between genetic and toxic factors.

PATHOGENESIS OF PD

In spite of the above-described efforts in identifying the etiology of PD, affected patients to date are diagnosed only when the symptoms of the disease have already appeared. Therefore, it is important to identify the mechanisms involved in the cellular death in order to halt or slow down the progression of the disease once it is already established.

In an attempt to unravel the mechanisms implicated in the neurodegenerative process in PD, a large number of presumed pathogenic factors have been put forward, including dopamine metabolism, mitochondrial dysfunction, free radicals, cell death by apoptosis, excitotoxicity, defect in trophic factors, and many others. Some of these are discussed below.

Dopamine Metabolism

Dopamine is the neurotransmitter of the SNc neurons controlling normal motor function. It seems, however, that dopamine is not essential for the normal development of the SNc system, as observed in mice lacking tyrosine hydroxylase (TH), the enzyme catalyzing the first and rate-limiting step of catecholamine biosynthesis. It is worthy to note that although these mutant mice have almost no dopamine production, they show a normal cytoarchitecture of the SNc dopaminergic system (29,30) as evidenced by immunostaining for DOPA decarboxylase, another enzyme in this synthetic pathway. Interestingly, between 2% and 22% of wild-type catecholamine concentrations are found in the brains of these mutant (31) mice, likely as the result of alternative synthetic pathways such as that involving tyrosinase, another enzyme that converts tyrosine to L-DOPA but that does so during melanin synthesis.

In the mature brain, although it is clear from PD that dopamine is essential for motor control, it has been frequently suggested that dopamine at the same time may exert deleterious effects that may participate in the progression of the disease. Evidence in support of this view is still lacking *in vivo* in that there is no definitive demonstration that individuals who erroneously receive high doses of dopamine precursor, L-DOPA, for a pro-

longed period fare worse because of the drug. Similarly, the toxic effect of dopamine/L-DOPA has not been observed in studies using animals with intact nigrostriatal pathway. More confusing is the situation in rats with moderate SNc damage produced by the neurotoxin 6-hydroxydopamine, which generated contradictory results (32,33). The issue of dopamine-mediated toxicity is more compelling when one looks at the large core of *in vitro* studies dealing with this question. For instance, it has been shown that 200 μM of L-DOPA can cause a 50% reduction in the number of dopaminergic neurons in postnatal midbrain cultures (34). This toxic effect seems to be mediated by the production of free radicals because it is prevented by the overexpression of copper/zinc superoxide dismutase (SOD1) (34), a key free radical–scavenging enzyme.

Another intriguing aspect related to the dopamine metabolism that has not yet been solved concerns the link between the vulnerability of SNc dopaminergic neurons and the prominent content in black pigmentation called neuromelanin in these neurons (35). It has been reported that (a) the dopamine-containing cell groups of the normal human midbrain differ markedly from each other in the percentage of neuromelanin-pigmented neurons they contain; (b) the estimated cell loss in these cell groups in PD is directly correlated with the percentage of neuromelanin-pigmented neurons normally present in them; and (c) within each cell group in PD brains, there is greater relative sparing of nonpigmented than of neuromelanin-pigmented neurons (36). These results suggest a selective vulnerability of the neuromelanin-pigmented subpopulation of mesencephalic dopaminergic neurons in PD. To date, however, the role of neuromelanin within dopaminergic cells and its origin are not known. A new insight in this field comes from the observation that, in postnatal midbrain cultures exposed to low doses of L-DOPA, dopaminergic neurons accumulate a black pigment with the same characteristics as neuromelanin (36a). This finding indicates that the formation of neuromelanin is clearly related to the presence of L-DOPA/dopamine, and thus, this *in vitro* cellular system may represent a new tool with which to study the actual role played by neuromelanin in the neurodegenerative process. Another unresolved issue inherent to dopaminergic neuron degeneration in PD is the potential contribution of Lewy bodies in the death of these neurons. Along this line, Dr. Sulzer's group has also found that incubation of monoaminergic clonal PC-12 cells with L-DOPA induces ubiquitinated intracellular inclusions reminiscent of Lewy bodies. This exciting finding may enable us to identify the factors involved in the formation of the Lewy bodies as well as to determine their actual role in the neurodegenerative process.

Dopamine metabolism by monoamine oxidase or by autooxidation leads to the formation of hydrogen peroxide, superoxide radicals, and several reactive quinones and semiquinones that could contribute to the heightened state of oxidative stress in PD (37). Neuromelanin within dopaminergic neurons can bind ferric iron and reduce it to its reactive ferrous form (35). Taken together, these results show that the SNc, because of its dopamine and neuromelanin content, is a designated target for oxidative attack. This view leads us now to discuss the important questions of oxidative stress and of mitochondrial dysfunction in PD (37).

Oxidative Stress and Mitochondrial Dysfunction

Several lines of evidence suggest that the SNc in PD is the site of an oxidative stress (37). As mentioned above, several powerful oxidants are produced in the course of normal metabolism, including hydrogen peroxide, superoxide, peroxyl and hydroxyl, and even nitric oxide (NO). These molecules may cause cellular damage by reacting with nucleic acids, proteins, lipids, and other molecules. Indeed, in the SNc of parkinsonian patients, there is evidence of increased malondialdehyde and hydroperoxidase, which suggests lipid peroxidation, increased carbonyl proteins suggesting oxidized proteins, increased

8-hydroxy-2-deoxyguanosine suggesting DNA damage, elevation of iron levels, increase in γ-glutamyl transpeptidase activity, and diminished reduced glutathione. The possibility that oxidative stress participates in the pathogenesis of PD offers therapeutic strategies based in the use of antioxidant agents in order to provide neuroprotection. These may include free radical scavengers, glutathione-enhancing agents, iron chelators, and drugs that interfere with the oxidative metabolism of dopamine. To date, clinical trials have been performed with vitamin E and deprenyl but have failed to show definite neuroprotection.

One main source of reactive oxygen species is the mitochondria. It is thus relevant to mention that a reduction in the activity of the complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain in PD brains has been reported (38). This defect could subject cells to oxidative attack as well as energy failure. Furthermore, it seems that the mitochondrial defect found in PD is generalized and not confined to the brain, as reduced complex I activity has been reported in platelets from PD patients. In addition, hybrid cells, in which mitochondrial DNA has been destroyed and repopulated with mitochondrial DNA from the platelets of PD patients, reproduce the defect in complex I activity (39). The latter finding suggests that the observed complex I deficit originates from an alteration in the mitochondrial rather than the nuclear genome.

It is of importance to indicate that although mitochondrial dysfunction and oxidative metabolism may well be critical components in the cascade of deleterious events leading to the death of SNc dopaminergic neurons, surprisingly none of the data available to date did address the question as to whether these abnormalities represent a primary or secondary events. Indeed, all of these data are merely circumstantial and correlative observations reported in autopsied brains in which most of the dopaminergic cells have already been destroyed, and thus the mechanistic value of autopsy findings must be taken with a great deal of caution.

Programmed Cell Death

In recent years, there has been growing interest in the manner in which neuronal cells degenerate. In this context, the concept that programmed cell death (PCD) may play a role in the pathogenesis of neurodegenerative disorders has emerged as an important hypothesis. PCD represents an active form of cell death in which intrinsic cellular genetic programs are activated, leading to cellular "suicide." This form of death must be distinguished from the presumed passive cellular death resulting from a noxious effect or harsh environmental factors. PCD is also referred to as apoptosis because apoptosis is probably the most common morphologic type of PCD. However, it is important to mention that apoptosis refers to a specific set of morphologic features and is not the sole and unique morphologically defined form of death encountered in PCD (40). For instance, apoptosis is defined by the association of cell body and nucleus shrinkage, chromatin clump formation, DNA fragmentation, and condensation of cytosol and nucleosol, often with preservation of organelles and phenotypic markers. The question of whether apoptosis occurs in neurodegenerative disorders should not be regarded as an esoteric academic problem but rather as a line of research that can shed light into the pathogenesis of PD as well as open new therapeutic avenues.

It has been reported that apoptosis occurs in the substantia nigra during normal development in rodents (41,42). It has also been demonstrated that this phenomenon is time dependent, paralleling the time-course of synaptogenesis, is modulated by factors derived from the target (and/or postsynaptic neurons), and occurs in dopaminergic neurons *per se* as evidenced by TH immunostaining (41–44). Occurrence of SNc PCD has also been examined in mature brains by studying experimental models of PD. Along this line, it has been reported that intrastriatal injection of 6-hydroxydopamine (6-OHDA) in developing animals results in the induction of apoptosis in SNc dopaminergic neurons (45). This effect

seems to be explained by the destruction of dopaminergic terminals by 6-OHDA, thus interfering with target support, rather than a direct action of the toxin-inducing apoptosis. The ability of intrastriatal 6-OHDA to induce apoptotic death is developmentally dependent, with a major induction of death during the first two postnatal weeks but only a minor effect at later postnatal times. Furthermore, at later postnatal days, 6-OHDA-induced cell death presented two different morphologies, apoptotic and nonapoptotic. This suggests either that the toxin induces cell death by two different mechanisms or that the same fundamental mechanism induces an apoptotic morphology in less mature animals and a nonapoptotic morphology in more mature animals. Studies with MPTP in mice have reported mixed results. We initially reported that acute administration of MPTP, in which the drug was given in four separate doses administered every 2 hours, resulted in a nonapoptotic cell death in the SNc (46). More recently, Tatton and Kish have reported (47) in a chronic model of MPTP administration (30 mg/kg per day for five consecutive days) the occurrence of apoptosis in phenotypically defined dopaminergic neurons. Therefore, PCD plays a role in the MPTP mouse model of PD, depending on the administration schedule of the neurotoxin. Finally, in PD, the situation is more complex in that there is mixed evidence concerning whether apoptotic morphology can be identified in the postmortem PD brains of patients (48). It is important to note that apoptotic cell death seems to take place within a short period of time, making its identification difficult in a chronic degenerative disease, and that the quality of the autopsied material might not allow high-quality morphologic studies to be performed.

The MPTP Mouse Model of PD

The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California exhibited a severe and irreversible akinetic rigid syndrome analogous to PD (49). Subsequently, it was found that this syndrome was induced by the self-administration of a synthetic heroin analog whose synthesis had been heavily contaminated by a by-product, MPTP (5). Since then, MPTP has been used extensively as a model of PD (5,50,51). From neuropathologic data, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD (52). Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNc than in the ventral tegmental area (53,54) and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus (55). On the other hand, the eosinophilic intraneuronal inclusions, Lewy bodies, so characteristic of PD, have thus far not convincingly been observed in MPTP-induced parkinsonism (56). However, MPTP has never been recovered from postmortem brain samples or body fluids of PD patients, consistent not with MPTP causing PD but with its being an excellent experimental model of PD. Accordingly, it can be speculated that elucidating the molecular mechanisms of MPTP should lead to important insights into the pathogenesis and treatment of PD.

The metabolism of MPTP is a complex, multistep process (57). After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood–brain barrier and, once in the brain, this protoxin is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridimium ($MPDP^+$) (by the enzyme monoamine oxidase type B) and then to MPP^+. Thereafter, MPP^+ gains access to dopaminergic neurons by binding to plasma membrane dopamine transporter (DAT) (58). 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 (59) or ablation of DAT gene in mutant mice (60) completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP (61).

Inside dopaminergic neurons, MPP^+ can be concentrated by an active process within the mitochondria (62), where it impairs mitochondrial respiration by inhibiting complex I

of the electron transport chain (63–65). The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, leading to a deficit in ATP formation. It appears, however, that complex I activity must be reduced by at least 70% to cause severe ATP depletion (66) and that, in contrast to the situation *in vitro, in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (67). Another consequence of complex I inhibition by MPP^+ is an increased production of free radicals, especially of superoxide (68–70). The importance of MPP^+-related superoxide production in the dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of SOD1 are significantly more resistant to MPTP (71). However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury (72). Instead, superoxide is believed to exert many or most of its toxic effects through the generation of other reactive species such as hydroxyl radical, whose oxidative properties can ultimately kill cells (72).

Superoxide can also react with NO to produce peroxynitrite, a potent oxidant (73). In light of this and of our previous work on superoxide (71), we (74) and others (75,76) have assessed the role of NO in the MPTP neurotoxic process. These studies show that inhibition of NO synthase (NOS) by 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice (77), attenuates, in a dose-dependent fashion, MPTP-induced dopaminergic toxicity (74,75). The protective effect of the NOS antagonist 7-NI against MPTP-induced dopaminergic damage was subsequently demonstrated in monkeys (76).

Neuronal NOS (nNOS) is the predominant isoform of NOS in the brain (78,79). Both by its abundance and its localization, nNOS appears to be an excellent candidate for producing NO for MPTP; in agreement with this possibility is our demonstration that mutant mice deficient in nNOS are partially protected against MPTP (74). The finding that mice are better protected by the NOS antagonist 7-NI than by the lack of nNOS expression suggests that isoforms other than nNOS may also be involved in MPTP neurotoxic process. Consistent with this view, it should be mentioned that inducible NOS (iNOS), which is not or is only minimally expressed in normal brains (80,81), is dramatically up-regulated after injury including that produced by MPTP (82). Indeed, early in the course of MPTP-induced dopaminergic neuron degeneration, there is an increase in midbrain iNOS activity within the robust glial reactions that occur in the SNc following the administration of this toxin (82). Consistent with the important role of iNOS in the MPTP neurotoxic process is our demonstration that mutant mice deficient in iNOS are more resistant to MPTP (82).

Among the various forms of damage produced by peroxynitrite, the presumed culprit in MPTP-mediated toxicity, is the oxidation of phenolic rings in proteins, and in particular of tyrosine residues (83), to form nitrotyrosine as the most important product (84). Thus, detection and quantification of nitrotyrosine provide important indirect evidence that peroxynitrite is involved in a pathologic process. Relevant to the participation of peroxynitrite in the MPTP model, it has been demonstrated that MPTP significantly increases striatal levels of nitrotyrosine in mice (75,85). Aside from its role as a marker, nitrotyrosine can be a harmful modification, as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity (86,87) and prevent phosphorylation of tyrosine residues important for signal transduction (88,89). This cascade of events appears quite relevant to MPTP's mode of action, as we have demonstrated that, following MPTP administration to mice, TH becomes inactivated by tyrosine nitration (90). Furthermore, peroxynitrite can damage, through oxidative processes, many vital cellular elements other than proteins (72). Among these, DNA is of unique importance because it is the repository for genetic information and is present in a single copy. Oxidants such as peroxynitrite can cause a

range of DNA damage (72), which can possibly occur in the MPTP model as well as in PD. Indeed, our preliminary data generated in collaboration with Dr. M. F. Chesselet (Department of Neurology, UCLA) indicate that MPTP causes conspicuous DNA damage such as strand breaks in SNc neurons in mice.

CONCLUSION

This summary of a quite prolific decade has attempted to outline the findings and the direction of PD research, which we believe should lay the groundwork for the research that will take place during the coming new millennium. As illustrated above, unquestionable progress has been made toward discovering the etiology and the pathogenesis of the disease. In light of this, and although much work is still before us, we should enter this new era with significant hope and enthusiasm for finding a cure for PD.

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COX-2 and Neurodegeneration in Parkinson's Disease

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ABSTRACT: **Parkinson's disease (PD) is a common neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Recent observations link cyclooxygenase type-2 (COX-2) to the progression of the disease. Consistent with this notion, studies with the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show that inhibition and ablation of COX-2 markedly reduce the deleterious effects of this toxin on the nigrostriatal pathway. The similarity between this experimental model and PD strongly supports the possibility that COX-2 expression is also pathogenic in PD.**

KEYWORDS: **inflammation; neurotoxicity; neurodegeneration; MPTP; Parkinson's disease; reactive oxygen species; superoxide dismutase**

INTRODUCTION

Inflammatory processes associated with an increased expression of cyclooxygenase (COX) and elevated prostaglandin E_2 (PGE_2) levels have been linked to a variety of neurodegenerative disorders, including Parkinson's disease (PD), amyotrophic lateral sclerosis, and Alzheimer's disease.[1] COX, which converts arachidonic acid to PGH_2, the prostaglandin precursor of PGE_2 and several others, comes in eukaryotic cells in two main isoforms: COX-1, which is constitutively expressed, and COX-2, which is inducible.[2] COX-2 is rapidly upregulated at inflammatory sites and appears to be responsible for the formation of proinflammatory PGs.[2] Thus, COX-2 may contribute to the neurodegenerative process that is seen in Parkinson's disease[3] and that are the focus of this manuscript.

COX-2 BRAIN LOCALIZATION: EMPHASIS ON THE NIGROSTRIATAL PATHWAY

COX-2 mRNA or protein is usually not detectable outside of a handful of discrete areas of the brain, where it is found primarily in neurons.[4,5] COX-2 immunoreactiv-

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ity is observed mainly in distal dendrites and dendritic spines and, apparently, exclusively in excitatory neurons such as glutamatergic neurons.[4] Consistent with this description, we found no evidence of definite COX-2 immunoreactivity either in normal mice or in human postmortem dopaminergic structures at the level of both the substantia nigra and the striatum, which correspond to the site of origin and of projection of the nigrostriatal neurons.

Conversely, COX-2 becomes expressed in most neurons following a variety of insults.[5] To a much lesser extent, COX-2 can also be upregulated by injury and disease in astrocytes and, more rarely, in microglial and endothelial cells.[5] These considerations are perfectly in agreement with our COX-2 immunostaining data in PD postmortem samples and in the 1-methyl-4-phenyl-1,2,3,6-trerahydropyridine (MPTP) mouse model of PD.[6] Indeed, although we did not see clear COX-2 immunoreactivity in any of the controls, except over the neurophil, strong COX-2 immunostaining was seen in cells in PD and MPTP midbrain samples. Almost all positive cells exhibited a neuronal morphology, and only a few resembled astrocytes. None appeared as microglial cells. By double immunostaining, we were able to show that the majority of COX-2–positive neurons in the MPTP mice were dopaminergic. Although Knott *et al.*[7] have found more abundant astrocytic COX-2 immunoreactivity in PD samples than we have (which could be related to technical differences), the two studies appear to agree that the majority of COX-2–positive cells in PD brains are neuronal.

COX-2 INDUCTION IN PD AND MPTP BRAINS

Among the many factors capable of inducing COX-2 expression are found many inflammatory cytokines, such as tumor necrosis factor (TNF)–α and interleukin (IL)-1β, as well as glutamate through the activation of the NMDA receptor and, presumably, inducible nitric oxide synthase (iNOS).[2] Relevant to PD and its experimental model MPTP are the demonstrations that many of the aforementioned factors are significantly increased in the cerebrospinal fluid and the substantia nigra in these two pathological situations.[3] It is worth noting, however, that while COX-2 upregulation occurs mainly in neurons, the factors potentially responsible for this induction, as suggested above, may emanate from glial cells. This raises the possibility of an interesting deleterious interplay between neuron and glia in which the first neuron to die in PD would trigger a glial response that would, by releasing proinflammatory factors, induce the expression of COX-2 in neurons, enhancing the susceptibility of dopaminergic neurons to the degenerative process. According to this scenario, COX-2 would not initiate the demise of dopaminergic neurons, but rather facilitate it. If one were to accept this scenario, one might wonder how COX-2 participates in the neurodegenerative process. In the most obvious scenario, upon COX-2 induction in dopaminergic neurons, these cells start to produce significant amounts of PGE_2 that would amplify the glial response and the production of glia-derived deleterious mediators such as reactive oxygen species and proinflammatory cytokines. Relevant to this hypothesis is our demonstration that following MPTP administration a robust glial response develops[8] and that mitigating this reaction attenuates dopaminergic neuronal loss.[9] At this point, however, we are not aware of any demonstration that

COX-2–derived PGE_2 plays a signaling role in linking injured neurons to the activation of glial cells.

Another route by which COX-2 could contribute to the progression of nigrostriatal neurodegeneration is via the oxidation of dopamine by COX-2 and the consequent production of dopamine quinones. In this case, glia-derived inflammatory events would lead to COX-2 induction in neurons that would employ dopamine to generate reactive quinones.[10] These reactive dopamine quinones, which are widely known to react with nucleophiles,[11] can bind covalently with cystein residues in proteins to form protein-bound 5-S-cysteinyl dopamine, a type of posttranslational modification that can seriously affect protein functions.

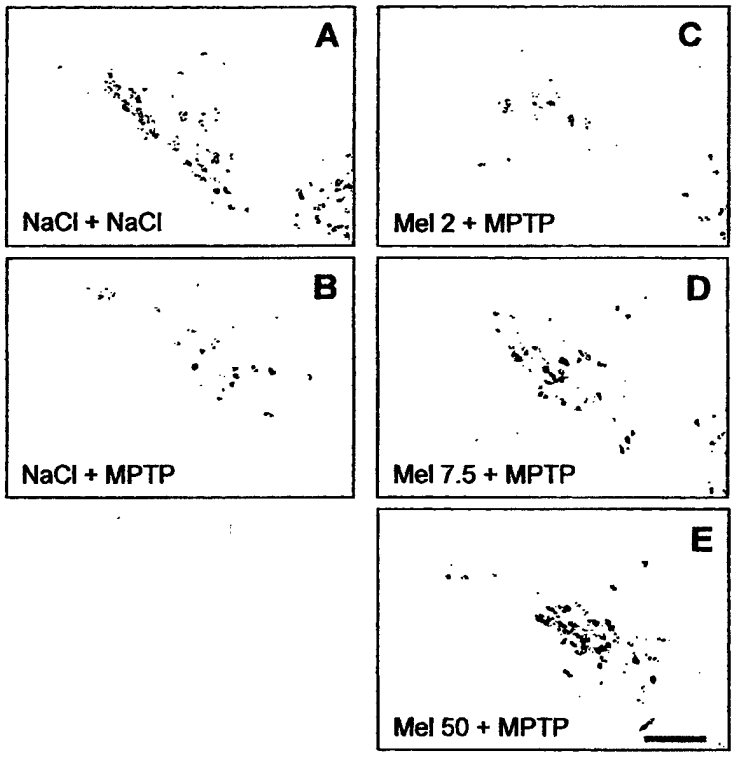

FIGURE 1. Effect of meloxicam on MPTP-induced SNpc dopaminergic neuronal death. **(A)** In saline-injected control mice, there are numerous SNpc tyrosine hydroxylase (TH)-positive neurons. **(B)** In mice treated with MPTP (30 mg/kg subcutaneous injection), the number of SNpc TH-positive neurons is reduced. **(C–E)** In mice treated with both MPTP and meloxicam, there is a noticeable attenuation of SNpc TH-positive neuronal loss. *Scale bar*: 200 μm. (Reproduced from Teismann & Ferger[13] with permission.)

TABLE 1. Effect of meloxicam on MPTP toxicity

	Saline	MPTP + saline	MPTP + meloxicam (2 mg/kg)	MPTP + meloxicam (7.5 mg/kg)	MPTP + meloxicam (50 mg/kg)
Tyrosine hydroxylase (cells/section)	76 ± 2	36 ± 4*	34 ± 6*	67 ± 4#	69 ± 3#
Nissl (cells/section)	94 ± 2	54 ± 6*	56 ± 9*	99 ± 3#	96 ± 56 ± 3#
Dopamine	13.91 ± 0.73	2.21 ± 0.40*	2.22 ± 0.53*	5.01 ± 0.47#	5.61 ± 0.35#
DOPAC	0.99 ± 0.06	0.23 ± 0.03*	0.31 ± 0.04*	0.43 ± 0.03#	0.39 ± 0.03#
HVA	1.30 ± 0.07	0.53 ± 0.06*	0.63 ± 0.08*	0.84 ± 0.05#	0.86 ± 0.04#

NOTE: Counts of tyrosine-hydroxylase positive neurons and Nissl in three sections at the third cranial nerve and dopamine, DOPAC, HVA content after saline or MPTP (30 mg/kg s.c.) in meloxicam (0, 2, 7.5, 50 mg/kg i.p.)-pretreated mice. *$P < 0.05$, fewer than saline-control mice. #$P < 0.05$, more than MPTP-injected mice and not different from control mice. Values are means ± SEM (n = 8–12 per group). (From Teismann & Ferger;13 used with permission.)

ROLE OF COX-2 IN THE MPTP MOUSE MODEL OF PD

To demonstrate whether COX-2 actually plays a deleterious role in PD, we and other investigators have examined the effects of COX-2 inhibition on MPTP-induced dopaminergic neurotoxicity. In an earlier report, acetylsalicylic acid and salicylic acid provided protection against MPTP neurotoxicity in mice, whereas diclofenac failed to do so.[12] Because the failure to observe neuroprotection by diclofenac could be due to poor brain entry, we have revisited the issue using meloxicam, a specific COX-2 inhibitor with better brain penetration.[13] In this subsequent study, the authors found that MPTP caused a significant reduction in striatal dopamine levels as well as in dopaminergic neuron numbers in the substantia nigra, which was markedly attenuated by meloxicam (FIG. 1 and TABLE 1). In another MPTP study, mutant mice deficient in COX-2 were used instead of pharmacological inhibitors. This study generated essentially the same outcomes.[14] These latter data confirm the significant role of COX-2 in MPTP-induced neurodegeneration.

CONCLUSION

COX-2 has emerged as a potential pathogenic factor in several neurodegenerative disorders, including PD. Several studies have shown that COX-2 protein is upregulated in dopaminergic neurons in PD and in its experimental model, MPTP. Although the actual mechanism by which COX-2 is involved in the nigrostriatal neurodegeneration remains to be elucidated, the fact that both the inhibition and abrogation of COX-2 in MPTP-treated mice attenuates significantly the loss of dopaminergic neurons provides compelling evidence of its role in the pathogenesis of PD.

ACKNOWLEDGMENTS

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NOTE ADDED IN PROOF

Since the submission of this manuscript, we have published an article [Teismann, P., *et al.* 2003. Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration. Proc. Natl. Acad. Sci. USA **100**(9): 5473–5478] further supporting a critical role for COX-2 in both the pathogenesis and selectivity of the PD neurodegenerative process. In this paper, we show that COX-2 is upregulated in brain dopaminergic neurons of both PD and MPTP mice and that COX-2 induction occurs through a JNKc-Jun–dependent mechanism after MPTP administration. We demonstrate that targeting COX-2 does not protect against MPTP-induced dopaminergic neurodegeneration by mitigating inflammation. Instead, we provide evidence that COX-2 inhibition/ablation prevents the formation of the oxidant species dopamine-quinone, which has been implicated in the pathogenesis of PD.

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The 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model

A Tool to Explore the Pathogenesis of Parkinson's Disease

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ABSTRACT: **Experimental models of dopaminergic neurodegeneration play a critical role in our quest to elucidate the cause of Parkinson's disease (PD). Despite the recent development of "genetic models" that have followed upon the discovery of mutations causing rare forms of familial PD, toxic models remain at the forefront when it comes to exploring the pathogenesis of sporadic PD. 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 once this neurotoxin causes intoxication, it induces in humans a syndrome virtually identical to PD. For the past two decades, the complex pharmacology of MPTP and the key steps in the MPTP neurotoxic process have been identified. These molecular events can be classified into three groups: First, those implicated in the initiation of toxicity, which include energy failure due to ATP depletion and oxidative stress mediated by superoxide and nitric oxide; second, those recruited subsequently in response to the initial neuronal perturbations, which include elements of the molecular pathways of apoptosis such as Bax; and, third, those amplifying the neurodegenerative insult, which include various proinflammatory factors such as prostaglandins. Herein, these different contributing factors are reviewed, as is the sequence in which it is believed these factors are acting within the cascade of events responsible for the death of dopaminergic neurons in the MPTP model and in PD. How to target these factors to devise effective neuroprotective therapies for PD is also discussed.**

KEYWORDS: **apoptosis; cell death; nitric oxide; neurotoxicity; neurodegeneration; MPTP; Parkinson's disease (PD); reactive oxygen species; superoxide dismutase**

INTRODUCTION

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 Par-

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kinson's disease (PD).[1] 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 that, unknown to anyone, were contaminated with MPTP.[2] In humans and nonhuman primates, depending on the regimen used, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD; in nonhuman primates, however, a resting tremor characteristic of PD has been demonstrated convincingly only in the African green monkey.[3] 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.[4,5] 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.[6,7] Except for four cases,[7,8] no human pathological material has been available for study; thus, the comparison between PD and the MPTP model is limited largely to nonhuman primates.[9] Neuropathological data show that MPTP administration causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD,[10] yet there is a resemblance that goes beyond the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons. Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area[11,12] 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.[13,14] 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, have not, thus far, been convincingly observed in MPTP-induced parkinsonism;[9] however, in MPTP-injected monkeys, intraneuronal inclusions reminiscent of Lewy bodies have been described.[15] Despite these imperfections, MPTP continues to be regarded as an excellent animal model of sporadic PD, and the belief is that studying MPTP toxic mechanisms will shed light on meaningful pathogenic mechanisms implicated in 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 have been monkeys, rats, and mice.[16] 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 neuropathological features. Herein, we will restrict our discussion to mice, since they have emerged as the preferred animals to explore cellular and molecular alterations produced by MPTP, in part because lines of engineered animals that are so critical to these types of investigations are available only in mice.[17]

MPTP MODE OF ACTION

As illustrated in FIGURE 1, the metabolism of MPTP is a complex, multistep process.[18] After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood-brain barrier. Once in the brain, the protoxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine

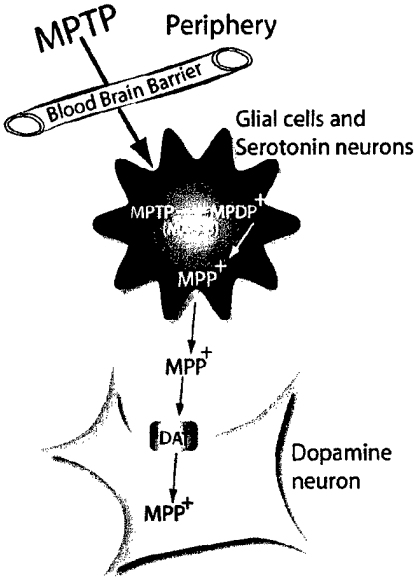

FIGURE 1. Schematic diagram of MPTP metabolism. After its systemic administration, MPTP crosses the blood–brain barrier. Once in the brain, MPTP is converted to $MPDP^+$ by monoamine oxidase B within nondopaminergic cells, and then to MPP^+ by an unknown mechanism. Thereafter, MPP^+ is released, again by an unknown mechanism, in the extracellular space. From there, MPP^+ is taken up by the dopamine transporter and thus enters dopaminergic neurons.

oxidase B within nondopaminergic 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 enter cells, but depends on the plasma membrane carriers to gain access to dopaminergic neurons. MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT),[19] as well as for norepinephrine and serotonin transporters. 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[20] or ablation of the DAT gene in mutant mice[21] completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP.[22]

Once inside dopaminergic neurons, MPP^+ can follow at least three routes (FIG. 2): (1) it can bind to the vesicular monoamine transporters (VMAT), which will translocate MPP^+ into synaptosomal vesicles;[23] (2) it can be concentrated within the mitochondria;[24] and (3) it can remain in the cytosol and interact with different cytosolic enzymes.[25] 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

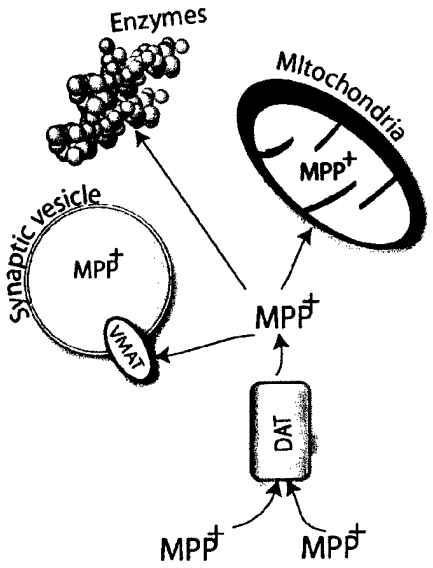

FIGURE 2. Schematic diagram of MPP^+ intracellular pathways. Inside dopaminergic neurons, MPP^+ can bind to the vesicular monoamine transporters, be translocated into synaptosomal vesicles, be concentrated by an active process within the mitochondria, and remain in the cytosol and interact with different cytosolic enzymes.

MPP^+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells.[23] 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.[26] These findings indicate that there is a clear inverse relationship between the capacity of MPP^+ sequestration (that is, VMAT density) and the magnitude of MPTP neurotoxicity. Inside dopaminergic neurons, MPP^+ can also be concentrated within the mitochondria (FIG. 2),[24] where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain[27,28] through its binding at or near the site of the mitochondrial poison rotenone.[29,30]

MPTP MECHANISM OF ACTION

Currently, it is believed that the neurotoxic process of MPTP is made up of a cascade of deleterious events, which can be divided into early and late neuronal perturbations and secondary nonneuronal 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. It appears, however, that complex I activity must be reduced >70% to cause severe ATP depletion in nonsynaptic mitochondria[31] and that, in contrast to *in vitro* MPTP, *in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels,[32] raising the question as to whether an 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.[33–35] A recent demonstration[36] showed that early ROS production can also occur in this model from the autooxidation of dopamine resulting from an MPP^+-induced massive release of vesicular dopamine to the cytosol. 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 nontransgenic littermates.[37] 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)[38–41] produced in the brain by both the neuronal and the inducible isoforms of the enzyme NO synthase.[42,43] Comprehensive reviews of the source and the role of NO in the MPTP model can be found in Przedborski and Vila[1] and in Przedborski and Dawson.[44]

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 in fact always recruited regardless of MPTP regimen, only prolonged administration of low-to-moderate doses of MPTP is associated with definite morphologically defined apoptotic neurons.[5,45] Supporting the implication of apoptotic molecular factors in the demise of dopaminergic neurons after MPTP administration is the demonstration that the proapoptotic protein Bax is instrumental in this toxic model.[46] Overexpression of the antiapoptotic Bcl-2 also protects dopaminergic cells against MPTP-induced neurodegeneration.[47,48] 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 SNpc following the administration of MPTP.[49,50] Additional caspases are also activated in MPTP-intoxicated mice such as caspase-8, which is a proximal effector of the tumor necrosis factor receptor (TNFr) family death pathway.[51] Interestingly, however, in the MPTP mouse model it is possible that caspase-8 activation is consequent to the recruitment of the mitochondria-dependent apoptotic pathway and not, as in many other pathological settings, to the ligation of TNFr.[52] Other observations supporting a role of apoptosis in the MPTP neurotoxic process include the demonstration of the resistance to MPTP of the fol-

lowing: mutant mice deficient in p53,[53] a cell cycle control gene involved in programmed cell death; mice with pharmacological or genetic inhibition of c-Jun N terminal kinases;[54–56] or mice that received a striatal adenoassociated virus vector delivery of an Apaf-1–dominant negative inhibitor.[57] 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.[58] 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[59] suggests an ongoing degenerative process and thus challenges the notion that MPTP produces a "hit and run" kind of damage. Therefore, this important observation[59] 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. With 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 glial fibrillary acidic protein (GFAP) expression remains upregulated even after the main wave of neuronal death has passed.[60–62] These findings suggest that, in the MPTP mouse model,[63] the astrocyte activation 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 upregulation.[64] Remarkably, activation of microglia, which is also quite strong in the MPTP mouse model,[60–62,65] occurs earlier than that of astrocytes and, more important, reaches a maximum before the peak of dopaminergic neurodegeneration.[62] In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc clearly occurs within a time frame 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, reactive nitrogen species (RNS), proinflammatory 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.[66] Among specific deleterious factors, cyclooxygenase type-2 (Cox-2) has emerged as an important determinant of cytotoxicity associated with inflammation.[67,68] In the normal brain, Cox-2 is significantly expressed only in specific subsets of forebrain neurons that are primarily glutamatergic in nature,[69] which suggests a role for Cox-2 in the postsynaptic signaling of excitatory neurons. However, under pathological conditions, especially those associated with a glial response, Cox-2 expression in the brain can increase significantly, as does the level of its products (for example, prostaglandin E_2, or PGE_2), which are responsible for many of the cytotoxic effects of inflammation. Interestingly, Cox-2 promoter shares many features with inducible nitric oxide synthase (iNOS) promoter;[70] thus, these two enzymes are often coex-

pressed in disease states associated with gliosis. Therefore, it is not surprising to find Cox-2 and iNOS expressed in SNpc glial cells of postmortem PD samples;[71] PGE_2 content is also elevated in SNpc from PD patients.[72] Of relevance to the potential role of prostaglandin in the pathogenesis of PD is the demonstration that the pharmacological inhibition of both Cox-2 and Cox-1[73] and the genetic ablation of Cox-2 attenuates MPTP neurotoxicity.[74]

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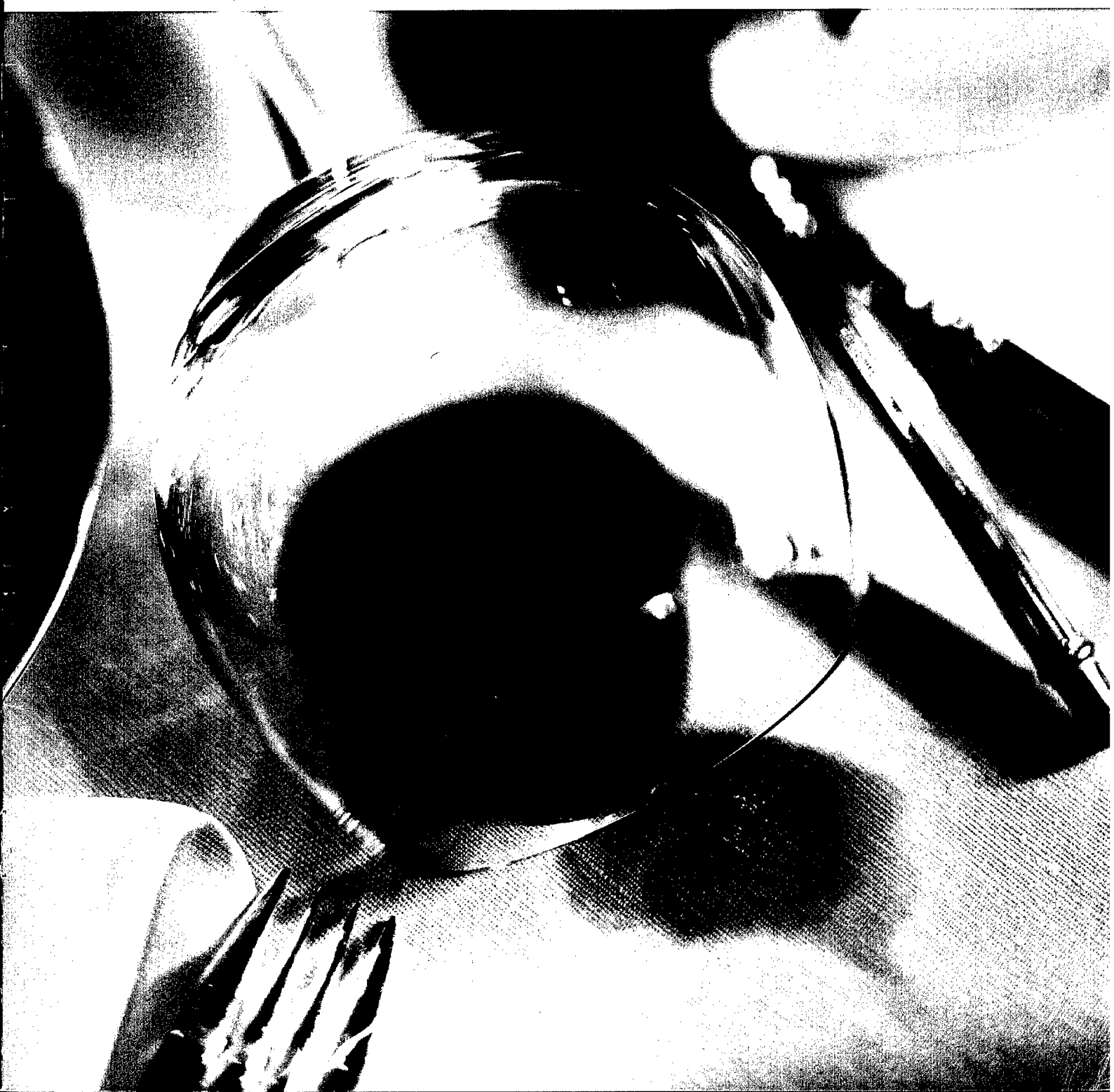

TARGETING PROGRAMMED CELL DEATH IN NEURODEGENERATIVE DISEASES

Miquel Vila and Serge Przedborski*‡§*

Molecular pathways of programmed cell death (PCD) are activated in various neurodegenerative disorders including Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease. In these diseases, PCD might be pathogenic, and targeting it might mitigate neurodegeneration. To identify potential neuroprotective targets within the PCD machinery, the expression and activity of some of its components have been altered by genetic or pharmacological means in experimental models of neurodegenerative diseases. The results of these studies have provided leads for the development of neuroprotective strategies for these progressive, disabling and often fatal disorders.

NEUROLOGICAL DISEASES

TUNEL TECHNIQUE
This technique enables the visualization of cells undergoing apoptosis by labelling the broken ends of the double-stranded DNA with biotin-conjugated dUTP, using the enzyme terminal deoxynucleotidyl transferase.

Programmed cell death (PCD) is a physiological process in which molecular programs that are intrinsic to the cell are activated to cause its destruction. This process is a fundamental property of all multicellular organisms and is crucial for their development, for organ morphogenesis, for tissue homeostasis and for defense against infected or damaged cells. The importance of PCD is emphasized by its remarkable degree of evolutionary conservation. However, excessive PCD can cause unwarranted cell death, which might lead to diseases such as immunodeficiency and neurodegeneration.

The term PCD is often used interchangeably with 'apoptosis' — a morphological form of cell death that is characterized by membrane blebbing, shrinkage of the cell body, nuclear condensation and DNA fragmentation. However, apoptosis is only one morphological form of PCD[1]; the molecular pathways linked to PCD are implicated in cell-death processes, the morphological diversity of which extends beyond apoptosis[2,3]. At times, even necrosis, which is traditionally considered to be a 'passive' death process (that is, death that does not rely on intracellular signalling pathways), has been prevented by anti-PCD compounds[4]. In this article, we group under the term PCD (also sometimes referred to as 'active cell death'[5]) all cell death forms that involve active intracellular processes, and use 'apoptosis' only in reference to the morphology of dying cells.

Over the past ten years, three experimental waves have characterized the study of PCD in neurodegeneration. Initially, the focus was on the search for apoptotic cells in *post mortem* tissues. This effort was undertaken in relation to several neurodegenerative disorders and gave rise to conflicting results. One lesson learned from these investigations was that looking for apoptosis in *post mortem* human tissue is complicated by many conceptual and technical factors. First, it is difficult to detect apoptosis owing to the presumed low daily rate of neuron loss in neurodegenerative disorders and the presumed rapid disappearance of apoptotic cells. Second, *post mortem* specimens typically derive from advanced stages of the disease, when most of the neurons that are affected by the pathological process are already lost. Third, most morphological *post mortem* studies rely on the so-called terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) TECHNIQUE to document the presence of apoptotic cells. However, we now know that TUNEL is not specific for apoptosis, especially in human *post mortem* tissue, in which factors such as hypoxia can produce TUNEL-positive, non-apoptotic DNA damage[6].

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Box 1 | **Programmed cell death and Alzheimer's disease**

Amyloid-β (Aβ) — a peptide that accumulates in the brains of people with Alzheimer's disease (AD) and forms amyloid plaques — directly induces apoptosis of cultured neurons[143]. Therefore, many researchers have looked for signs of programmed cell death (PCD) in Alzheimer patients. Along this line, fragmentation of nuclear DNA, detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)[144], has been detected in brains of people with AD, although this technique lacks specificity. More specific biochemical evidence that PCD might occur in AD is provided by the detection of activated caspases 3, 8 and 9 in hippocampal neurons of brains affected by AD[145–148]. Moreover, pharmacological or molecular inhibition of particular members of the caspase family, such as caspases 2, 3, 8 and 12, has been reported to offer partial or complete protection against Aβ-induced apoptotic cell death *in vitro*[25,149–151]. Aβ is derived from γ-secretase-mediated processing of the amyloid precursor protein (APP), but it has been shown, in both cultured cells and brains affected by AD, that APP can also be cleaved by caspases, such as caspase 3, at sites distinct from the classic secretase-processing sites[152]. Caspase-mediated cleavage of APP not only releases Aβ, but can also release a carboxy-terminal peptide that is a potent inducer of apoptosis[153]. Similarly, caspase 3-cleaved fragments of tau, a microtubule-associated protein that is the primary protein component of the filaments found in the brains of people with AD, have also been detected in *post mortem* samples[148]. Despite all of these data, there is no evidence of caspase activation or apoptotic cell death in animal models of AD and, therefore, there is no evidence *in vivo* for a potential beneficial effect of blocking PCD pathways in AD.

Given these problems, many investigators have stopped using an exclusively morphological approach, and now include techniques that assess molecular components of the PCD machinery. Although this combined approach has often shed light on the state of PCD in neurodegenerative diseases, none of these *post mortem* findings have established a role for PCD in the pathogenic process.

More recently, investigators have lost interest in the demonstration of PCD-associated cellular and molecular changes in human tissues, and they now focus on showing the actual role of PCD in the neurodegenerative process. To achieve this goal, key PCD molecules have been manipulated (either in transgenic or knockout studies), or inhibited by pharmacological agents or viral vectors in experimental models of neurodegenerative disorders. These studies have not only identified PCD components that either promote or prevent neuronal death, but they have also disclosed molecular targets for the development of drugs for preventing and treating neurodegenerative disorders.

In this article, after discussing the molecular composition of the PCD machinery, we review the results of manipulating such molecular pathways on the fate of neurons in experimental models of three prominent neurodegenerative disorders: Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). Although some work has been done on PCD in Alzheimer's disease[7] (BOX 1), the benefit of targeting PCD in experimental models of Alzheimer's disease remains to be determined; so, we will not discuss Alzheimer's disease here. Similarly, we will not discuss PCD in stroke[8], as this disorder falls outside the scope of an article on neurodegenerative diseases[9].

ZYMOGENS
The inactive precursors of enzymes — often transformed into the active enzyme by partial proteolysis.

Molecular pathways of PCD

The key mediators of PCD are the proteolytic enzymes called 'caspases', which cleave their substrates after specific aspartic acid residues. Caspases exist as ZYMOGENS (procaspases) in almost every animal cell, and they become activated in response to intracellular signalling pathways that are triggered by various cellular perturbations, such as DNA damage and withdrawal of trophic support. The family of mammalian caspases comprises 15 members, which can be divided into initiators (procaspases 2, 8, 9 and 10) and effectors (procaspases 3, 6 and 7) of PCD.

The initiators are the first caspases to become activated in the PCD cascade. They have long amino-terminal prodomains that contain specific protein–protein interaction motifs. Through these domains, initiator caspases 8 and 9 are activated after being aggregated by the adaptor molecules FADD (Fas-associating protein with death domain)[10] and Apaf1 (apoptotic protease-activating factor 1)[11], respectively. On activation, initiator caspases can cleave effector procaspases into their active forms, which are responsible for events such as mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation and, eventually, cell death.

The Bcl2 family of proteins, which are implicated in the regulation of PCD, comprises members that have either anti-PCD (such as Bcl2 and Bcl-x_L) or pro-PCD (such as Bax and Bak) effects[12]. Structurally, they all share some degree of similarity and can have up to four Bcl2-homology domains (BH1–BH4). Besides the many Bcl2 members that contain BH domains, such as Bcl2 *per se* and Bax, there are molecules that share sequence homology only with the BH3 domain, such as Bid or Bim. These BH3-only proteins can act as intracellular death ligands, proximal to multidomain Bcl2 members, and can connect with proximal signal transduction pathways[13]. Multidomain Bcl2 members can preserve or disrupt mitochondrial integrity by regulating the release of mitochondrial apoptogenic factors such as cytochrome *c*, Smac/Diablo or apoptosis-inducing factor (AIF). Bcl2 can also inhibit initiator caspases by a mitochondrial-independent mechanism[14].

The death receptor (or extrinsic) PCD pathway. The extrinsic PCD pathway (FIG. 1) is recruited on activation of cell-surface death receptors such as Fas/CD95 and the tumour necrosis factor receptor 1 (TNFR1). Death-receptor activation is initiated by specific ligands called death activators — the Fas ligand binds to Fas, and TNFα binds to TNFR1. On binding, the intracellular 'death domains' on these receptors associate with an adaptor protein that contains 'death effector domains', Fas associates with FADD, and TNFR1 associates with FADD and TRADD (TNFR-associated protein with death domain). Adaptor proteins then recruit procaspase 8, leading to its activation. Activated caspase 8 can then activate other caspases, either directly or indirectly, by cleaving Bid (FIG. 1). The extrinsic PCD pathway is especially instrumental in pathological conditions in which inflammation is a prominent feature. Because there is a growing appreciation that inflammation is a

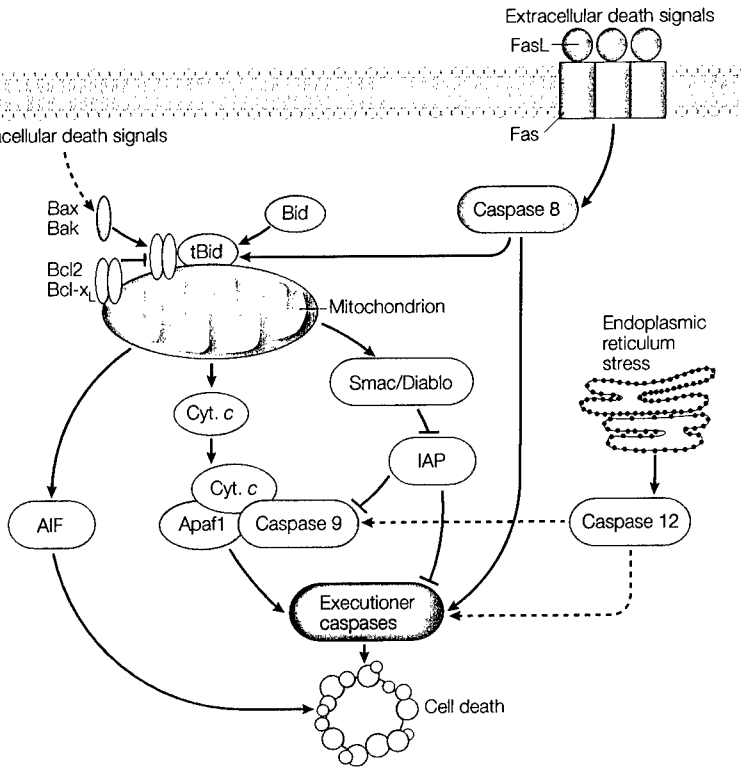


Figure 1 | **Molecular pathways of programmed cell death.** Extracellular signals through cellular death receptors, such as Fas, and intracellular signals, including damage to subcellular constituents or endoplasmic reticulum stress, both trigger genetically programmed pathways of programmed cell death (PCD). The two main PCD pathways result in the activation of downstream executioner caspases and cell death. AIF, apoptosis-inducing factor; Cyt., cytochrome; FasL, Fas ligand; IAP, inhibitor of apoptosis; tBid, truncated Bid.

feature of neurodegeneration with potential pathogenic significance[15], targeting the extrinsic PCD pathway in neurodegenerative diseases is a warranted approach. In addition to being activated by the extrinsic pathway, procaspase 8 also seems to be cleaved by a mechanism that depends on the intrinsic pathway[16].

The mitochondrial (or intrinsic) PCD pathway. In the intrinsic PCD pathway, death receptor-independent stimuli can trigger PCD by inducing translocation of pro-PCD molecules (such as Bax) to the mitochondria, with the subsequent release of mitochondrial apoptogenic factors (such as cytochrome *c*) to the cytosol (FIG. 1). Once released from the mitochondria, cytochrome *c* interacts with two other cytosolic protein factors[11], Apaf1 and procaspase 9, to activate caspase 3. The formation of this multimeric Apaf1–cytochrome *c* complex might serve to increase the local concentration of procaspases for intermolecular cleavage, and to set a relatively high threshold of caspase activation so that an occasional leakage of cytochrome *c* will not cause cells to undergo PCD[17]. Smac/Diablo is another mitochondrial intermembrane protein that is released into the cytosol on induction of PCD[18,19]. Once in the cytosol, Smac/Diablo interacts with several inhibitors of apoptosis (IAPs), therefore relieving the inhibitory effect of IAPs on initiator (such as caspase 9) and effector caspases (such as caspase 3)[20,21]. In contrast to cytochrome *c* and Smac/Diablo, the release of AIF and endonuclease G from the mitochondrial intermembrane space does not lead to caspase activation[22,23]. Under certain death-inducing stimuli, AIF translocates from the mitochondria to the nucleus where it induces caspase-independent, large-scale DNA fragmentation[22]. Similarly, endonuclease G, which is normally involved in the replication of mitochondrial DNA, can translocate to the nucleus on induction of PCD, and can induce fragmentation of nuclear DNA[23]. It has been reported that wah-1, the AIF homologue in *Caenorhabditis elegans*, associates and cooperates with endonuclease G to promote DNA degradation and apoptosis[24].

Stress in the endoplasmic reticulum (ER), including the disruption of calcium homeostasis and accumulation of unfolded proteins in the ER, can also result in PCD[25] through activation of caspase 12. Active caspase 12 can, in turn, cleave caspase 9 (REF. 26). Pro-PCD members of the Bcl2 family — such as Bax and Bak — operate at the ER to maintain calcium homeostasis and regulate ER-dependent PCD[27].

Targeting PCD in Parkinson's disease

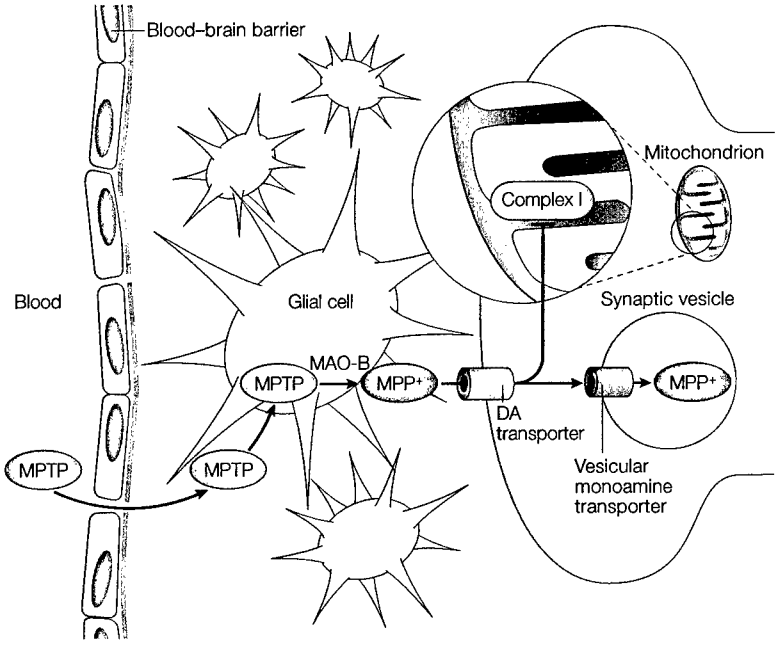
Parkinson's disease (PD) is a common neurodegenerative disorder of unknown cause, characterized by resting tremor, slowness of movement, rigidity and postural instability[28]. PD symptoms are attributed to the loss of dopamine (DA)-containing neurons in the substantia nigra pars compacta (SNPC). In the United States alone, this disease affects about one million people[28]. Although several approved drugs alleviate PD symptoms, none of them seem to stop or slow the neurodegenerative process.

Initially, the demonstration of increased numbers of TUNEL-positive DA neurons in the brains of patients with PD has been used to support the occurrence of apoptosis in this disease[29]. Subsequent studies, using a greater variety of morphological tools, have either succeeded[30–32] or failed[33–35] to find more apoptotic neurons in *post mortem* tissue from PD patients, leading to lively discussions in the field about whether apoptosis in PD is a myth or a reality. Moving away from morphological assessments, immunolocalization of Bax shows that a greater percentage of DA neurons in the SNPC were positive for this pro-PCD protein in brains of patients with PD as compared with controls[36]. In addition, Bax content seemed higher in the remaining DA neurons[37], consistent with an ongoing neurodegenerative process. DA neurons with increased expression and subcellular redistribution of the anti-PCD protein Bcl-x$_L$, and with increased activity of the effector protease caspase 3, have been found in greater abundance in the SNPC of people with PD as compared with controls[38,39], although these findings could not be independently confirmed[35]. Other PCD-related alterations detected in the brain of patients with PD include the activation of caspase 8 (REFS 40,41) and caspase 9 (REF. 41). But despite this body of descriptive data, the evidence cannot be regarded as an unequivocal demonstration that PCD has a pathogenic role in PD.

To address this crucial issue, it is necessary to use experimental models that allow manipulations of the PCD machinery and to assess their impact on neuronal

Box 2 | **The MPTP model of Parkinson's disease**

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of a meperidine analogue with potent heroin-like effects that can induce a parkinsonian syndrome in humans almost indistinguishable from Parkinson's disease (PD)[154]. Since the discovery that MPTP causes parkinsonism in humans and non-human primates, as well as in various other mammalian species, it has been used extensively as a model of PD. From neuropathological data, MPTP administration causes damage to the nigrostriatal dopamine (DA) pathway identical to that seen in PD, with the exception of the intraneuronal inclusions known as Lewy bodies. It is worthwhile noting that *post mortem* brain samples from patients with PD show a selective defect in the mitochondrial electron transport chain complex that is affected by MPTP[155,156]. The metabolism of MPTP is a complex, multistep process (see figure). After its systemic administration, MPTP, which is a pro-toxin, rapidly crosses the blood–brain barrier and is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) in non-DA cells, and then, probably by spontaneous oxidation, to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. MPP^+ is then taken up by DA transporters, for which it has high affinity. Once inside DA neurons, MPP^+ is concentrated by an active process within the mitochondria, where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain. The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, resulting in an increased production of free radicals, which causes oxidative stress and activation of programmed cell death molecular pathways.



death. For the past twenty years, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD has generated important insights into PD pathogenesis and the role of PCD in PD (BOX 2). In addition to the MPTP model, the chronic infusion of rotenone to rats, which recapitulates most of the pathological hallmarks of PD[42], is emerging as an invaluable model to investigate PD pathogenesis. But compared to MPTP, the rotenone model is technically challenging and might not be as specific for the SNPC DA neurons[43] as initally claimed[42]. Genetic models of PD, based on the overexpression of α-synuclein, have also been developed (see later discussion)[44–48], but the lack of consistent SNPC DA neurodegeneration makes these models more suitable for studying pathogenic processes other than DA neuronal death.

Alterations in energy metabolism, generation of reactive oxygen species (ROS) and perturbations in calcium homeostasis occur within hours of MPTP administration — well before any significant neuronal death has occurred[49] — raising the possibility that these events are not the main effectors of cell death. Instead, they could be intracellular signals that can set into motion deleterious molecular cascades — such as PCD activation — which are ultimately responsible for the demise of DA neurons. The prolonged administration of relatively low doses of MPTP to mice leads to morphologically defined apoptotic DA neurons[50]. Under this regime of MPTP intoxication, Bax is strongly upregulated in SNPC DA neurons[51], whereas Bcl2 is downregulated. In this model, activation of Bax induces the recruitment of the mitochondrial PCD pathway, with the subsequent activation of caspase 9 and caspase 3 (REFS 38,41). The key role of Bax in MPTP-induced neurotoxicity is illustrated by the demonstration that mutant mice deficient in Bax are resistant to the toxicity of MPTP[51]. Overexpression of *Bcl2* also protects DA cells against MPTP-induced neurodegeneration[52,53].

How the deregulation of Bcl2 family members occurs after MPTP administration is unknown. It is improbable that MPTP directly alters Bax expression and conformation. Instead, it is more plausible that MPTP activates intracellular signalling pathways, which, in turn, cause Bax upregulation and its post-translational activation (for example, Bax oligomerization and internalization into mitochondrial membranes). The tumour supressor protein p53 is one of the rare molecules known to regulate *Bax* expression[54], and p53 is activated after MPTP intoxication[55], probably in response to MPTP-induced DNA damage[56]. Inhibition of p53 attenuates MPTP-induced Bax upregulation and the degeneration of DA neurons[57]. In addition, p53 null mice are resistant to the MPTP-induced death of DA neurons[58]. Activation of the Jun N-terminal kinase (JNK) pathway has also been observed after MPTP administration[59,60]. Moreover, pharmacological blockade of JNK activation with CEP-1347/KT-7515 (REF. 61) or CEP-11004, or its inhibition by adenoviral gene transfer of the JNK-binding domain of JNK-interacting protein 1 (REF. 60) resulted in a marked attenuation of MPTP-induced neurodegeneration. *In vitro* evidence indicates that JNK activation caused by DNA damage is required for the mitochondrial translocation of Bax and the resulting recruitment of the mitochondrial PCD pathway[62,63]. These data indicate that, in the MPTP mouse model of PD, both p53 and JNK might act in concert to cause Bax induction and the post-translational changes that are mandatory to its pro-PCD role.

The intrinsic PCD pathway is recruited after MPTP administration[41], and blockade of this PCD pathway by an intrastriatal injection of an adeno-associated viral (AAV) vector containing a DOMINANT-NEGATIVE form of Apaf1 prevents the MPTP-induced activation of caspase 3 and the ensuing SNPC neuronal death[64]. By contrast, data on the recruitment and the importance of the extrinsic PCD pathway in the MPTP mouse model is still lacking.

DOMINANT NEGATIVE
A mutant molecule that can form a heteromeric complex with the normal molecule, knocking out the activity of the entire complex.

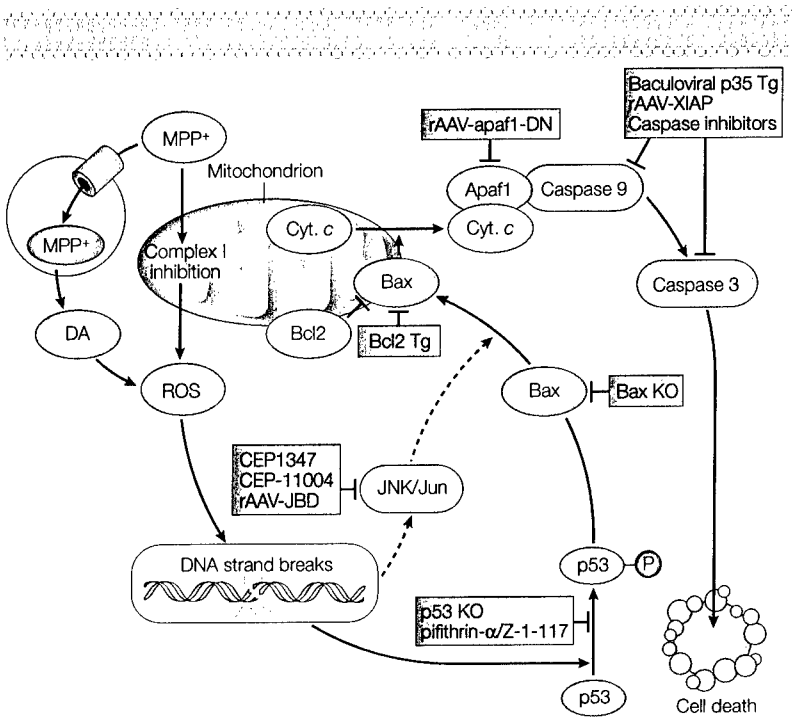


Figure 2 | **Proposed mechanism of MPTP-induced programmed cell death.** 1-methyl-4-phenylpyridinium (MPP^+), the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), impairs mitochondrial respiration in dopaminergic neurons by inhibiting complex I of the electron transport chain. Inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, resulting in an increased production of reactive oxygen species (ROS). MPP^+ can also redistribute vesicular dopamine (DA) to the cytosol. There, DA readily auto-oxidizes, thereby generating ROS. Both mitochondrial and cytolsolic MPP^+-related ROS produtions damage cellular elements, including DNA, and probably alter the expression of redox-sensitive transcription factors. ROS and damaged DNA activate p53, which induces upregulation of Bax. Bax is subsequently translocated into the mitochondria, probably by mediation of Jun N-terminal kinase (JNK), where it induces the release of cytochrome (cyt.) *c* to the cytosol and the ensuing caspase activation and cell death. Approaches aimed at targeting different key elements of this cascade (red boxes) result in an attenuation of MPTP-induced neurodegeneration. DN, dominant-negative; JBD, JNK-binding domain; KO, knockout; rAAV, adeno-associated virus vector delivery; tBid, truncated bid; Tg, transgenic, XIAP; X-chromosome-linked inhibitor of apoptosis.

Approaches that are aimed at inhibiting PCD at the level of the effector caspases have given inconsistent results. Adenoviral gene transfer of X-chromosome-linked inhibitor of apoptosis (XIAP) — a protein caspase inhibitor — prevents MPTP-induced SNPC neuronal death, but does not prevent the loss of striatal DA nerve fibres[65]. By contrast, transgenic mice overexpressing p35 — a general caspase inhibitor — in neurons showed attenuated MPTP-induced DA cell death and striatal DA depletion[41]. The discrepancy between these two studies might be explained by the more comprehensive inhibition of caspases mediated by p35 compared with XIAP, as p35 not only inhibits executioner caspases (such as caspases 3 and 7), but also upstream initiator caspases, such as caspases 8 and 9. In both studies, however, DA cell death was assessed early after MPTP administration, raising the question of whether the obtained effect indicates an actual protection or merely a delay in the cell death process. Similar to the findings with XIAP, some *in vitro* studies indicate that it is possible to produce resistance to cell death selectively at the level of the cell body. The broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), and peptide inhibitors of caspases 2, 3 and 9 attenuate the loss of DA-containing ventral midbrain cell bodies (but not neurites) exposed to the active metabolite of MPTP — 1-methyl-4-phenylpyridinium (MPP^+) — in culture[66]. This observation indicates that the molecular pathways that lead to the destruction of the soma might differ from those that govern axonal degeneration[67]. In another study[40], zVAD-fmk failed to protect cultured ventral midbrain neurons from the effect of MPP^+, but instead triggered a morphological switch from apoptosis to necrosis. Minocycline, a clinically approved tetracycline derivative that inhibits microglial activation and presumably also inhibits caspase 1 and caspase 3 (REF. 68), mitigates MPTP-induced neurodegeneration[69,70]. Caspase 8, an initiator caspase in the extrinsic PCD pathway, is also activated in MPTP-treated mice and in patients with PD[40,41]. Paradoxically, activation of caspase 8 in the MPTP model occurs after the recruitment of the intrinsic, and not the extrinsic, PCD pathway[41], indicating that caspase 8 might participate in the amplification, rather than in the initiation of the PCD in SNPC DA neurons.

Another molecule that has been linked to neurodegeneration in PD is α-synuclein, mutations of which cause a familial form of PD[71,72]. α-Synuclein is also an important component of the neuropathological hallmark of the disease — the intracellular inclusions known as Lewy bodies[73]. In MPTP-treated animals, α-synuclein accumulates and becomes nitrated in the cytosol of SNPC DA neurons[74,75]. Ablation of α-synuclein in mice prevents MPTP-induced neurodegeneration[76]. Although a direct link between α-synuclein and DA cell death is not yet well established, expression of mutant α-synuclein in cell cultures promotes apoptosis[77], and cytochrome *c* stimulates the aggregation of α-synuclein *in vitro*[78,79]. Other pathways might also be involved in triggering PCD in PD. For example, in the substantia nigra from patients with PD, the levels of the antioxidant glutathione are reduced[80] — an event that can induce PCD in neurons[81].

Together, these data indicate that the molecular pathways of PCD are involved in the death of SNPC DA neurons in experimental models of PD (FIG. 2). They support the contention that alterations in the components of the PCD machinery that have been identified in the brain of people with PD are of pathological importance. It is noteworthy that targeting PCD upstream of its execution phase results in a marked attenuation of neurodegeneration, whereas interfering at a more downstream level, such as caspase activation, produces variable results. This is of therapeutic relevance because, once the caspase executioner program is in place, its inhibition might only delay cell death. As the symptoms of PD are caused by the loss of DA terminals in the striatum, preventing the death of SNPC cell bodies without preventing the degeneration of their axons is unlikely to be a helpful therapeutic strategy. Neurons might have two self-destruction programmes, one for PCD in the cell body and a second

Box 3 | The transgenic *SOD1* model of amyotrophic lateral sclerosis

After the discovery that mutations in the gene that encodes the cytosolic free radical scavenging enzyme copper/zinc superoxide dismutase (SOD1) are responsible for the familial form of amyotrophic lateral sclerosis (ALS)[157,158], it has been shown that the transgenic expression of different *SOD1* mutants in mice[83–85] and rats[86] replicates the clinical and pathological hallmarks of ALS. The age at onset of symptoms and the life span of these transgenic animals varies across different lines, depending on the mutation and its level of expression, but when symptoms appear, they invariably include motor abnormalities that progress with the same pattern as ALS[84,159]. The first motor abnormality in mice is a fine tremor, in at least one limb, when the animal is held in the air by the tail[159]. Thereafter, weakness and atrophy of proximal muscles, predominating in the hindlimbs, develop progressively. At the end-stage of the disease (~140 days), mice transgenic for mutant SOD1 (G93A) are severely paralyzed and can no longer feed or drink on their own[159]. These end-stage mice have a profound loss of motor neurons (~50%), many dystrophic neurites, marked gliosis, some Lewy body-like intracellular inclusions and motor neurons filled with phosphorylated neurofilaments[83,159–162].

one for the axon[67]. So, a combination of anti-PCD strategies might be required to obtain optimal clinical benefit from such neuroprotective approaches.

Targeting PCD in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a relentless, fatal disorder that is characterized by a loss of the motor neurons in the cerebral cortex and spinal cord[82]. The progressive decline of muscular function results in paralysis, speech and swallowing disabilities, emotional disturbance and, ultimately, respiratory failure, causing death within 2–5 years after the onset of the disease. So far, only a few approved treatments (such as mechanical ventilation and riluzole) prolong survival in patients with ALS to some extent.

Whereas about 90% of ALS cases are sporadic (they show no genetic linkage), about 10% are inherited[82]. Of these, about a fifth carry a dominant mutation in superoxide dismutase 1 (*SOD1*). Transgenic expression of different human ALS-linked *SOD1* mutations in mice[83–85] and rats[86] replicates the clinical and pathological hallmarks of ALS (BOX 3), regardless of whether the free-radical scavenging activity of SOD1 is increased, normal or almost absent[83–87]. This observation, combined with the fact that mutant mice deficient in SOD1 do not develop any motor neuron disease[88], indicates that the cytotoxicity of mutant SOD1 is a GAIN-OF-FUNCTION effect[89]. Transfected neuronal cells that express mutant SOD1 cDNA undergo apoptosis[90], as do PC-12 cells transfected with mutant SOD1 (REF. 91) and primary neurons from transgenic mice that express mutant SOD1 (REF. 92). These *in vitro* data led investigators to consider whether mutant SOD1 kills motor neurons by activating PCD. However, inhibition of PCD in transgenic mice that express mutant SOD1 delays, but does not permanently prevent, neurodegeneration[93,94]. This indicates that the recruitment of PCD in this experimental model of ALS does not result from a direct effect, but from an indirect effect of the mutant protein on the PCD machinery[95]. In keeping with this view, mutant SOD1 has the propensity to form intracellular aggregates, the presence of which, in the cytosol of motor neurons, might impair microtubule-dependent axonal transport[96]. It might therefore be possible that mutant SOD1, by stimulating protein aggregation, causes important motor neuron perturbations that, in turn, trigger PCD.

Although there are numerous reports on the neuropathological changes in the spinal cord of people with ALS, only a handful provide fine morphological descriptions of the dying motor neurons[97,98]. Some degenerating neurons have some features reminiscent of apoptosis, but none of these dying neurons can confidently be labelled as apoptotic. In mice transgenic for mutant SOD1, clear apoptotic cells are seen in the spinal cord, but are they are rare[99] and can be immunoreactive for neurofilament or glial fibrillary acid protein[100], indicating that both neuronal and glial cells are dying by apoptosis in this model. Non-apoptotic PCD variants, such as PARAPTOSIS[3] also seem to occur in this animal model of ALS[101]. The search for other markers of apoptosis in ALS has generated conflicting results. In one *post mortem* study, DNA fragmentation was detected by TUNEL in the spinal cord motor neurons from patients with ALS, but not from controls[102]. In two other studies, DNA fragmentation was detected not only in the motor cortex and spinal cord of people with ALS, but also in controls, although to a lesser degree[103,104]. In a subsequent study, internucleosomal DNA fragmentation was detected in the motor cortex and spinal cord of patients with ALS, but not in spared regions, such as the somatosensory cortex[98]. Here, DNA fragmentation was documented in the anterior horn of the spinal cord and in the motor cortex of patients by gel electrophoresis[98] — a highly specific technique that is not frequently used to identify apoptosis in the nervous system because of its lack of sensitivity. In contrast to these positive findings, other investigators, using similar techniques and tissue samples, have failed to provide any evidence of internucleosomal cleavage of DNA in *post mortem* tissue from patients with ALS or from animal models of the disease[105,106]. Two other apoptotic markers, the Le^Y antigen and fractin, are highly expressed in the spinal cord of people with ALS[102] and transgenic *SOD1* mice[99], respectively, but neither marker has been detected in control subjects. Likewise, the levels of the PCD-related protein Par-4 (prostate apoptosis response-4) were increased in the spinal cord of patients with ALS and in mice transgenic for mutant SOD1, compared with their respective controls[107].

The study of known molecular mediators of PCD, irrespective of the morphology of the dying cells, in *post mortem* ALS samples and in mice transgenic for mutant SOD1 has provided more consistent results. In the lumbar cord of patients with ALS and transgenic *SOD1* mice, the mRNA content of *Bcl2* is decreased, whereas the level of *Bax* mRNA is increased as compared with controls[108,109]. At the protein level, spinal expression of Bcl2 and Bcl-x_L is either unchanged[110] or decreased[98,109], whereas that of Bax is increased[98,103,109] both in patients and *SOD1* mice. Because different *SOD1* mutations do not cause exactly the same neuropathology, it is important to stress that a similar pattern of changes of pro- and anti-PCD Bcl2 family members has been found in the

GAIN-OF-FUNCTION
A mutation that confers either a previously inexistent activity to the affected protein or increases a pre-existing funtion.

PARAPTOSIS
A form of programmed cell death that is related to apoptosis. It is transcription dependent and features swelling of the endoplasmic reticulum and mitochondria. However, it does not depend on caspase activation, except for caspase 9, lacks internucleosomal DNA cleavage, and does not show other morphological hallmarks of apoptosis such as nuclear fragmentation.

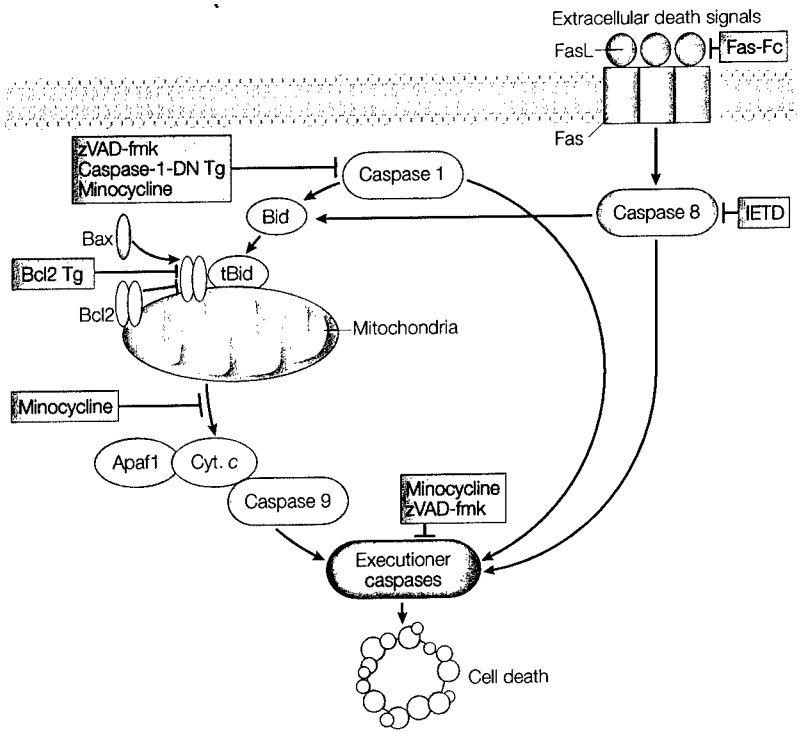


Figure 3 | **Targeting programmed cell death in amyotrophic lateral sclerosis.** In a transgenic mouse model of amyotrophic lateral sclerosis (ALS), initiator caspases activate downstream effector caspases mainly through the recruitment of the mitochondrial programmed cell death pathway. Interfering with different molecular elements of this cascade (red boxes), and particularly with caspase activation, significantly delays disease onset and mortality in this experimental model of ALS. Cyt., cytochrome; DN, dominant negative; FasL, Fas ligand; IETD Ile-Glu-Thr-Asp-fluoromethylketane ; tBid, truncated Bid; Tg, transgenic; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

spinal cords of another line of mice transgenic for SOD1 that harbour a different *SOD1* mutation[111]. None of these alterations, however, are seen in young asymptomatic transgenic mice, but they become progressively more manifest as the neurodegenerative process evolves[109]. In the spinal cord of both patients and affected transgenic *SOD1* mice, Bax is not only upregulated, but it is also mainly expressed in its deleterious homodimeric conformation[98,109]. In mice, Bax is relocated from the cytosol to the mitochondria[98,112]. These data indicate that, in ALS, the finely tuned balance between cell death antagonists and agonists is skewed towards death. Consistent with this idea, overexpression of Bcl2 mitigates neurodegeneration in both *in vitro* and *in vivo* models of ALS[91,93] and prolongs survival in transgenic *SOD1* mice[93]. As in the MPTP model, Bax upregulation in ALS is associated with p53 activation[111,113]. However, in contrast to the MPTP model, targeting p53 did not confer any protection against mutant SOD1-mediated neurodegeneration[114,115].

The pro-PCD protein Bid is expressed in the spinal cord of transgenic *SOD1* mice and is cleaved into its most active form during the progression of the disease[116]. However, whereas cleaved Bid is seen in mice transgenic for mutant SOD1 as early as the beginning of symptoms, activated caspase 8, which is known to cleave Bid, is detected only at the end-stage of the disease[116]. This observation indicates that cleavage of Bid might occur in this ALS model by another mechanism, perhaps involving caspase 1 (REF. 116), the inhibition of which prolongs the life-span of these animals[117]. Evidence for a prominent recruitment of the mitochondrial PCD pathway (that is, release of cytochrome *c* from the mitochondria to the cytosol) has been found in the spinal cord of patients and transgenic *SOD1* mice[112]. Moreover, pharmacological inhibition of cytochrome *c* release delays disease onset and extends survival of transgenic *SOD1* mice[118]. Activation of effector caspases, such as caspases 3 and 7, has also been reported in the spinal cord of patients with ALS[98] and of transgenic *SOD1* mice in a time-dependent manner that parallels the progression of the neurodegenerative process[99,100]. An instrumental role for caspases in ALS-related neurodegeneration is evidenced by the demonstration that zVAD-fmk attenuates mutant SOD1-mediated cell death in transfected PC-12 cells[91] and in transgenic *SOD1* mice[94], resulting in a delayed disease onset and mortality in these animals[94]. It has also been reported that mRNA and protein levels of the intrinsic caspase inhibitor XIAP are decreased in the spinal cord of symptomatic *SOD1* mice, and that induction of XIAP *in vitro* rescues cells harbouring mutant SOD1 from death by inhibiting the activation of caspase 3 (REF. 119). Activation of caspase 8, which mediates the death receptor pathway triggered by Fas, has been implicated in ALS in cultured embryonic motor neurons[120]. In this study, apoptotic cell death induced by trophic factor deprivation was rescued by Fas-Fc, an antibody fragment that blocks interactions between Fas and FasL, and by the caspase-8 inhibitor Ile-Glu-Thr-Asp-fluoromethylketone (IETD-fmk)[120]. In the presence of neurotrophic factors, exogenous Fas activators such as soluble FasL or anti-Fas antibodies triggered apoptosis of half of the purified motor neurons that were blocked by IETD-fmk[120]. Motor neurons isolated from transgenic mice that overexpress different ALS-linked SOD1 mutants showed increased susceptibility to activation of the Fas-triggered death pathway[121]. However, *in vivo* studies in mice transgenic for mutant *SOD1* indicate that activation of caspase 8, like induction of TNFα (REF. 122), occurs in the spinal cord only near end-stages of the cell-death process[116]. This observation indicates that the extrinsic PCD pathway in this model might make a late contribution to the neurodegenerative process.

Together (FIG. 3), the data that we have reviewed here indicate that interfering with PCD delays neuronal death and prolongs survival in experimental models of ALS. Eventually, these mice die despite the different interventions that we have discussed, indicating that targeting the PCD cascade can slow the death process but cannot abrogate it.

Targeting PCD in Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by choreic involuntary movements that result from selective neuronal loss in the striatum and the cerebral cortex. HD is caused by expansions of the trinucleotide CAG in

the *huntingtin* gene, producing a protein containing polyglutamine repeats[123]. Transgenic mice that overexpress a fragment of human huntingtin with an extended polyglutamine region show reduced survival, intraneuronal aggregates and behavioural deficits similar to HD[124]. Of these transgenic mice lines, the R6/2 line has been the most thoroughly studied. The length of the polyglutamine repeat is inversely correlated with the age of disease onset. HD is fatal, with a mean survival after onset of 15–20 years, and, at present, there is no effective treatment.

Although it is not known how mutant huntingtin promotes cell death, a self-amplification cascade of caspase activation has been proposed to participate. In *post mortem* brain tissue from patients with HD, TUNEL-positive cells, the morphology of which is reminiscent of what is found in apoptosis, have been detected in the neostriatum[125,126]. There is also evidence of the activation of caspases 1 and 8 (REFS 127,128) in the brain of patients with HD. Activation of caspase 1 was assessed by determining the levels of one of its substrates, interleukin-1β, and this might therefore reflect an inflammatory response rather than an activation of PCD pathways. Also, it is not clear whether activation of caspase 1 occurred in neurons or other cell types.

In experimental models of HD, there is evidence for the activation of caspases 1 and 3 (REF. 127). Furthermore, administration of ZVAD-fmk[127] or minocycline[68] — which inhibit caspases 1 and 3 — delays disease progression and mortality in the R6/2 transgenic mouse model of HD. Expression of a dominant-negative mutant form of caspase 1 in R6/2 mice extends survival and delays the appearance of neuronal inclusions, receptor alterations and the onset of symptoms[127]. Transgenic mice that express the caspase 1 dominant-negative mutation are also more resistant to neurotoxins that have been used to model HD, such as malonate and 3-nitropropionic acid[129]. Dietary restriction suppresses activation of caspase 1, reduces brain atrophy and the formation of huntingtin aggregates, delays disease onset and increases survival in another transgenic mouse model of HD, produced by the expression of a human amino-terminal truncated huntingtin with 82 polyglutamine repeats (HD-N171-82Q)[130].

Although these findings indicate that caspases might be valuable targets for therapeutic intervention in HD, how mutant huntingtin triggers PCD remains an enigma. It has been shown[131] that expression of extended polyglutamine repeats *in vitro* can directly activate initiator and effector caspases, such as caspases 3, 8 and 9. Moreover, wild-type huntingtin can attenuate PCD in cultured cells by preventing activation of caspase 3 through inhibition of the processing and activity of caspase 9 (REF. 132). However, the relationship between expanded polyglutamine repeats and PCD pathways seems more complex because, in addition to triggering the activation of PCD, some polyglutamine-containing proteins are caspase substrates. It has been shown that caspase 3 can cleave wild-type and mutant huntingtin proteins *in vitro*, thereby generating truncated fragments[133–135]. Truncated fragments that contain expanded polyglutamine repeats show increased toxicity and propensity to aggregate, compared with the full-length protein[133,134,136]. Huntingtin fragments have been identified in the brain of people with HD[137–140], but also in controls, indicating that caspase-mediated cleavage of wild-type huntingtin might occur as a normal physiological event. However, it is thought that cleavage of mutant huntingtin would release fragments with the potential for increased toxicity and accumulation, owing to the presence of the expanded polyglutamine tract[140].

Caspase 8 is another caspase that might be involved in the pathogenesis of HD. This caspase is recruited to

Table 1 | ***In vivo* and *in vitro* approaches to attenuate neurodegeneration in experimental models by targeting programmed cell death**

Disease	Experimental model	Genetic manipulation	Viral vector delivery	Pharmacological inhibition
PD	MPTP-treated mice	Bax KO[51] of JIP-1 (REF. 60)	JNK binding domain CEP-1347/KT-7515 (JNK)[61]	Pifithrin-α/Z-1-117 (p53)[57]
		Bcl2 Tg[52,53]		CEP-11004 (JNK)[163]
		p53 KO[58]	Apaf1 (REF. 64)	Minocycline (caspases1, 3, cyt. *c*)[69,70]
		Baculoviral p35 Tg[41]	XIAP[65]	
	Mesencephalic dopaminergic neurons in culture			zVAD-fmk/M-920 (REF. 66) zVDVAD-fmk (caspase 2)[66] zDEVD-fmk/M-725 (caspase 3)[66] zLEHD-fmk (caspase 9)[66]
ALS	Transgenic *SOD1* mice	Bcl2 Tg[93]		zVAD-fmk[94]
		Caspase 1-DN Tg[117]		Minocycline (caspase 1, 3, cyt. *c*)[118]
	Embryonic motor neurons in culture			IETD-fmk (caspase 8)[120] Fas-Fc (Fas-FasL)[120]
HD	Transgenic R6/2 mice	Caspase 1-DN Tg[127]		zVAD-fmk[127] Minocycline (caspase 1, 3, cyt. *c*)[68]
	Striatal neurons in culture	Wild-type huntingtin transfection[132]		

ALS, amyotrophic lateral sclerosis; cyt., cytochrome; DN, dominant-negative; FasL, Fas Ligand; HD, Huntington's disease; IETD, Ile-Glu-Thr-Asp-fluoromethylketane; JNK, Jun N-terminal kinase; JIP, JNK interacting protein-1; KO, knockout; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease ; SOD1, superoxide dismutase; Tg, transgenic; XIAP, X-chromosome-linked inhibitor of apoptosis; zDEVD-fmk, benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; zLEHD-fmk, benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zVDVAD-fmk, benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone.

intracellular aggregates and is subsequently activated in neuronal cells that express an expanded polyglutamine repeat[128]. In this system, caspase inhibition prevents cell death but not inclusion formation[128]. Furthermore, activated caspase 8 has been detected in the insoluble fractions of brains of people with HD but not of controls, further supporting the idea that caspase 8 is recruited to aggregates and subsequently activated[128]. It has also been proposed that caspase 8 activation in HD is mediated by the formation of pro-PCD heterodimers between Hip1 (huntingtin interacting protein 1) and Hippi (Hip1 protein interactor), which is favoured by the disease-associated polyglutamine expansion[141]. This non-receptor-mediated pathway for activating caspase 8 might trigger PCD through components of the extrinsic cell-death pathway and contribute to neuronal death in HD.

Together, these data indicate a complex association between expanded polyglutamine reapeat-containing proteins and caspase activation, indicating that caspases might also be a useful target for therapeutic intervention in HD.

Conclusions and perspectives

The different studies that we have reviewed here indicate that key molecular components of PCD are recruited in PD, ALS and HD. Much of the data on the occurrence of PCD in these diseases has been obtained by studying *post mortem* human brain samples. However, the information on the temporal relationship between these molecular changes, and their importance in the pathologic cascade, emanates largely from the use of animal models. Although not all of the diseases that we have reviewed show identical PCD changes, two general conclusions can be formulated. First, activation of PCD molecular pathways is a consistent feature of neurodegeneration. Second, PCD is not the sole mediator of cell demise in these disorders, but is a key component within a coalition of deleterious mechanisms that are responsible for the degenerative process.

The question of whether PCD pathways are involved in the demise of neurons in neurodegenerative diseases is more than academic, as it has important implications for the rational development of therapeutic strategies (TABLE 1). However, although the molecular complexity of the PCD cascade potentially offers many opportunities for its modulation, such pathways contain few conventional drug targets, such as enzymes and receptors. Therefore, researchers are focusing on other strategies to affect the components of the PCD pathway[142]. One approach is the modulation of the expression of key molecular components of the PCD machinery by gene and antisense therapy, but these technologies still need further development. Also, the problem of whether PCD can be selectively modulated in a specific organ or cell type, without adverse effects on others, remains to be solved. This issue is especially important in the context of chronic processes such as neurodegeneration that would require a sustained anti-PCD treatment. This problem could eventually be solved by fusing anti-PCD molecules with other molecules that are tagged to be recognized by tissue-specific receptors or uptake systems (for example, DA transporters). Alternatively, the problem could be tackled by synthesizing anti-PCD molecules as inactive pro-molecules that would be enzymatically activated in specific tissues or cell types (for example, by tyrosine hydroxylase in DA neurons).

One of the few potential pharmacological approaches that could modulate PCD involves inhibitors of caspase activity. According to the data that we have reviewed here, caspase inhibition might indeed delay cell death in different experimental models of neurodegeneration. However, targeting PCD at such a downstream point seems insufficient to stop the degenerative process. Nevertheless, it should be possible to combine caspase inhibition with other treatments directed at upstream events, as we believe that activation of PCD in neurodegenerative diseases is not a primary event. Instead, we think it is a 'suicide' decision, taken by cells that are affected by disease-related abnormalities such as inflammation, mitochondrial dysfunction, oxidative stress, or misfolding and aggregation of proteins. Targeting PCD alone is therefore unlikely to be sufficient to obtain a beneficial therapeutic effect. Optimal neuroprotection might require administration of a pharmacological cocktail directed against several pathogenic events.

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Invited Review

Pathogenic Role of Glial Cells in Parkinson's Disease

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Abstract: Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc). The loss of these neurons is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes. This glial response may be the source of trophic factors and can protect against reactive oxygen species and glutamate. Alternatively, this glial response can also mediate a variety of deleterious events related to the production of pro-oxidant reactive species, and pro-inflammatory prostaglandin and cytokines. We discuss the potential protective and deleterious effects of glial cells in the SNpc of PD and examine how those factors may contribute to the pathogenesis of this disease. © 2002 Movement Disorder Society

Key words: astrocyte; gliosis; IL-1β, iNOS; microglia; MPTP; neurodegeneration; Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder characterized mainly by resting tremor, slowness of movement, rigidity, and postural instability[1] and associated with a dramatic loss of dopamine-containing neurons in the substantia nigra pars compacta (SNpc).[2] Currently, the number of PD patients has been estimated at ~1,000,000 in North America with ~50,000 newly affected individuals each year.[1] To date, the most effective treatment for PD remains the administration of a precursor of dopamine, L-dopa, which, by replenishing the brain in dopamine, alleviates almost all PD symptoms. The chronic administration of L-dopa, however, often causes motor and psychiatric side effects, which may be as debilitating as PD itself.[3] Moreover, as of yet there is no evidence that L-dopa therapy can impede the neurodegenerative process in PD. Thus, there is an urgent need to acquire a better understanding of both etiologic (i.e., causes) and pathogenic (i.e., mechanisms of cell death) factors implicated in the neurodegenerative process of PD; not only to prevent the disease, but also to develop therapeutic strategies aimed at halting its progression. Although etiological factors (e.g., mutant α-synuclein, mutant parkin, and several others that remain to be identified) are presumably pivotal in the initiation of the demise of SNpc dopaminergic neurons in PD, it has been recognized increasingly that additional factors underlie the propagation of the neurodegenerative process. To elucidate such factors, and consequently to develop new therapies, the neuropathology of PD has been revisited in search of abnormalities that could shed light on these additional pathogenic culprits. In keeping with this goal, it is important to mention that aside from the dramatic loss of dopaminergic neurons, the SNpc is also the site of a glial reaction in both PD and experimental models of PD.[4–7]

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Gliosis is a prominent neuropathological feature of many diseases of the brain whose sole and unique function has been thought, for many years, to be the removal of cellular debris. Mounting evidence indicates that the role played by gliosis in pathological situations may not be restricted to its "housekeeping" function but may also include actions that significantly and actively contribute to the death of neurons, especially in neurodegenerative disorders like PD. Yet, several lines of evidence indicate that gliosis may actually exert very different effects in the diseased brain as, depending upon the situation, it may mediate either beneficial or harmful events. We summarize the observations regarding gliosis in PD and in experimental models of PD as well as outline recent findings regarding the potential role of gliosis in the overall neurodegenerative process that occurs in PD.

Before discussing the question of gliosis in PD, it is important to remind the reader that glia cells are composed of macroglia that includes astrocytes and oligodendrocytes, and microglia. As pointed out by Wilkin and Knott,[8] oligodendrocytes, which are involved in the process of myelination, have not been implicated in PD, whereas both astrocytes and microglial cells have. Accordingly, the focus of this review will be on astrocytes and microglial cells. Astrocytes are crucial, in the normal, undamaged adult brain, for the homeostatic control of the neuronal extracellular environment.[8] Conversely, little is known about microglial functions in the normal adult/mature brain. After an injury to the brain, astrocytes and microglial cells undergo various phenotypic changes that enable them to both respond to and to play a role in the pathological processes.[9,10] For instance, microglial activation is characterized by: proliferation, increased or de novo expression of marker molecules such as major histocompatibility complex antigens, migration, and eventually transformation into a macrophage-like appearance.[11]

Glial Reaction in PD

In normal brains, neither resting astrocytes nor microglial cells are evenly distributed.[12,13] The density of microglial cells is remarkably higher in the substantia nigra (SN) compared to other midbrain areas and brain regions such as the hippocampus.[14] This observation combined with the finding that SN neurons are much more susceptible to activated microglial-mediated injury[14] lend support to the idea that gliosis may play an especially meaningful role in PD.

The nigrostriatal pathway is the most affected dopaminergic system in PD. The neurons that form this pathway have their cell bodies in the SNpc and their nerve terminals in the striatum. Of particular relevance to this review is the finding that the loss of dopaminergic neurons in post-mortem parkinsonian brains is associated with a significant glial reaction.[4,5,15,16] Although the damage to dopaminergic elements is consistently more severe in the striatum than in the SNpc, the response of glial cells is consistently more robust in the SNpc than in the striatum.[5] This discrepancy can be explained by the fact that dopaminergic structures that are degenerating are in dominance in the SNpc, whereas they are in the minority in the striatum as dopaminergic synapses represent $<15\%$ of the entire pool of synapses in the striatum.[17,18] Aside from this topographical difference, the magnitudes of the astrocytic and microglial responses in parkinsonian brains are also very different. The SNpc of many but not all post-mortem PD cases exhibits, at best, a mild increase in the number of astrocytes and in the immunoreactivity for glial fibrillary acid proteins (GFAP)[4,16]; a full-blown reactive astrocytes have been observed only in a few instances.[4] The density of GFAP-positive astrocytes seems to be inversely related to the magnitude of dopaminergic neuronal loss across the different main dopaminergic areas of the brain in PD post-mortem samples.[12] This suggests that dopaminergic neurons within areas poorly populated with astrocytes are more prone to degenerate. Among the astrocytic pathologic features seen in PD, the count of α-synuclein positive-inclusions within SNpc astrocytes correlates positively with the severity of SNpc dopaminergic neuronal loss[19]; whether these inclusions have any pathogenic significance remains unknown. Unlike the astrocytic response, the activation of microglial cells in PD is consistently dramatic.[5,15,16] Microscopically, this microglial response in the SNpc culminates in those subregions most affected by the neurodegenerative process.[5,15,16] Moreover, activated microglial cells are predominantly found in close proximity to free neuromelanin in the neuropil and to remaining neurons, onto which they sometime agglomerate to produce an image of neuronophagia.[5]

Glial Reaction in Parkinsonian Syndromes

Among the various parkinsonian syndromes, PD does not have a monopoly on the association of nigrostriatal neurodegeneration and gliosis.[20] In most of these syndromes, the magnitude of SNpc DA neuronal loss is variable, Lewy bodies are often lacking, and, more importantly, neurodegenerative changes extent well beyond the SNpc and the dopaminergic neurons. Yet, there is some gliosis noted quite consistently at the level of the SNpc as well as at the level of the other affected regions of the brain. Interestingly, even in the initial reports on progressive supranuclear palsy[21] and striatonigral degen-

eration,[22] gliosis was already recognized as a prominent feature of the pathological changes seen in these syndromes. Likewise, histological examination showed frank gliosis in most of the familial forms of parkinsonian syndromes whether they are linked to unknown[23] or known gene defects such as mutations in parkin[24,25] or in α-synuclein.[26] To date, however, although the occurrence of gliosis in all of these conditions is clearly indicated, unlike in PD, no detailed description is provided regarding the glial response and especially whether it comprises astrocytes or microglia or both.

GLIAL REACTION IN EXPERIMENTAL MODELS OF PD

The neuropathological picture found in experimental models of PD is very similar to that found in PD itself. Among these models,[27] the 6-hydroxydopamine (6-OHDA) and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models are the ones that have been the most extensively used and, thus, not surprisingly these are the ones for which we have the largest amount of neuropathological information. Remarkably, the glial response found in rodents after the administration of 6-OHDA[7,28–33] is fairly comparable to that seen after the administration MPTP. Given this fact, and given that neuropathological data in humans are only available for the MPTP toxin, our discussion on the glial response in experimental models of PD will be restricted to the MPTP model. As a preamble to this discussion, it is worth providing a brief review of the MPTP model.[34] The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug-addicts in California were rushed to the emergency room with a severe bradykinetic and rigid syndrome.[35] Subsequently, it was discovered that this syndrome was induced by the self-administration of street batches of a synthetic meperidine analogue whose synthesis was heavily contaminated with a by-product, MPTP.[36] In the period of a few days after the administration of MPTP, these patients exhibited a severe akinetic rigid syndrome reminiscent of PD, and L-dopa was tried with great success, relieving the symptoms of these patients. Since the discovery that MPTP causes parkinsonism in human and nonhuman primates as well as in various other mammalian species, this neurotoxin has been used extensively as a model of PD.[34,37,38] For MPTP utility and safety see Przedborski and colleagues' technical review.[39]

In human and nonhuman primates, MPTP produces an irreversible and severe parkinsonian syndrome that replicates all of the cardinal clinical features of PD. Although it is believed that the neurodegenerative process in Parkinson's disease occurs over several years, however, the most-active phase of neuronal death after MPTP administration is presumably completed over a short period of time, producing a clinical condition consistent with "end-stage PD" in a few days. Brain imaging and neuropathological data suggest that after the acute phase of neuronal death, nigrostriatal dopaminergic neurons continue to succumb at a much lower rate for many years after MPTP exposure.[40,41] From a neuropathological standpoint, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD with a resemblance that goes beyond the degeneration of nigrostriatal dopaminergic neurons. For instance, as in PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area[42,43] and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus, at least in monkeys treated with low dose of MPTP,[44] but apparently not in acutely intoxicated humans.[45] Two typical neuropathological features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, other pigmented nuclei such as the locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions called, "Lewy bodies," which are so characteristic of PD, have thus far not been convincingly observed in any of the MPTP-intoxicated humans or animals.[46] More relevant to the topic of this review is the fact that in the few MPTP-intoxicated individuals who came to autopsy, post-mortem examination shows a marked glial reaction in the SNpc whose magnitude seems to parallel that of dopaminergic neuronal loss.[41] In all three autopsy cases, reactive astrocytes, activated microglial cells, and images of neuronophagia are abundantly seen in the SNpc.[41]

The aforementioned studies indicate that the glial response in the SNpc is fairly similar between humans with PD and those intoxicated by MPTP, although a more significant astrocytic reaction is seen in the latter.[41] From a neuropathological standpoint, microglial activation and especially neuronophagia is indicative of an active, ongoing process of cell death. Although this contention is consistent with the fact that PD is a progressive condition, it challenges the notion that MPTP produces a "hit-and-run" kind of damage and rather suggests that a single acute insult in the SNpc could set in motion a self-sustaining cascade of events with long-lasting deleterious effects. Yet neither post-mortem studies in PD nor in MPTP-intoxicated individuals can provide information about the temporal relationship between the loss of dopaminergic neurons and the glial reaction in the SNpc. The situation is quite different in rodents. For instance, looking at mice injected with MPTP and killed

at different time points thereafter, it seems 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 up-regulated even after the main wave of neuronal death has passed.[6,47,48] These findings suggest that, in the MPTP mouse model,[49] the astrocytic reaction is consecutive to the death of neurons and not the reverse. This is supported by the demonstration that blockage of 1-methyl-4-phenylperydinium (MPP^+, the active metabolite of MPTP)[49] uptake into dopaminergic neurons not only completely prevents SNpc dopaminergic neuronal death but also GFAP up-regulation.[50] Remarkably, activation of microglial cells, which is also quite strong in the MPTP mouse model,[6,47,48,51] occurs much earlier than that of astrocytes and more importantly reaches a maximum before the peak of dopaminergic neurodegeneration.[48] In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc 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. We examine through which beneficial or detrimental mechanisms the glial response in PD can possibly play out in the neurodegenerative process.

Protective Effect of Glial Cells in PD

Glial response to injury may in fact have beneficial effects that, in the case of PD, could attenuate neurodegeneration. Among the different mechanisms by which glial-derived neuroprotection could be mediated, the first that comes to mind involves the production of trophic factors.

It is well recognized that many mature and, even more so, immature tissues and cell types, including glial cells, possess trophic properties that are essential for the survival of dopaminergic neurons. Relevant to this is the observation that striatal oligodendrocyte-type 2 astrocytes greatly improve the survival and phenotype expression of mesencephalic dopaminergic neurons in culture, while simultaneously decreasing the apoptotic demise of these neurons.[52] Although the actual identity of this glial-related trophic factor remains to be established, several others have already been well characterized. Among these, glial-derived neurotrophic factor (GDNF), which can be released by activated microglia, seems to be the most potent factor in supporting SNpc dopaminergic neurons during their period of natural, developmental death in post-natal ventral midbrain cultures.[53] It is also worth emphasizing that GDNF induces dopaminergic nerve fiber sprouting in the injured rodent striatum,[54] and that this effect is markedly decreased when GDNF expression is inhibited by intrastriatal infusion of antisense oligonucleotides.[55] Furthermore, GDNF, delivered either by infusion of the recombinant protein or by viral vectors, has been shown to markedly attenuate dopaminergic neuronal death and to significantly boost dopaminergic function within injured neurons in both MPTP-treated monkeys and mice.[56–58] Unfortunately, in humans with PD, much less enthusiastic results have been obtained thus far, in that repetitive intraventricular injections of recombinant GDNF to one advanced parkinsonian patient was poorly tolerated and failed to halt the progression of the disease.[59] Brain-derived neurotrophic factor (BDNF) is another trophic factor that can be released by activated microglia and which can support the survival and process outgrowth of dopaminergic structures in the striatum.[54] The usefulness of BDNF as a potential neuroprotective factor has been well documented in several experimental models of PD,[60–62] but whether it can produce similar beneficial effects in human PD remains to be demonstrated.

Glial cells may also protect dopaminergic neurons against degeneration by scavenging toxic compounds released by the dying neurons. Dopamine can produce reactive oxygen species (ROS) through different routes.[63] 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 glial cells.[64] Glia, which can avidly take up extracellular glutamate, may mitigate the presumed harmful effects of the subthalamic excitotoxic input to the SN[65] that is hyperactive in PD.[66] Taken together, the data reviewed here support the contention that glial cells could have neuroprotective roles in PD. Whether any of those actually dampen the neurodegenerative process in parkinsonian patients remains to be demonstrated.

Deleterious Role of Glial Cells in PD

As we will see, there are also many compelling findings that support the contention that glial cells could be harmful in PD. In this context, the spotlight seems to be more on activated microglial cells and less on reactive astrocytes. The importance of activated microglial cells in the neurodegenerative process is underscored by the following demonstrations in rats[67]: 1) the stereotaxic injection of bacterial endotoxin lipopolysaccharide (LPS) into the SNpc causes a strong activation of microglia throughout the SN followed by a marked degeneration of dopaminergic neurons; and 2) the pharmacolog-

ical inhibition of microglial activation prevents LPS-induced SNpc neuronal death. Similarly, LPS-induced microglial activation leads to injury of dopaminergic cell line MES 23.5 and dopaminergic primary ventral midbrain neurons in culture.[68]

Activated microglial cells can produce a variety of noxious compounds including ROS, reactive nitrogen species (RNS), pro-inflammatory prostaglandins, and cytokines. Among the array of reactive species, lately the lion's share of attention has been given to RNS due to the prevalent idea that nitric oxide (NO)-mediated nitrating stress could be pivotal in the pathogenesis of PD.[69–73] So far, however, none of the characterized isoforms of NO synthase (NOS) have been identified within SNpc dopaminergic neurons; hence, NO involved in the nitrating stress of PD most likely originates from other neurons or glial cells, as we hypothesized previously.[69] It is particularly relevant to mention that numerous glial cells in the SNpc of both PD patients[74] and MPTP-treated mice,[48,51] but not of controls, express high levels of inducible NOS (iNOS). Upon its induction, this NOS isoform produces high amounts of NO[75] as well as superoxide radicals[76]—two reactive species that can either directly or indirectly promote neuronal death by inflicting oxidative damage.

Prostaglandins and their synthesizing enzymes, such as cyclooxygenase type-2 (Cox-2), constitute a second group of potential culprits. Indeed, Cox-2 has emerged as an important determinant of cytotoxicity associated with inflammation.[77,78] In the normal brain, Cox-2 is significantly expressed only in specific subsets of forebrain neurons that are primarily glutamatergic in nature,[79] suggesting a role for Cox-2 in the postsynaptic signaling of excitatory neurons. Under pathological conditions, especially those associated with a glial response, Cox-2 expression in the brain can increase significantly, as does the level of its products (e.g., prostaglandin E_2), which are responsible for many of the cytotoxic effects of inflammation. Interestingly, Cox-2 promoter shares many features with iNOS promoter[75] and thus, these two enzymes are often co-expressed in disease states associated with gliosis. Therefore, it is not surprising to find Cox-2 and iNOS expressed in SNpc glial cells of post-mortem PD samples[80]; PGE_2 content is also elevated in the SNpc from PD patients.[81] Of relevance to the potential role of prostaglandin in the pathogenesis of PD, is the demonstration that the pharmacological inhibition of both Cox-2 and Cox-1 attenuates MPTP toxicity in mice.[82]

A third group of glial-derived compounds that can inflict damage in PD is the pro-inflammatory cytokines. Several among these, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are increased in both SNpc tissues and cerebrospinal fluids of PD patients[83–85] although some of the reported alterations may be related to the chronic use of the anti-PD therapy L-dopa.[86] Nevertheless, at autopsy, convincing immunostaining for TNF-α, IL-1β, and interferon-γ (IFN-γ) is observed in SNpc glial cells from PD patients.[87] These cytokines may act in PD on at least two levels. First, although they are produced by glial cells, they can stimulate other glial cells not yet activated, thereby amplifying and propagating the glial response and consequently the glial-related injury to neurons. Relevant to this scenario are the following demonstrations[87]: glial-derived TNF-α, IL-1β, and IFN-γ activate other microglial cells that start to express the macrophage cell surface antigen Fc∈R11 (CD23). Activation of CD-23 on these newly activated microglial cells induces iNOS expression and the subsequent production of NO that, in turn, can amplify the production of cytokines within glial cells (e.g., TNF-α) and can diffuse to neighboring neurons. Second, glial-derived cytokines may also act directly on dopaminergic neurons by binding to specific cell surface cytokine receptors (e.g., TNF-α receptor). 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. In connection with this, 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.[88] In relation to apoptosis, Bax, a potent pro-apoptotic protein, is up-regulated after MPTP administration and its ablation prevents the loss of SNpc dopaminergic neurons in this experimental model[89]; and caspase-3, a key agent of apoptosis, is activated in post-mortem PD samples.[90]

CONCLUSION

We have tried to succinctly discuss the issue of glial response in PD and how this cellular component of PD neuropathology, which has been neglected far too long, plays out in the overall neurodegenerative process (Fig. 1). Although we have tried to provide the reader with a balanced view on the issue, it is our opinion that, given the available evidence to date, data supporting a detrimental role of the glial response in PD outweigh those supporting a beneficial role. We also believe that, should the glial response in PD indeed be implicated in the neurodegenerative process, it is unlikely that any aspect of the glial response initiates the death of SNpc dopaminergic neurons, but quite possibly propagates the neurodegenerative process. This view, if confirmed, could

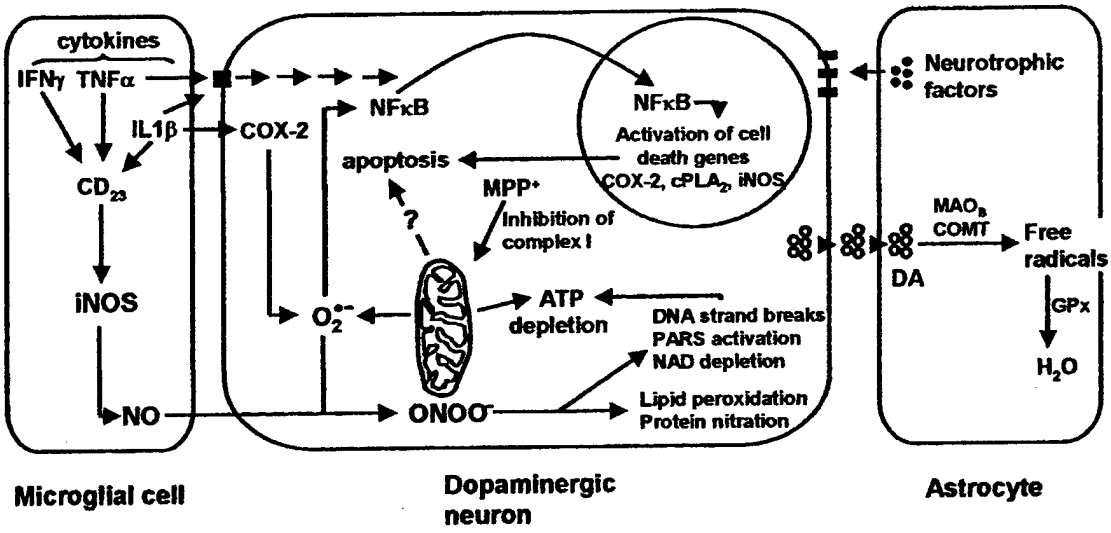

FIG. 1. Potential involvement of glial cells in the pathogenesis of Parkinson's disease. Activated microglial cells may contribute to dopaminergic neurodegeneration by releasing cytotoxic compounds such as cytokines. Cytokines may exert a direct effect on dopaminergic neurons by activating transduction pathways that lead to apoptosis or, alternatively, by inducing the expression of COX-2 within dopaminergic neurons and iNOS within glial cells that, in turn, stimulate the formation of superoxide ($O_2^{\bullet-}$) and nitric oxide (NO), respectively. NO is membrane permeable and can diffuse to neighboring dopaminergic neurons. If the neighboring cell has elevated levels $O_2^{\bullet-}$, there is an increased probability that $O_2^{\bullet-}$ will react with NO to form peroxynitrite ($ONOO^-$), which can damage lipids, proteins, and DNA. Damaged DNA stimulates Poly(ADP-ribose) synthase (PARS) activity, which depletes further ATP already compromised by the inhibition of the mitochondrial complex I by MPP^+. Other glial cells, such as astrocytes, may have a neuroprotective effect on dopaminergic neurons by producing neurotrophic factors, such as GDNF, or by metabolizing dopamine by monoamino oxidase-B (MAO_B) or catechol-*O*-methyl transferase (COMT), then eliminating free radicals using glutathione peroxidase (GPx).

have far-reaching therapeutic implications, as targeting a specific aspect of the glial-related cascade of deleterious events may prove successful in slowing or even halting further neurodegeneration in PD.[91]

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SERIES INTRODUCTION

Neurodegeneration: What is it and where are we?

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"Neurodegeneration" is a commonly used word whose meaning is believed to be universally understood. Yet finding a precise definition for neurodegeneration is much more arduous than one might imagine. Often, neurodegeneration is only casually mentioned and scarcely discussed in major medical textbooks and is even incompletely defined in the most comprehensive dictionaries. Etymologically, the word is composed of the prefix "neuro-," which designates nerve cells (i.e., neurons), and "degeneration," which refers to, in the case of tissues or organs, a process of losing structure or function. Thus, in the strict sense of the word, neurodegeneration corresponds to any pathological condition primarily affecting neurons. In practice, neurodegenerative diseases represent a large group of neurological disorders with heterogeneous clinical and pathological expressions affecting specific subsets of neurons in specific functional anatomic systems; they arise for unknown reasons and progress in a relentless manner. Conversely, neoplasm, edema, hemorrhage, and trauma of the nervous system, which are not primary neuronal diseases, are not considered to be neurodegenerative disorders. Diseases of the nervous system that implicate not neurons per se but rather their attributes, such as the myelin sheath as seen in multiple sclerosis, are not neurodegenerative disorders either, nor are pathologies in which neurons die as the result of a known cause such as hypoxia, poison, metabolic defects, or infections.

Among the hundreds of different neurodegenerative disorders, so far the lion's share of attention has been given only to a handful, including Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS). Many of the less common or publicized neurodegenerative disorders, though no less devastating, have remained essentially ignored.

The most consistent risk factor for developing a neurodegenerative disorder, especially AD or PD, is increasing age (1). Over the past century, the growth rate of the population aged 65 and beyond in 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, possibly the proportion of persons suffering from some kind of neurodegenerative disorder. This prediction is at the center of growing concerns in the medical community and among lawmakers, for one can easily foresee the increasing magnitude of emotional, physical, and financial burdens on patients, caregivers, and society that are related to these disabling illnesses. Compounding the problem is the fact that while, 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 process. In keeping with this, the development of effective preventive or protective therapies has been impeded by the limitations of our knowledge of the causes and the mechanisms by which neurons die in neurodegenerative diseases. Despite this bleak outlook, several neurobiological breakthroughs have brought closer than ever the day when the secrets of several neurodegenerative disorders will be unlocked and effective therapeutic strategies will become available. In this Perspective series, selected genetic and molecular advances relevant to the biology of neurodegeneration — e.g., to apoptosis, oxidative stress, and mitochondrial dysfunction — will be reviewed. While some of these will be discussed in terms of generic mechanisms underlying neuronal death, others will be discussed in the context of a specific disease such as ALS or HD. From the various Perspectives in this series, readers may obtain a comprehensive update on prominent neurodegenerative conditions from both a clinical and a molecular viewpoint. As a preamble to the series, however, it would be useful to discuss some general notions related to neu-

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Nonstandard abbreviations used: Alzheimer disease (AD); Parkinson disease (PD); Huntington disease (HD); amyotrophic lateral sclerosis (ALS); neurofibrillary tangle (NFT); superoxide dismutase-1 (SOD1); programmed cell death (PCD).

rodegeneration that should help set the stage for the more detailed articles to follow.

Classification of neurodegenerative diseases

The number of neurodegenerative diseases is currently estimated to be a few hundred, and, among these, many appear to overlap with one another clinically and pathologically, rendering their practical classification quite challenging. The issue is further complicated by the fact that, in diseases such as multisystem atrophy in which several areas of the brain are affected, different combinations of lesions can give rise to different clinical pictures (2). Furthermore, the same neurodegenerative process, especially at the beginning, can affect different areas of the brain, making a given disease appear very different from a symptomatic standpoint. Despite these difficulties, the most popular categorization of neurodegenerative disorders is still based on the predominant clinical feature or the topography of the predominant lesion, or often on a combination of both. Accordingly, neurodegenerative disorders of the CNS may, for example, be first grouped into diseases of the cerebral cortex, the basal ganglia, the brainstem and cerebellum, or the spinal cord. Then, within each group, a given disease may be further classified based on its main clinical features. For instance, the group of diseases that predominantly affect the cerebral cortex may be divided into dementing (e.g., AD) and nondementing conditions. Of note, while AD is by far the most frequently cited cause of dementing cerebral cortex pathology (3), dementia can apparently be observed in at least 50 different diseases (4). Moreover, dementia is not exclusively observed in neurodegenerative disorders; it is also frequently observed in ischemic, metabolic, toxic, infectious, and traumatic insults of the brain.

Diseases that predominantly involve the basal ganglia (a series of deep nuclei situated at the base of the forebrain, including the caudate nucleus putamen, globus pallidus, substantia nigra, subthalamic nucleus, red nucleus, and some thalamic and brainstem nuclei) are essentially characterized by abnormal movements. Yet, based on the phenomenology of the abnormal movements, diseases of the basal ganglia can be classified as hypokinetic or hyperkinetic. Hypokinetic basal ganglia disorders are epitomized by PD, in which the amplitude and velocity of voluntary movements are diminished or, in extreme cases, even nonexistent, causing the patient to become a prisoner within his or her own body. Aside from PD, parkinsonism – which refers to an association of at least two of the following clinical signs: resting tremor, slowness of movements, stiffness, and postural instability – is found in a variety of other diseases of the basal ganglia as well. In some (e.g., striatonigral degeneration), there is only parkinsonism, but in others, often called parkinson-plus syndromes, there is parkinsonism plus signs of cerebellar ataxia (e.g., olivopontocerebellar atrophy), orthostatic hypotension (e.g., Shy-Drager syndrome), or paralysis of vertical eye movements (e.g., progressive supranuclear palsy). Because, early on, parkinsonism may be the only clinical expression of parkinson-plus syndromes, it is difficult to reach an accurate diagnosis before the patient reaches a more advanced stage of the disease. This problem is well illustrated by the fact that more than 77% of patients with parkinsonism are diagnosed in life as having PD (5), but as much as a quarter of these are found at autopsy to have lesions incompatible with PD (6). At the other end of the spectrum are the hyperkinetic basal ganglia disorders, which are epitomized by HD and essential tremor. In these two conditions, excessive abnormal movements such as chorea or tremor are superimposed onto and interfere with normal voluntary movements. Although hyperkinetic basal ganglia disorders are probably as diverse as are hypokinetic basal ganglia disorders, their accurate classification, even during life, is less problematic, in part because specific disease markers such as gene mutations exist for several of these syndromes.

Classification of neurodegenerative diseases of the cerebellum and its connections is particularly challenging because of the striking overlap among the various pathological conditions. Indeed, some diseases of the cerebellum can readily be grouped into three main neuropathological types: cerebellar cortical atrophy (lesion confined to the Purkinje cells and the inferior olives), pontocerebellar atrophy (lesion affecting several cerebellar and brain structures), and Friedreich ataxia (lesion affecting the posterior column of the spinal cord, peripheral nerves, and the heart). However, several other diseases of the cerebellum and its connections cannot be situated in one of these categories such as dentatorubral degeneration, in which the most conspicuous lesions are in the dentate and red nuclei, and Machado-Joseph disease, in which degeneration involves the lower and upper motor neurons, the substantia nigra, and the dentate system.

Among the neurodegenerative diseases that predominantly affect the spinal cord are ALS and spinal muscular atrophy, in which the most severe lesions are found in the anterior part of the spinal cord, and the already cited Friedreich ataxia, in which the most severe lesions are found in the posterior part of the spinal cord. Finally, there is one group of neurological diseases that are often, but not always, considered neurodegenerative because of their chronic course and unknown etiopathogenesis but that, unlike those described above, show no apparent structural abnormalities. These include torsion dystonia, Tourette syndrome, essential tremor, and schizophrenia. Various brain-imaging studies and electrophysiological investigations have revealed significant functional abnormalities in all of these singular neurodegenerative disorders but have not yet enabled us to unravel their chemical neuroanatomical substrates.

Over the past two decades, significant advances in neurohistological techniques such as immunohistochemistry have replaced or supplemented many of the classical histological approaches. Unquestionably, these new techniques have improved the sensitivity and specificity of neuropathological diagnostic criteria and consequently the accuracy of classification of neu-

rodegenerative disorders. However, despite the fact that these new methods can now determine the presence of particular inclusions or deposits or the degree of gliosis in specific brain areas, none of the already refined classifications of neurodegenerative diseases is entirely satisfactory. On the other hand, the incorporation of state-of-the-art basic science techniques such as gene array, PCR, Western blot, and laser-guided microdissection into our arsenal of neuropathological diagnostic tools should provide, in the near future, new clues as to how to effectively classify neurodegenerative diseases. Based on the use of some of these novel technologies, a new school of thought favors classification according not to the diseases' neuropathological hallmarks, but rather to their molecular characteristics. In this novel approach, neuropathological entities that used to belong to very distinct categories are lumped together because of a common molecular defect. For example, HD, spinal cerebellar atrophy and myotonic dystrophy fall into the category of the trinucleotide-repeat diseases (7); Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, and fetal familial insomnia fall into the category of the prion diseases (8); PD, progressive supranuclear palsy, and diffuse Lewy body dementia fall into the category of the synucleinopathies (9); and corticobasal degeneration, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), and Pick disease fall into the category of the tauopathies (10). Although the jury is still out on whether this new classification will alleviate the problems previously encountered, we believe that it promises to be less ambiguous and more clinically and therapeutically practical.

What causes neurodegeneration?

With few exceptions, the causes of neurodegenerative diseases are essentially unknown, and even when they have been identified, the mechanisms by which they initiate the disease remain, at best, speculative. For example, while the etiology of HD was identified more than two decades ago, we still do not know with certainty how mutant huntingtin provokes the disease.

One of the most ferocious debates surrounding the etiology of neurodegenerative disorders concerns the relative roles of genetic and environmental factors in the initiation of these diseases. Some neurodegenerative disorders have a clear familial occurrence, suggesting a genetic basis. Among these affected families, the disease runs as an autosomal dominant trait, as in HD and dentatorubral pallidoluysian atrophy. Less frequently, the disease runs as an autosomal recessive trait (e.g., familial spastic paraparesis), an X-linked trait (e.g., spinal and bulbar muscular atrophy), or even a maternally inherited trait (e.g., mitochondrial Leber optic neuropathy). In addition to these "pure" genetic neurodegenerative diseases, others are essentially sporadic but show a small contingent of patients in whom the illness is inherited. This is true for PD, AD, and even ALS, of which about 10% of all cases are unequivocally familial. Although rare, these familial cases represent powerful resources to elucidate the molecular bases and, more importantly, the neurodegeneration mechanisms of their respective sporadic variants.

For those in whom the disease is truly sporadic, which is the vast majority of patients, it appears that any genetic contribution to the neurodegenerative process is minimal (11). Instead, toxic environmental factors may be the prime suspects in initiating neurodegenerative processes. Supporting this view is the observation that some neurodegenerative conditions arise in geographic or temporal clusters. This is the case for the PD-ALS complex, which is, presumably, due to a toxic compound contained in *Cycas circinalis*, an indigenous plant commonly ingested as a food or medicine by the Chamorros of Guam (12). Intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a by-product of the synthesis of a meperidine compound, is also known to produce a severe and irreversible parkinsonian syndrome, which is almost identical to PD (13). While many other examples of toxic exposure–related neurological conditions exist, all occur within a specific geographic, social, or professional context, which is missing from the medical history of most patients suffering from a neurodegenerative disorder. Moreover, several large-scale epidemiological studies have failed to show any definitive association between environmental factors and occurrence of diseases such as PD (14). Collectively, these findings argue that sporadic cases are neither clearly genetic nor clearly environmental, but that, possibly, they result from a combination of genetic and environmental causes. In this vein, the demonstration of a nonsyndromic familial deafness linked to a mitochondrial point mutation (15) provides a compelling argument. In this study, family members who harbored the mutation developed a hearing impairment only if exposed to the antibiotic aminoglycoside, illustrating the significant pathogenic interactions between genetics and the environment. The possibility that such dual mechanisms represent a valuable pathogenic scenario underlying sporadic neurodegeneration warrants serious consideration.

Cell demise in neurodegeneration

As mentioned above, only in a very small group of so-called neurodegenerative conditions are no apparent neuropathological changes found. In all others, overt neuropathology, mainly in the form of a focal loss of neurons with reactive gliosis, is seen. Residual neurons may exhibit varying morphologies ranging from an almost normal appearance to a severe distortion with a combination of abnormal features such as process attrition, shape and size alterations of the cell body and nucleus, organelle fragmentation, dispersion of Nissl bodies, cytoplasmic vacuolization, and chromatin condensation. In several neurodegenerative disorders, spared neurons can also present with various types of intracellular proteinaceous inclusions, which, in the absence of any definite known pathogenic role, are quite useful in differentiating neurodegenerative disorders. This is particularly clear in the case of the variants of PD, which can be stratified based on the presence or the absence of the intraneuronal inclusions called Lewy bodies (16).

The diversity of cell death morphology in neurodegeneration is often neglected, and many authors still consider only two types, apoptosis and necrosis. The former is universally recognized to be active in the sense of being mediated by intracellular signaling pathways, and the latter is traditionally considered passive. There is increasing evidence that this dichotomy is too rigid, especially for neurons, and readers interested in this debate are encouraged to consult the comprehensive review written by Peter Clarke (17). In brief, there is mounting evidence that the mode of cell death in the nervous system, as it can be defined by morphological features, is much more diverse than initially thought (18, 19). At least four main types of distinct neuronal death have been defined: apoptotic, necrotic, autophagic, and cytoplasmic; the detailed morphological description of each of these goes beyond the scope of this article but can be found in ref. 18. The main reason why attention to these different forms of cell death may be clinically important is that several of the distinct forms of cell demise are controlled by distinct molecular mechanisms (20–23). Another widespread misconception relating to the mode of cell death is the belief that only necrosis elicits inflammation. While the inflammatory reaction is indeed generally stronger in regions of necrosis than, for example, in regions of apoptosis, this may simply reflect the greater number of cells dying in necrotic regions. Moreover, those who claim that non-necrotic forms of cell death such as apoptosis do not elicit inflammation are referring to "exudative" inflammation (24), whose hallmarks include the infiltration of the diseased tissue by blood-borne cells including neutrophils and monocytes. Yet, in the CNS and especially in neurodegeneration, even for necrosis the inflammatory response is largely local, meaning that it is mainly made of resident microglia and astrocytes as seen in apoptosis. It is thus fair to conclude that while the intensity of the glial reaction may vary among the forms of cell death detailed in ref. 17, the occurrence of gliosis is not a hallmark of necrosis only.

With the few exceptions indicated above, neurodegenerative disorders have in common that they affect specific subpopulations of neurons at the level of specific structures of the nervous system.

In some neurodegenerative diseases, such as olivopontocerebellar atrophy, multiple brain structures within the nervous system are affected, usually at separate sites, while nearby and often intermingled neuronal subtypes are spared. In these so-called system neurodegenerative diseases, the spatial pattern of the lesions often becomes better defined as the disease progresses. It is clear that the distribution of blood vessels is not essentially responsible for determining the spatial pattern of the lesions. On the other hand, in many system neurodegenerative diseases, as is emphasized in ref. 25, the different lesions appear to be functionally and anatomically interconnected. Such a "linked" degeneration is observed in ALS, in which both the corticospinal track and the spinal cord lower motor neurons are affected, and in progressive supranuclear palsy, in which both the globus pallidus and the subthalamic nucleus are lesioned. Although such transneuronal degeneration is a well-recognized phenomenon (26), very little is known about its molecular basis except that this trans-synaptic demise seems to occur by programmed cell death (27, 28). As is emphasized by Oppenheimer and Esiri (25), transneuronal degeneration does not account for all of the combinations of lesions that are found in system neurodegenerative diseases. For example, the authors point out that in Friedreich ataxia there is degeneration of the spino cerebellar tracts and the dentate nuclei, but not of the Purkinje cells, which, supposedly, constitute the link between these two lesions.

Not in all neurodegenerative disorders are large numbers of nervous system structures at risk. Indeed, in several neurodegenerative diseases the lesions appear to be restricted to one or a few brain regions, especially at the beginning of the disease. This is particularly well illustrated in spinal muscular atrophy, in which the degenerative process is limited to a loss of lower motor neurons; and in ALS, in which damage to the upper and lower motor neurons may represent the sole neuropathological change at the beginning of the disease, and other areas, including the substantia nigra, may become affected later (29). Still, the initial site of neuropathology remains the most severely affected throughout the disease, forming a sort of neurodegenerative gradient. In diseases like striatonigral degeneration, the neurodegenerative process is quite symmetrical from the onset, while in others like PD, one side of the brain is usually more severely affected than the other. This is clearly noticeable clinically and is demonstrable by brain-imaging techniques (30).

The locations of the principal lesions have been well established in most if not all known neurodegenerative disorders, but it remains difficult to determine the extent of degeneration that affects more than one group of neurons and, consequently, to define the exact neuropathological topography of certain diseases. This problem stems from at least three issues. First, lesions are often missed through incomplete examination of the brain and spinal cord. Second, quantitative morphology in postmortem samples seldom uses the rigorous counting methods necessary to generate reliable neuron numbers (31). Third, sick neurons, which will not necessarily die, often lose the phenotypic markers used to identify and count them (32). For these reasons, reported estimations regarding the distribution and the magnitude of neuronal loss in neurodegenerative disorders may, with perhaps a few exceptions, have to be taken with a grain of salt.

Onset and progressive course of the disease

Most patients suffering from a neurodegenerative disorder know approximately when their symptoms began. Because, almost invariably, there is significant cellular redundancy in neuronal pathways, the onset of symptoms does not equate with the onset of the disease. Instead, the beginning of symptoms corresponds to a neurodegenerative stage at which the number of residual neurons in a given pathway falls below the

number required to maintain normal functioning of the affected pathway. This means that the onset of the disease occurs at some earlier time, which, depending on how fast the neurodegenerative process evolves, can range from a few months to several years. In most instances, the lack of presymptomatic markers and of knowledge about the true kinetics of cell demise precludes our ability to determine disease onset.

It is also interesting to consider why a sudden worsening of a patient's condition is sometimes observed. Although we cannot exclude that the neurodegenerative process may suddenly accelerate, especially under the influence of intercurrent deleterious factors such as infection, it is more likely that the rate of neuronal death remains about the same throughout the natural course of the disease. Yet the relationship between the clinical expression of a disease and the number of residual neurons does not have to be linear or even constant. So a patient may remain clinically unchanged during a prolonged period of time, despite a loss of many cells, and then abruptly deteriorate as the number of neurons drops below a functional threshold.

All neurodegenerative disorders progress slowly over time, often taking several years to reach the end stage. Does this observation indicate that sick neurons succumb to the disease only after a protracted agony? It must be remembered that neuronal degeneration corresponds to an asynchronous death, in that cells within a population die at very different times. As a corollary at any given time, only a small number of cells are actually dying; among these, many, if not all, are at various stages along the cell death pathway. However, standard clinical, radiological, and biochemical measurements, which are so critical to assessing the disease, generate information on the entire population of cells. Therefore, the rate of change in any of the usual clinical parameters essentially reflects the decay of the entire population of affected cells and provides very little insight into the pace at which the death of an individual cell occurs. Still, if one looks at the large body of in vitro data, it appears that, once a cell gets sick, the entire process of death proceeds rapidly. Given these facts, the protracted clinical progression may reflect a small number of neurons dying rapidly at any given point in time.

Fatality in neurodegenerative disorders

Neurodegeneration is thought to shorten the life expectancy of affected patients. If this concept is unfortunately true in many instances, it should be emphasized that not all "mortal" neurodegenerative disorders are fatal per se. Only those in which the affected neurological structures impair ability to control or execute such vital functions as respiration, heart rate, or blood pressure are unquestionably deadly. These include ALS, in which the loss of lower motor neurons innervating respiratory muscles leads the patient to succumb to respiratory failure. Alternatively, in diseases like Friedreich ataxia, the association of neurodegeneration with heart disease (33) can also cause the death of the patient, although, in this case, death is due not to any neuronal loss but instead to serious cardiac problems such as congestive heart failure. In most other neurodegenerative disorders, death is attributed neither to the disease of the nervous system nor to associated extra-nervous system degeneration but rather to the resulting motor and cognitive impairments that increase the risk of fatal accidental falling, aspiration pneumonia, pressure skin ulcers, malnutrition, and dehydration. Also, to our knowledge, there is no evidence that neurodegeneration increases the odds of developing comorbidity, such as with cancer, stroke, or heart attack, which remain the leading causes of death in industrialized countries. In conclusion, while a few specific neurodegenerative disorders directly cause death, most instead facilitate the occurrence of secondary health problems that carry a high mortality rate. Although this distinction may seem a matter of semantics, we believe that it is significant, not only for the management of patients, but also for our understanding of the actual consequences of the neurodegenerative process.

Neurodegeneration and aging

Many elderly individuals exhibit mild motor and cognitive alterations reminiscent of those found in neurodegeneration. This observation gave birth to the popular idea that aging might be a "benign" form of neurodegeneration. This idea was supported by the notion, widely accepted until recently, that normal aging, like neurodegeneration, is inevitably associated with neuronal death. From as early as the 1950s, decreased numbers of neurons in different regions of the brain were reported in aged humans with no overt neurological or psychiatric conditions (34). Subsequent studies have estimated these losses to be as high as 100,000 neurons per day, which could easily explain the cognitive decline and decrease in brain weight traditionally associated with normal aging (34). However, with the development of more accurate procedures for counting neurons, this view has been modified over the last several years, particularly as stereological procedures for estimating neuron numbers have been applied to aging research (35). As reviewed by Morrison and Hof, the application of stereological techniques has shown in several species, including humans, that the age-related decline in neuron number via neuronal death is not significantly involved in normal aging, at least with respect to the neocortex and to the hippocampal subregions most implicated in memory, such as entorhinal cortex and CA1 (35). These results, therefore, challenge the notion that neurodegeneration invariably occurs in normal aging.

If significant neuronal loss is lacking, some other pathological features of neurodegeneration, such as the presence of Lewy bodies, so typical of PD, and neurofibrillary tangles (NFTs) and senile plaques, so typical of AD, can be detected in brains of asymptomatic aged individuals (36, 37). The critical question thus becomes: Do these changes occur "normally" during aging or reflect a "presymptomatic" stage of these diseases? Because it is impossible to perform longitudinal neuropathological studies, it is impossible to deter-

mine whether these individuals would have developed full disease expression if they had lived longer. In fact, hitherto, there been no definitive evidence supporting such a progression (35). Instead, neuropathological and functional brain-imaging studies have revealed striking quantitative and qualitative differences between aged nondemented and demented individuals (35, 38), suggesting that aging and neurodegeneration may represent very distinct entities. For instance, in the nondemented elderly, no NFTs are observed in the frontal and temporal cortices and only a few NFTs are found in the entorhinal cortex and the hippocampal CA1 subregion, even in the absence of any neuronal loss (35). Conversely, in the demented elderly, even with the mildest cognitive impairments, some NFTs are observed in the frontal and temporal cortices and high densities of NFTs are found in the entorhinal cortex and the hippocampal CA1 subregion together with significant neuronal loss (35).

Therefore, the definition of normal aging is critical to any conclusion about the effect of the passage of time on the brain. Despite intense clinical-neuropathological correlative investigations, to date, experts remain unsure about whether the fact that a change is commonplace makes it normal and, conversely, whether changes, however slight, that are known to be associated with definite diseases of the nervous system are necessarily pathological. It is still difficult to know exactly to what extent neurons are damaged or lost in aged humans as a result exclusively of the passage of time. It seems clear, however, that the impact of the passage of time on the number of neurons is much less important than was previously believed, and that compelling evidence is lacking to support the idea that aging is a form of neurodegeneration *at minima*.

Conclusion

Current classifications of neurodegenerative diseases are based on a clinicopathological approach, i.e., defined by a presentation of symptoms and signs linked to neuropathological findings. Without undermining the usefulness of the clinicopathological approach for diagnosis and treatment in current neurological practice, this approach to classification should be reassessed. As indicated earlier in this review, it would probably be more meaningful to classify neurodegenerative diseases by their molecular characteristics, redefining the diseases as the consequence of biochemical processes, some of which may operate in more than one disease. By doing so, we may reveal pathogenic mechanisms that are common to some of these diseases, and open new therapeutic avenues that may be effective in several unrelated neurodegenerative diseases.

In this introductory review, we have tried to provide an overview of the complexity of the field of neurodegeneration, as well as to lay the groundwork for the upcoming set of articles that will complete this Perspective series.

As we have mentioned, HD has received at great deal of attention in the field of neuroscience, as it is a prototypic model of a genetic neurodegenerative disease. While it is well established that a triplet-repeat CAG expansion mutation in the huntingtin gene on chromosome 4 is responsible for HD, Anne B. Young (39) will bring us on the chaotic trail of research that aims to define the normal functioning of this newly identified protein, as well as to elucidate the intimate mechanism by which the mutant huntingtin kills neurons. Although much remains to be done, this article provides us with an update on the most salient advances made in the past decade in the field of HD, suggests pathological scenarios as to how mutant huntingtin may lead to HD, and, most importantly, discusses the many steps in the process of functional decline and cell death that might be targeted by new neuroprotective therapies (39).

While HD is by nature a genetic condition, PD is only in rare instances an inherited disease. Despite this scarcity, many experts in the field of neurodegeneration share the belief that these rare genetic forms of PD represent unique tools to unravel the molecular mechanisms of neurodegeneration in the sporadic form of PD, which accounts for more than 90% of all cases. Accordingly, Ted Dawson and Valina Dawson review, in their Perspective, the different genetic forms of PD identified to date (40). They then summarize the current knowledge on the normal biology of two proteins, a-synuclein and parkin, whose mutations have been linked to familial PD (40). The authors also discuss how these different proteins may interact with each other and how, in response to the known PD-causing mutations, they may trigger the neurodegenerative processes (40).

The recognition that many neurodegenerative diseases are associated with some sort of intra- or extracellular proteinaceous aggregates has sparked major interest in the idea that these amorphous deposits may play a pathogenic role in the demise of specific subsets of neurons in various brain diseases. Along this line, what could be a better example of "proteinopathic" neurodegenerative disease than AD, which features NFTs and senile plaques? In this context, Todd Golde (41) reviews the presumed role of amyloid β protein (Aβ) in the initiation of AD and outlines the molecular scenario by which Aβ may activate the deleterious cascade of events ultimately responsible for dementia and cell death in AD. In light of this information the author discusses the different therapeutic approaches that may be envisioned for AD (41). He also summarizes the state of our knowledge about risk factors and biomarkers for AD that can be used to detect individuals at risk for developing the disease, and to follow its progression once it has developed (41).

Interestingly, in another dreadful neurodegenerative disease, ALS, of which about 2% of the cases are related to a mutation affecting the enzyme superoxide dismutase-1 (SOD1), the presence of abnormal protein aggregates has also been hypothesized to participate in the neurodegenerative process. Nevertheless, the evidence supporting such a role in ALS is much more tenuous than in AD, and many appealing alternative pathogenic hypotheses have been put forward to explain the mechanism by which spinal cord motor neurons die in ALS, especially in response to mutant SOD1. For

instance, Guégan and Przedborski, in their article (42), approach the pathogenesis of ALS in general and of familial ALS linked to SOD1 mutations in particular, through the lends of programmed cell death (PCD) (42). In this article, we review the large core of data that pertain to the question of PCD in ALS, covering morphological and molecular findings that support the contribution of this molecularly regulated form of cell death to ALS neurodegeneration (42). We also discuss how particular molecular components of the PCD machinery can be targeted in ALS to develop new neuroprotective strategies for the treatment of this invariably fatal disease (42).

Moving away from disease-specific pathological mechanisms, Eric Schon and Giovanni Manfredi (43) address the topic of mitochondrial defects as a potential generic deleterious mechanism in neurodegeneration. In this article, the authors remind us that while there are well-defined mitochondrial diseases, which express themselves most often as myopathies or encephalopathy or both, mitochondrial defects have been implicated in a dazzling number of neurodegenerative diseases as well (43). To help the reader to better apprehend the difficulty in readily recognizing the signature of a mitochondrial component in neurodegenerative diseases, Schon and Manfredi review key molecular principles that govern mitochondrial biology (43). Based on this information, they then discuss in depth the fundamental issues of how the proposed mitochondrial alterations in neurodegenerative diseases may arise and whether these defects are the cause or the consequence of the neurodegenerative processes (43).

To continue with generic cytotoxic mechanisms, Harry Ischiropoulos and Joseph S. Beckman review, in their Perspective (44), the many lines of evidence supporting a role of oxidative and nitrative processes in the pathogenesis of numerous neurodegenerative diseases. These authors review the various cellular pathways that may be at the origin of the oxidative stress in neurodegeneration (44). They remind us that the most recent data have also identified nitric oxide-derived reactive nitrogen intermediates as critical contributors of protein modification and cell injury, and that consideration should be also given to inappropriate regulation of iron and other divalent redox metals, such as copper, as well as to redox-inactive zinc (44). Ischiropoulos and Beckman conclude by taking the stance that oxidative processes are critical in the pathogenic mechanisms of neurodegenerative diseases, and that promising therapeutic interventions geared toward mitigating oxidative processes may represent valuable therapeutic avenues for both acute and chronic neurodegeneration (44).

As this brief summary shows, each article in this Perspective series will discuss in depth a selected aspect of neurodegeneration. While each will focus on a very different topic, all will share a common theme: the neurobiology of neurodegeneration, and translational research that, in our opinion, represents the most effective way to bring basic science discoveries to clinical trials.

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Free Radical and Nitric Oxide Toxicity in Parkinson's Disease

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Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal features include tremor, slowness of movement, rigidity, and postural instability (1). Epidemiological data indicate that currently about one million individuals are affected with PD in North America alone and that about 50,000 new cases arise every year (1). Pathologically, PD is characterized primarily by a dramatic degeneration of the nigrostriatal pathway (2). The latter is formed by dopamine-producing neurons whose cell bodies, located in the substantia nigra pars compacta (SNpc), project their axons all the way up to the basal ganglia, where they release dopamine, thereby ensuring dopaminergic neurotransmission. As part of the neurodegeneration of the nigrostriatal pathway, both cell bodies and to an even greater extent striatal nerve terminals degenerate (2). Aside from these prominent features, other aspects of the pathology of PD include the presence of intraneuronal proteinaceous inclusions called Lewy bodies (3). Despite the large body of knowledge about PD, why and how nigrostriatal dopaminergic neurons die in this disease remains an enigma.

Over the years, several pathogenic hypotheses have been proposed in attempts to explain the mechanisms of neuronal loss in PD. Among these, the lion's share has been given to the oxidative stress hypothesis (4), which proposes that the fine-tuned balance between the production and destruction of reactive oxygen species is skewed, resulting in oxidative damage that leads to severe cellular dysfunction and ultimately to cell death. Countless studies have been published in support of this presumed pathogenic scenario (4). Among the plethora of reactive species capable of mediating oxidative damage in PD, mounting evidence points to peroxynitrite as a main culprit (5). Peroxynitrite is a highly reactive, tissue-damaging species that results from the combination of two other reactive species, namely, superoxide and nitric oxide (NO) (Fig. 8.1A). Because of the remarkable reactivity of peroxynitrite, there is very little doubt that it can inflict a variety of oxidative damage such as oxidative modifications of proteins, DNA, and lipids on dopaminergic neurons in the brains of parkinsonian patients.

In this chapter, we summarize the current highlights regarding the potential deleterious role of peroxynitrite in the pathogenesis of PD through the use of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model, and we review the following points. First, what evidence is there that peroxynitrite is produced in the MPTP mouse model of PD? Second, are both superoxide and NO really required in the proposed deleterious scenario? Third, what is the source of the NO that is involved in the presumed deleterious reaction? And, fourth, does peroxynitrite cause detectable damage after the administration of MPTP?

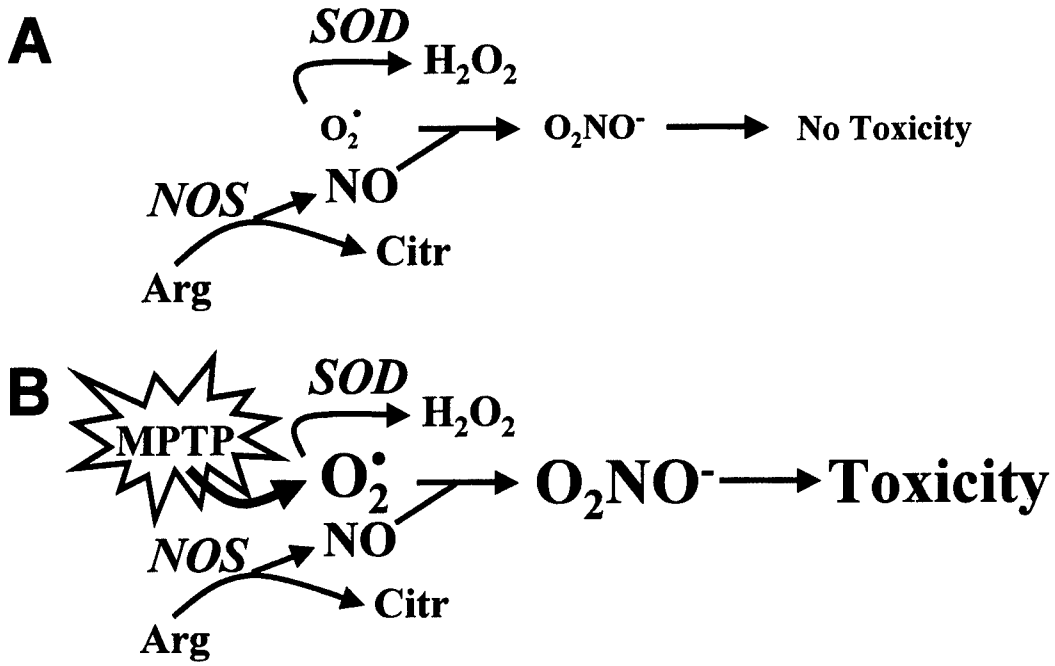

FIGURE 8.1. Superoxide radical ($O_2^{\bullet}$) reacts with nitric oxide (NO) to produce peroxynitrite (O_2NO^-). In normal situations **(A)**, superoxide dismutase detoxifies most of the produced superoxide radicals. On the other hand, NO, which is produced by the oxidation of L-arginine into L-citrulline, is present in high amounts. Because of this, little peroxynitrite is formed, and thus, minimal toxicity is attributable to this reaction. After methylphenyltetrahydropyridine (MPTP) administration **(B)**, the level of $O_2^{\bullet}$ increases dramatically and, thus, more peroxynitrite is produced and significant cytotoxicity now occurs.

MPTP MOUSE MODEL OF PARKINSON'S DISEASE

As a preamble of our discussion, it is worth providing a brief review of the MPTP model (6). That MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California were rushed to the emergency room with a severe bradykinetic and rigid syndrome (7). Subsequently, it was discovered that this syndrome was induced by the self-administration of street batches of a synthetic meperidine analog, whose synthesis was heavily contaminated by a byproduct, MPTP (8). In the period of a few days after the administration of MPTP, these patients exhibited a severe and irreversible akinetic rigid syndrome akin to PD, and levodopa was tried with great success, relieving the symptoms of these patients. Since the discovery that MPTP causes parkinsonism in human and nonhuman primates, as well as in various other mammalian species, this neurotoxin has been used extensively as a model of PD (6,9,10). For a technical review of MPTP utility and safety, please see Przedborski et al. (11).

In human and nonhuman primates, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the hallmarks of PD, including tremor, rigidity, slowness of movement, postural instability, and even gait freezing. The responses and the complications to traditional antiparkinsonian therapies are virtually identical to those seen in PD. However, although it is believed that the neurodegenerative process in PD occurs over several years, the most active phase of neuronal death after MPTP adminis-

tration is presumably completed over a short period of time, producing a clinical condition consistent with "endstage PD" in a few days. Still, brain imaging and neuropathological data suggest that after the acute phase of neuronal death, nigrostriatal dopaminergic neurons continue to succumb at a much lower rate for many years after MPTP exposure (12,13). From a neuropathological standpoint, MPTP administration causes damage to the dopaminergic pathways, which is identical to that seen in PD with a resemblance that goes beyond the degeneration of nigrostriatal dopaminergic neurons. For instance, as in PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area (14,15) and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus, at least in monkeys treated with low-dose MPTP (16), but apparently not in acutely intoxicated humans (17). On the other hand, two typical neuropathological features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, other pigmented nuclei such as the locus ceruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions, or Lewy bodies, so characteristic of PD, have thus far not been convincingly observed in MPTP-induced parkinsonism (18). Despite this impressive resemblance between PD and the MPTP model, MPTP has never been recovered from postmortem brain samples or body fluids of parkinsonian patients. Altogether, these findings are consistent with MPTP not causing PD, but being an excellent experimental model of PD. Accordingly, it can be speculated that elucidating the molecular mechanisms of MPTP should lead to important insights into the pathogenesis and treatment of PD.

IS PEROXYNITRITE PRODUCED IN THE MPTP MOUSE MODEL?

Together with its high reactivity, peroxynitrite is known to be very unstable and therefore to have a very short half-life. Consequently, it is virtually impossible to measure the actual content of peroxynitrite in biological samples collected and processed for laboratory measurements. To circumvent this problem, several investigators have exploited the fact that peroxynitrite can induce irreversible amino acid modifications such as the nitration of phenolic groups found in tyrosine residues (19). This example of modification, called tyrosine nitration, can affect tyrosine residues irrespective as to whether they are free or contained within proteins and is regarded as a faithful fingerprint of peroxynitrite involvement in a given pathological process. Over the years, several methods, both chromatographic and immuno-based, have been developed to measure nitrotyrosine content, thanks to the availability of specific antibodies raised against nitrotyrosine (20). To date, there is some evidence that nitrotyrosine formation increases in the brains of parkinsonian patients, particularly wherever Lewy bodies are found (21–23). To examine the question of nitrotyrosine formation in the demise of nigrostriatal dopaminergic neurons in a more dynamic fashion, the MPTP model of PD provides an invaluable tool. High-performance liquid chromatographic (HPLC) studies have shown that nitrotyrosine is increased in selected brain regions after MPTP administration to mice (24). Because of the recent concern regarding the specificity of the molecule detected by HPLC (25), we have used gas chromatography with mass spectroscopy, a method that combines chromatographic ability with the capacity to confirm with certainty the nature of the detected peak (26). Based on this method, we have demonstrated that 24 hours after the last injection of MPTP to mice, the level of nitrotyrosine increases dramatically in the ventral midbrain, the brain region that contains the SNpc, as well as in the striatum (Fig. 8.2) (26). In contrast, at the same time points and in the same animals, we showed that brain regions known *not* to be affected by MPTP (i.e., the cerebellum and the frontal cortex) failed to show any change in the levels of nitrotyrosine (Fig. 8.2) (26). Our data provide compelling evidence

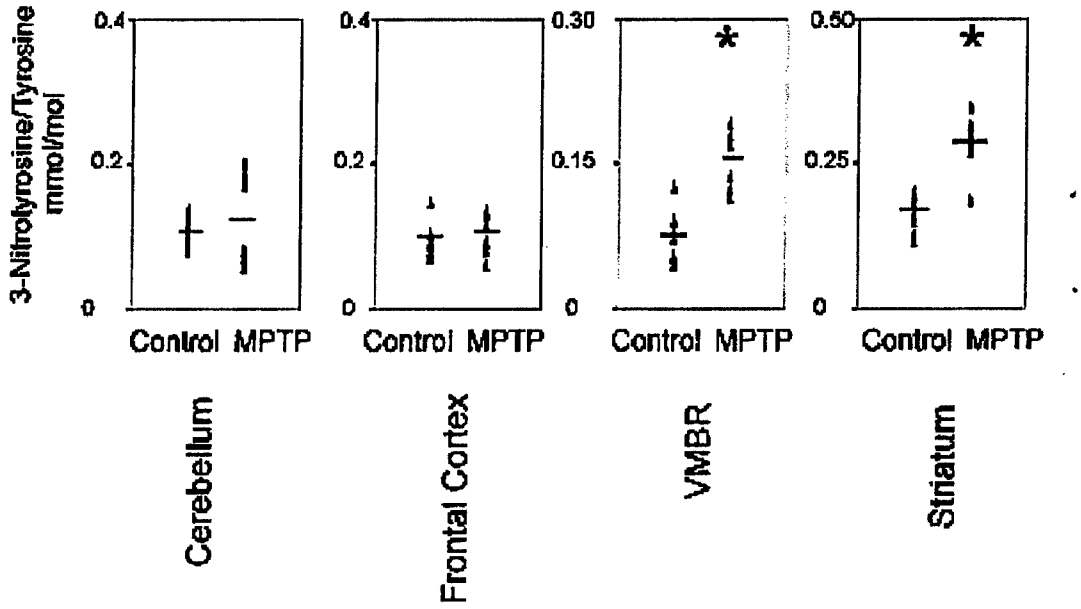

FIGURE 8.2. Gas chromatographic/mass spectroscopic quantification of nitrotyrosine 24 hours after methylphenyltetrahydropyridine (MPTP) administration in cerebellum, frontal cerebral cortex, ventral midbrain (VMBR), and striatum. (*Significantly higher than controls [$p < .05$, Student's t test].) (From Pennathur S, Jackson-Lewis V, Przedborski S, et al. Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o,o'-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–treated mice, a model of oxidative stress in Parkinson's disease. *J Biol Chem* 1999;274:34621–34628, with permission.)

that MPTP does increase nitrotyrosine formation and that these alterations are a reflection of a pathological process specific to MPTP (26). It can also be concluded that these findings strongly support the notion that peroxynitrite is involved in the MPTP-related cascade of deleterious events and possibly in the pathogenesis of PD.

ARE BOTH SUPEROXIDE AND NITRIC OXIDE REQUIRED?

Before embarking on this question, specifically in the MPTP model of PD, it is necessary to remind the reader that peroxynitrite, as stated before, results from the interaction between superoxide and NO (27). In a normal situation, superoxide is constantly produced in a large number of biological reactions within our cells, and its intracellular concentration is maintained at extremely low levels by an abundance of the enzyme superoxide dismutase (SOD), which destroys the superoxide radical (Fig. 8.1A). Conversely, NO is present in abundance, both within cells and in the extracellular space surrounding these cells, due to the production of this reactive species by several isoforms of the NO synthase (NOS) enzyme (Fig. 8.1A). Therefore, because in normal situations the level of superoxide is low, the basal level of peroxynitrite is also low, as is the level of oxidative damage inflicted by peroxynitrite (Fig. 8.1A.). In contrast, in PD as modeled by the MPTP neurotoxin, the level of superoxide increases significantly (Fig. 8.1B), presumably via the blockade of mitochondrial respiration and/or other mechanisms (28). Consequently, the intracellular concentration of superoxide now rises, as does the formation of peroxynitrite and the resulting magnitude of oxidative stress and cytotoxicity (Fig. 8.1B).

In light of this, one might ask whether it is true that superoxide is a rate-limiting factor in

this reaction. To address this important question, we have used transgenic mice that express two to three times more SOD in the brain (29) with the prospect that by increasing superoxide detoxification, less superoxide will be available to react with NO, thus less peroxynitrite will be formed and less toxicity will occur (Fig. 8.3A). The results of our study show that MPTP administration causes significant damage to the nigrostriatal pathway in non-transgenic littermates with normal activity of SOD in the brain (30). In contrast, in transgenic animals with increased activity of SOD in the brain, a similar regimen of MPTP causes only minimal damage to the dopaminergic neurons (30). Similar results were observed in transgenic mice expressing manganese SOD, another SOD isoform (31). These findings lead us to conclude that as predicted (Fig. 8.3A), adjusting the amount of superoxide radicals in the brain appears to be a key component in the MPTP neurotoxic process.

The second question regarding MPTP toxic biochemistry is whether there is also a need for NO (Fig. 8.3B). To address this second crucial question, several investigators, including ourselves, have modulated the amount of NO available for this reaction by targeting NOS, the enzyme responsible for NO formation. The use of different NOS antagonists (32–34) has convincingly demonstrated that

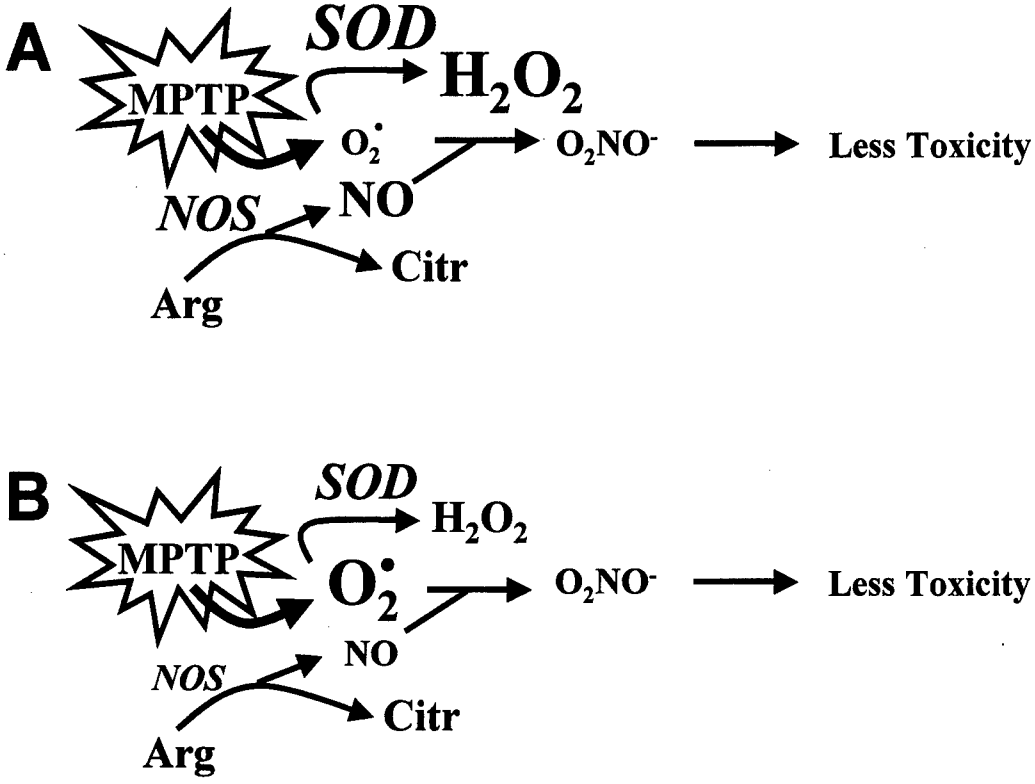

FIGURE 8.3. Effects of genetic interventions on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-related production of peroxynitrite (O_2NO^-) and cytotoxicity. By increasing superoxide dismutase *(SOD)* activity **(A)**, MPTP-related superoxide radical ($O_2^{\bullet}$) production is reduced. Thus, less superoxide radicals are available to react with nitric oxide *(NO)*; consequently, less peroxynitrite is produced, and less cytotoxicity occurs. By blocking nitric oxide synthase *(NOS)* **(B)**, the formation of NO is reduced. Thus, less NO is available to react with superoxide radicals, and consequently, less peroxynitrite is produced and less cytotoxicity occurs.

blockade of NOS, which reduces the production of NO, attenuates significantly MPTP-induced neurotoxicity. Collectively, all of these studies indicate that, as hypothesized, both superoxide and NO are necessary to the deleterious biochemical reaction involved in the MPTP-mediated demise of the nigrostriatal dopaminergic pathway (Fig. 8.3).

WHAT IS THE SOURCE OF NITRIC OXIDE INVOLVED IN MPTP NEUROTOXICITY?

Thus far, three different isoforms of NOS have been cloned and characterized (5). These include neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). All of these isoforms are present in the brain, though in variable abundance. The most abundant isoform nNOS is expressed in several neuronal subtypes, but interestingly, nNOS has not been identified in dopaminergic neurons of the nigrostriatal pathway. Yet, dopaminergic neurons of the nigrostriatal pathway are surrounded by an abundant network of neuronal cell bodies and fibers that contain nNOS (35), suggesting that any NO that will be used by dopaminergic neurons in MPTP neurotoxicity or in the pathogenesis of PD will have to originate from neighboring neurons. In contrast to nNOS, iNOS is normally not expressed in the brain; however, in pathological situations, particularly those associated with gliosis, iNOS can be induced. Consistent with this notion, in the case of PD and in the MPTP model, it has been demonstrated by immunohistochemical methods that glial cells at the level of the SNpc exhibit a robust expression of iNOS (36,37). As for eNOS in the brain, only very discrete populations of neurons seem to express this NOS isoform (38). Otherwise, eNOS is confined to the endothelial cells of blood vessels, which are abundant in all regions of the brain. In regions such as the substantia nigra and the striatum, we found no evidence that eNOS is expressed in neuronal cells (Du Chu Wu and Serge Przedborski, *personal observation, 1999)*. Nevertheless, in these two brain regions, dopaminergic structures entertain a close relationship with blood vessels whose walls exhibit robust eNOS immunoreactivity (Du Chu Wu and Serge Przedborski, *personal observation*). From a pharmacological standpoint, it is important to determine which of these isoforms is responsible for the production of NO in the pathogenesis of PD. Thanks to the development of genetically engineered animals in which the gene for each of the isoforms of NOS has been separately ablated, it became possible to answer this question in a precise fashion. Using mutant mice deficient in nNOS, we were able to demonstrate that ablation of nNOS markedly attenuates MPTP toxicity (32). Indeed, our data on dopamine content in the striatum of these mutant animals show that compared with their wild-type littermates, MPTP inflicts significantly less damage (32). These results indicate that by eliminating nNOS, MPTP neurotoxicity is partially, but not completely, blocked, which suggests that although nNOS plays a significant role in MPTP neurotoxicity, it is probably not the sole isoform of NOS implicated in this process.

Using iNOS knockout mice and their wild-type littermates, both Dehmer et al. (39) and Liberatore et al. (37) showed, first, that not only is there a robust glial reaction after MPTP administration, but also that there is an upregulation of iNOS. More important, these studies demonstrate that the administration of MPTP, through different regimens to iNOS knockout mice and their wild-type littermates, produces significantly less neuronal loss in mutant mice deficient in iNOS compared with their wild-type counterparts (37,39). Again, as for nNOS, toxicity is only attenuated and not prevented in iNOS-deficient mice. As for eNOS, using Western blotting techniques, we have preliminary data showing that the level of expression of eNOS is unaffected by MPTP administration, and more important, that when toxicity to MPTP is assessed in eNOS knockout animals, the ablation of this isoform has no significant impact on the demise of dopaminergic neurons. Collectively, these data suggest that in the

MPTP model, only iNOS and nNOS seem to play a significant role in the neurotoxic process. eNOS, although present, seems not to have a role here. It can also be extrapolated from these data that optimal neuroprotection may be obtained in the MPTP model and possibly in PD, only if both nNOS and iNOS are inhibited.

DOES PEROXYNITRITE CAUSE DETECTABLE DAMAGE AFTER MPTP ADMINISTRATION?

As stated already, peroxynitrite can damage virtually any cellular component, including proteins, lipids, and DNA, as well as dopamine (40). With respect to this chapter, we focus our discussion only on the MPTP-related peroxynitrite effects on proteins. Based on the chromatographic studies mentioned earlier in this chapter, we already know that MPTP causes detectable damage to proteins as a whole, as evidenced by nitrotyrosine levels (Fig. 8.2). This can not only be quantified on Western blot analysis but also visualized. Using this approach, we have demonstrated that after MPTP administration, several proteins with very different molecular masses are indeed nitrated (41). We also show that this phenomenon is time dependent, peaking between 6 and 12 hours after MPTP administration (41). This is not surprising because previously we have reported that MPTP cytotoxicity is also time dependent (42) and that biochemical correlates of MPTP toxicity such as adenosine triphosphate (ATP) depletion can be detected as early as 1 hour after MPTP administration (43). Remarkably, this Western blot study revealed a robust nitration only on some and not all resolved proteins (41), which is an unexpected finding, because virtually all proteins contain at least one tyrosine residue. Therefore, our Western blot data indicate that whereas all proteins *could* potentially be nitrated, only *specific* proteins seem to be nitrated after MPTP administration. To try to identify what specific proteins are nitrated after MPTP administration, we decided to assess the propensity of specific protein candidates to become nitrated after MPTP administration. Herein, we illustrate the case of two such protein candidates: tyrosine hydroxylase (TH), which is the rate-limiting enzyme in dopamine synthesis, and α-synuclein, a small presynaptic protein whose mutations have recently been implicated in the development of a familial form of PD. TH contains 16 tyrosine residues in its primary structure, and most of these are found in the vicinity of its catalytic site. It should also be mentioned that although nitrotyrosine has been presented so far in this chapter as a marker of peroxynitrite, nitrotyrosine can be neurotoxic in its own right. For instance, free nitrotyrosine when injected into the striatum has been shown to cause some nigrostriatal damage (44). In addition, because nitrotyrosine adds negative charges into a protein, which could have an impact on this protein's secondary or tertiary structure, nitrotyrosine could also, in the case of an enzyme, affect its catalytic activity. Relevant to this possibility, we have demonstrated that TH becomes heavily nitrated between 3 and 6 hours after the last injection of MPTP (Fig. 8.4) (41). We have also shown that coinciding with its nitration, TH looses its catalytic activity (Fig. 8.4) (41). Similarly, we have demonstrated that after MPTP administration, α-synuclein becomes heavily nitrated 4 hours after the last MPTP dose (Fig. 8.5.) (45). Interestingly, we found that at the same time point and under the same regimen of MPTP, proteins related to α-synuclein such as β-synuclein or synaptophysin are not nitrated (Fig. 8.5) (45). The possible functional implication of the nitration of α-synuclein is that this small presynaptic protein is known to be quite insoluble, and by perturbing its spatial organization, nitration can conceivably reduce α-synuclein's solubility, thus promoting its precipitation and the formation of aggregates. This view, although difficult to test in the MPTP mouse model, found support in a study performed by Giasson et al. (22), who showed that α-synuclein in the parkinsonian brain is indeed nitrated and that the nitrated form of α-synu-

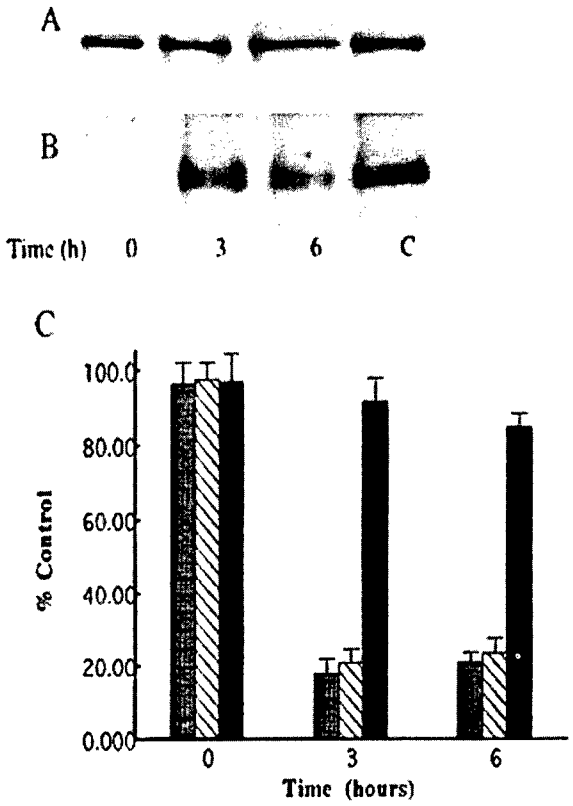

FIGURE 8.4. Inactivation of tyrosine hydroxylase (TH) by tyrosine nitration after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration. At selected times (0-, 3-, and 6-hour postexposure), TH was immunoprecipitated from the striatum of MPTP-injected mice and then visualized using an anti-TH antibody. **A:** Comparable amounts of immunoprecipitated TH were loaded onto the gel. To assess the presence of tyrosine nitration in immunoprecipitated TH, an anti-nitrotyrosine antibody was used. **B:** TH is markedly nitrated at 3 and 6 hours, but not at 0 hour after MPTP administration. As a positive control **(C)**, TH was immunoprecipitated from peroxynitrite-treated PC12 cells. Paralleling the time course of its nitration, TH enzymatic activity (*hatched bars*), and consequently, production of dopamine (*gray bars*) dropped dramatically at 3 and 6 hours post-MPTP administration **(C)**. Conversely, at those selected times, TH protein contents (*black bars*) did not differ significantly from those of healthy controls, indicating that TH is inactivated as a result of nitration. (From Ara J, Przedborski S, Naini AB, et al. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci USA* 1998;95:7659–7663, with permission.)

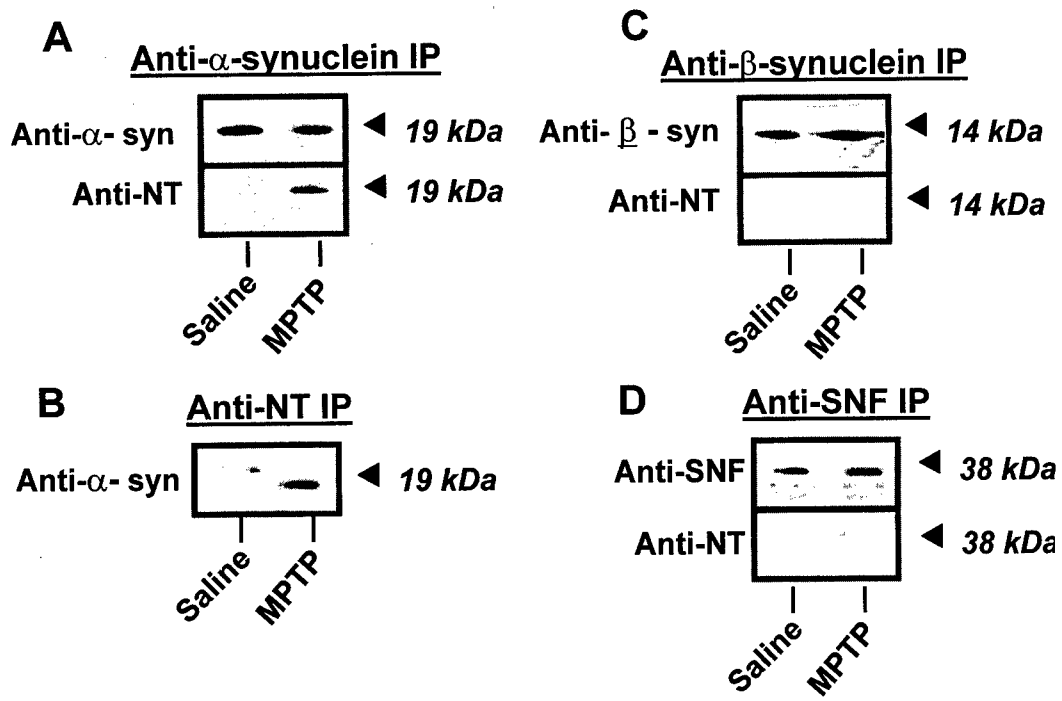

FIGURE 8.5. Tyrosine nitration of striatal α-synuclein, but not of β-synuclein, or synaptophysin, after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection to mice. After MPTP administration (4 hours after the last injection) or vehicle (saline), striatal proteins were immunoprecipitated using anti-synuclein antibody **(A)**; anti-nitrotyrosine **(B)**; anti-β-synuclein **(C)**; or anti-synaptophysin **(D)**. After having been resolved on gels, proteins were immunostained with anti-nitrotyrosine **(A,C,D [lower panels])**, anti-α-syn **(A [top panel] and B)**, anti-β-syn **(C [top panel])**, and anti-synaptophysin **(D [top panel])**. (From Przedborski S, Chen Q, Vila M, et al. Oxidative post-translational modifications of alpha-synuclein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease. *J Neurochem* 2001;76:637–640, with permission.)

clein is preferentially found in dystrophic neurites and Lewy bodies.

CONCLUSIONS

In light of the outlined data, we can propose a pathogenic scenario for PD based on the different steps in the MPTP model (Fig. 8.6). The first step of this scenario relies on the fact that one must agree with the idea that the initiating factor of the deleterious cascade is a molecule that shares similarities with the MPTP active metabolite 1-methyl-4-phenylpyridimium (MPP^+). As such, this putative molecule has to enter dopaminergic neurons via the plasma membrane dopamine transporter (DAT). Once inside dopaminergic neurons, MPP^+ acts on mitochondria, where it blocks mitochondrial respiration. This has two immediate consequences: (a) blockade of the production of ATP, and therefore, an ensuing energy crisis, and (b) an increased production of superoxide. As we indicated already, other mechanisms may also stimulate the production of superoxide after MPTP administration. At the same time, neighboring neurons that contain nNOS produce NO, and later, when gliosis develops, activated glial cells that contain iNOS contribute to the production of NO. NO, known to be quite stable compared to other reactive species, can travel several micrometers away from its site of production. NO, like water, can freely cross the plasma membrane, and thus, after being produced

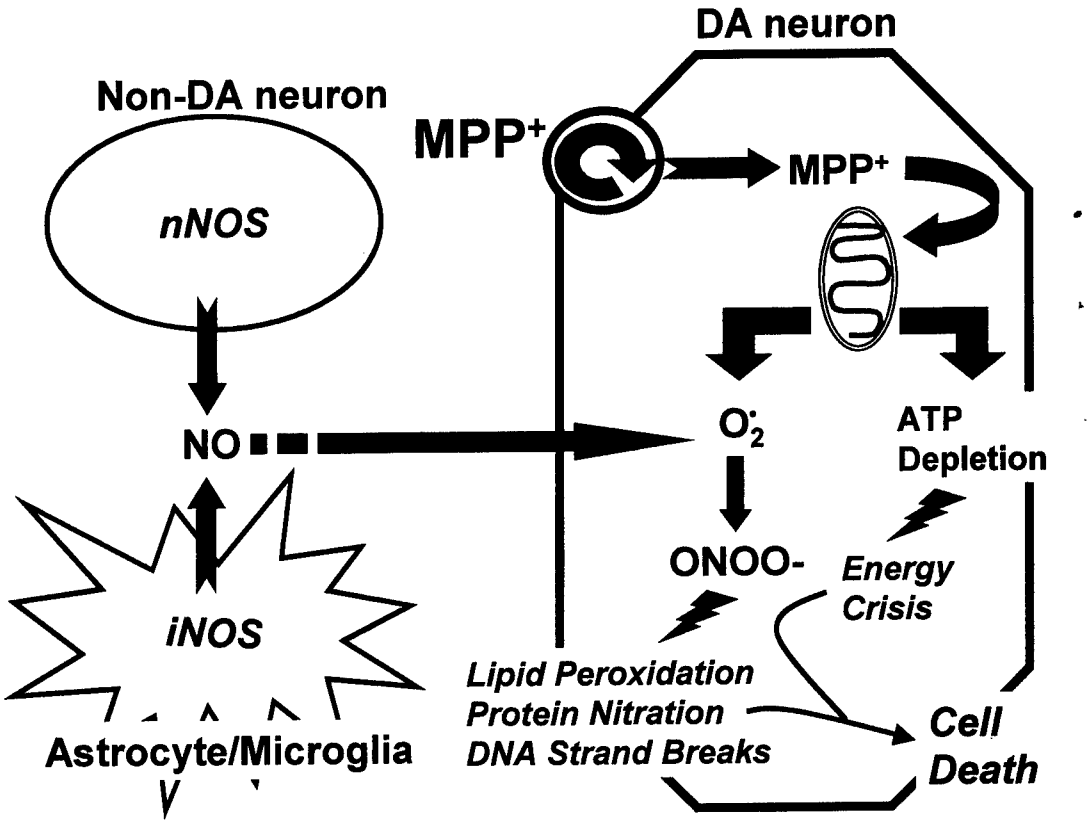

FIGURE 8.6. Proposed scenario of methylphenyltetrahydropyridine neurotoxic process and pathogenesis of Parkinson's disease.

and released extracellularly, NO can easily penetrate dopaminergic neurons (Fig. 8.6). There, NO reacts with superoxide to produce peroxynitrite. Peroxynitrite, in turn, will now inflict severe oxidative damage to lipids, DNA, and proteins such as the nitration of tyrosine residues. Together, oxidative damage and energy crisis lead to cellular dysfunction, which when built up over time can eventually reach a magnitude that is no longer compatible with life, thus neurons die (Fig. 8.6). Although this scenario is primarily relevant to MPTP neurotoxicity, we strongly believe that a similar sequence of events may well underlie the neurodegenerative process in PD.

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Review Article

Nitric Oxide and Reactive Oxygen Species in Parkinson's Disease

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Summary

Parkinson's disease is a neurodegenerative disorder of unknown pathogenesis. Oxidative stress has been proposed as one of several pathogenic hypotheses. Evidence for the participation of oxidative processes in the pathogenesis of Parkinson's disease have been obtained in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model by the use of genetically altered mice. MPTP administration has been shown to increase levels of superoxide both intracellularly, via the inhibition of mitochondrial respiration and other mechanisms and extracellularly, via the activation of NADPH-oxidase in microglia. In addition to superoxide, nitric oxide production by nNOS or by microglial iNOS also contributes to the MPTP neurotoxocity. Mice with endowed defences against superoxide or with deficiency in the nNOS and iNOS are protected from MPTP toxicity suggesting that formation of reactive oxygen and nitrogen intermediates both intracellularly and extracellularly contributes to the demise of dopaminergic neurons. Similar contribution of reactive nitrogen and oxygen species may well underlie the neurodegenerative processes in Parkinson's disease.

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Keywords Neurodegeneration: Parkinson's disease; MPTP; iNOS; ROS; NADPH-oxidase; peroxynitrite.

INTRODUCTION

Parkinson's disease (PD) is a common, neurodegenerative disorder whose cardinal features include tremor, slowness of movement, rigidity and postural instability (*1*). Epidemiological data indicate that, currently, about one million individuals are affected with PD in North America alone, and that about 50,000 new cases arise every year (*1*). Pathologically, PD is characterized primarily by a dramatic degeneration of the nigrostriatal pathway (*2*). The latter is formed by dopamine-producing neurons whose cell bodies, located in the substantia nigra pars compacta (SNpc), project their axons up to the basal ganglia, where they release dopamine, thereby assuring dopaminergic neurotransmission. As part of the neurodegeneration of the nigrostriatal pathway, both cell bodies and, to a greater extent, striatal nerve terminals, degenerate (*2*). Aside from these prominent features, other aspects of the pathology of PD include the presence of intraneuronal proteinacious inclusions called Lewy bodies (*3*). Despite the large body of knowledge about PD, why and how nigrostriatal dopaminergic neurons die in this disease remains an enigma.

Over the years, several pathogenic hypotheses have been proposed in attempts to explain the mechanisms of neuronal loss in PD. Among these putative pathophysiological mechanisms, the oxidative stress hypothesis (*4*) proposes that the balance between the production and destruction of reactive oxygen species is disrupted, permitting oxidants and reactive intermediates to inappropriately oxidize macromolecules resulting in cellular dysfunction and, ultimately, in cell death. To date, countless studies have been published in support of this presumed pathogenic scenario (*4*). Among the plethora of reactive species capable of mediating oxidative damage in PD, mounting evidence points to nitric oxide-derived reactive species such as peroxynitrite, nitrogen dioxide and other unrecognized potential reactive nitrogen species as the main culprits (*5*). The formation of these reactive intermediates requires the oxidation of nitric oxide or stable metabolites of nitric oxide such as nitrite or requires the reaction of nitric oxide with other radical species such as superoxide, for example, to form peroxynitrite. Because of the remarkable reactivity of these nitrogen species, biological molecules such as proteins, DNA and lipids in dopaminergic neurons in the brains of parkinsonian patients could be targeted for oxidation or other modifications, resulting in extensive cellular injury and ultimately in cell death.

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In this chapter, we will summarize the current findings regarding the potential role of nitric oxide-derived reactive nitrogen species in the pathogenesis of PD through the use of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model. The chapter will review the potential [illegible] of nitric oxide as well as superoxide and other reactive oxygen species, the evidence for the production of these reactive nitrogen intermediates in the MPTP mouse model of PD and the potential consequence and biological targets of these deleterious species following the administration of MPTP.

MPTP MOUSE MODEL OF PARKINSON'S DISEASE

As a preamble to this discussion, it is worth providing a brief review of the MPTP model (*6*). The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California were rushed to the emergency room with a severe bradykinetic and rigid syndrome (*7*). Subsequently, it was discovered that this syndrome was induced by the self-administration of a synthetic meperidine analogue whose synthesis was heavily contaminated by a by-product, MPTP (*8*). In the period of a few days following the administration of MPTP, these patients exhibited a severe and irreversible akinetic rigid syndrome akin to PD, and L-DOPA was tried with great success, relieving the symptoms in these patients. Since the discovery that MPTP causes parkinsonism in human and non-human primates as well as in various other mammalian species, this neurotoxin has been used extensively as a model of PD (*6, 9, 10*). For a technical review of MPTP utility and safety, please refer to Ref. (*11*).

In human and non-human primates, MPTP produces an irreversible and severe parkinsonian syndrome that recapitulates almost all of the hallmarks of PD including tremor, rigidity, slowness of movement, postural instability, and even gait freezing. The responses as well as the complications to traditional anti-parkinsonian therapies are virtually identical to those seen in PD. However, while it is believed that the neurodegenerative process in PD occurs over several years, the most-active phase of neuronal death following MPTP administration is completed over a short period of time, producing a clinical condition consistent with 'end-stage PD' in a few days. Still, brain imaging and neuropathological data suggest that, following the acute phase of neuronal death, nigrostriatal dopaminergic neurons continue to succumb at a much lower rate for many years after MPTP exposure (*12, 13*). From a neuropathological standpoint, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD with a resemblance that goes beyond the degeneration of nigrostriatal dopaminergic neurons. For instance, as in PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area (*14, 15*) and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus, at least in monkeys treated with low dose of MPTP (*16*), but apparently not in acutely intoxicated humans (*17*). On the other hand, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, other pigmented nuclei such as the locus coeruleus have been spared (*18*). Second, the eosinophilic intraneuronal inclusions Lewy bodies, characteristic of PD, have thus far not been convincingly observed in MPTP-induced parkinsonism (*18*). However, whether these absences of pathological features from MPTP reflect a difference in the neurotoxic mechanisms between PD and MPTP model or merely a difference in the dosage regimen that these animals and PD patients are exposed to a neurotoxin remains to be determined. Although thus far, MPTP has never been recovered from post-mortem brain tissues or body fluids of parkinsonian patients, indicating that MPTP is unlikely to cause PD, this neurotoxin provides an excellent experimental model for this disorder. Accordingly, it can be speculated that elucidating the molecular mechanisms of MPTP should lead to important insights into the pathogenesis and treatment of PD.

WHAT IS THE SOURCE OF NITRIC OXIDE INVOLVED IN MPTP NEUROTOXICITY?

Thus far, three different isoforms of NOS have been cloned and characterized (*5*). These include neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). All of these isoforms are present in the brain though in variable amounts. The most abundant isoform, nNOS, is expressed in several neuronal subtypes but, interestingly, thus far nNOS has not been detected in dopaminergic neurons of the nigrostriatal pathway. Yet, dopaminergic neurons of the nigrostriatal pathway are surrounded by an abundant network of neuronal cell bodies and fibres that contain nNOS (*19*), suggesting that any nitric oxide that will be used by dopaminergic neurons in MPTP neurotoxicity or in the pathogenesis of PD will have to originate from neighbouring neurons. In contrast to nNOS, iNOS is normally not expressed in the brain; however, in pathological situations, especially those associated with gliosis, iNOS can be induced. Consistent with this notion, in the case of PD as well as in the MPTP model, it has been demonstrated by immunohistochemical methods, that glial cells at the level of the SNpc exhibit a robust expression of iNOS (*20, 21*). As for eNOS in the brain, only very discrete populations of neurons seem to express this NOS isoform (*22*). Primarily, eNOS is confined to the endothelial cells of blood vessels, which are abundant in all regions of the brain. In regions like the substantia nigra and the striatum, we found no evidence that eNOS is expressed in neuronal cells (Wu and Przedborski, personal observation). Nevertheless, in these two brain regions, dopaminergic structures entertain a close relationship with blood vessels whose walls exhibit robust eNOS immunoreactivity (Wu and Przedborski, personal observation). From a pharmacological

standpoint, it is important to determine which of these isoforms is responsible for the production of nitric oxide in the pathogenesis of PD. The development of genetically engineered animals, in which the gene for each of the isoforms of NOS has been separately ablated, has made possible the investigations of the contribution of each isoform in the pathophysiology of MPTP. Using mutant mice deficient in nNOS, we were able to demonstrate that ablation of nNOS markedly attenuates MPTP toxicity (*23*). Indeed, the data on dopamine content in the striatum of these mutant animals show that, compared to their wildtype littermates, MPTP inflicts significantly less damage (*23*). These results indicate that nNOS produced nitric oxide contributes in part to the MPTP neurotoxicity and that nitric oxide derived from the other two NOS isoforms may also contribute in the MPTP neurotoxicity.

Schulz and collaborators (*24*) and we (*21*) show that not only is there a robust glial reaction but also an upregulation of iNOS expression and nitric oxide production after MPTP administration. More importantly, administration of MPTP, through different regimens, produces significantly less neuronal loss in mice deficient in iNOS compared to their wildtype counterparts (*21, 24*). Again, similar to the nNOS deficient mice, toxicity is only attenuated and not completely prevented in iNOS deficient mice. As for eNOS, using Western blot techniques, our unpublished data show that the level of expression of eNOS is unaffected by MPTP administration and, more importantly, when toxicity to MPTP is assessed in eNOS knockout animals, the ablation of this isoform has no significant impact on the demise of dopaminergic neurons. Collectively, these data suggest that, in the MPTP model, nNOS and iNOS but not eNOS play a significant role in the neurotoxic process. It can also be extrapolated from these data that optimal neuroprotection may be obtained in the MPTP model and possibly in PD, only if both nNOS and iNOS are inhibited.

EVIDENCE FOR THE FORMATION OF REACTIVE NITROGEN SPECIES IN MPTP TOXICITY MODEL

Reactive nitrogen species such as nitrogen dioxide and peroxynitrite are capable of both oxidizing and nitrating biological macromolecules. Formation of these reactive intermediates does require nitric oxide or its stable metabolite such as nitrite and could in part explain the contribution of nitric oxide to the toxicity of MPTP. The reaction of oxygen with nitric oxide, although kinetically slow, can generate nitrogen dioxide which can also be formed by the oxidation of nitrite by peroxidase and heme proteins. Peroxynitrite can be formed by the kinetically favourable reaction between superoxide and nitric oxide (*25*). One common reaction which could be considered as a biological 'fingerprint' for the formation of these reactive nitrogen species is the nitration of aromatic residues and particularly of tyrosine giving rise to 3-nitrotyrosine (*26*).

Over the years, both analytical (based on GC/MS separation and detection) as well as immunological methods have been developed to quantify, detect and localize nitrotyrosine in the MPTP model (*27*). Data obtained from selective analytical methodology such as the HPLC have revealed that nitrotyrosine levels are increased in selected brain regions following MPTP administration to mice (*28*). Using GC/MS, 24 h after the last injection of MPTP to mice, the level of nitrotyrosine increases dramatically in the ventral midbrain, the brain region that contains the SNpc, as well as in the striatum (*29*). In contrast, at the same time points and in the same animals, we showed that brain regions that are not affected by MPTP, i.e., the cerebellum and the frontal cortex, fail to show any change in the levels of nitrotyrosine (*29*). These data provide compelling evidence that MPTP does increase nitrotyrosine formation and that these alterations are a reflection of a pathological process specific to MPTP (*29*). The use of immunological approaches such as two-dimensional separation of brain proteins followed by Western blotting with anti-nitrotyrosine antibodies reveal that several proteins with different molecular masses are nitrated between 3 and 12 h after MPTP administration (*30*). The early occurrence of nitration events is not surprising since previously we have reported that MPTP cytotoxicity is also time-dependent (*31*) and that biochemical correlates of MPTP toxicity such as ATP depletion can be detected as early as 1 h after MPTP administration (*32*). Remarkably, this analysis also reveal a robust nitration of some but not all resolved proteins (*30*), which is an unexpected finding since virtually most proteins contain tyrosine residues suggesting that while all proteins *could* potentially be nitrated, only *specific* proteins are nitrated following MPTP administration. With the utility of immunoprecipitation, two proteins are identified as prominent targets for nitration after MPTP administration; tyrosine hydroxylase (TH), which is the rate-limiting enzyme in dopamine synthesis, and α-synuclein, a small presynaptic protein whose mutations have recently been implicated in the development of a familial form of PD. The data indicated that TH is nitrated at 3 h and 6 h after the last injection of MPTP (*30*) and coinciding with this nitration, a loss of catalytic activity in generating L-DOPA, is observed (*30*). Nitration of TH has also been observed in cells treated with 1-methyl-4-phenylpyridium (MPP^+), the active metabolite of MPTP, and low molecular weight molecules capable of effectively removing superoxide and hydrogen peroxide prevent the nitration of TH (*33*).

Four hours after the last MPTP administration, another protein that is modified by nitration is α-synuclein (*34*). Interestingly, at the same time point and under the same regimen of MPTP, proteins related to α-synuclein such as β-synuclein or synaptophysin are not nitrated (*34*). The *in vivo* consequence of tyrosine nitration of α-synuclein is still under investigation but interestingly Giasson and collaborators (*35*) showed that nitrated α-synuclein is preferentially found in dystrophic neurites and in Lewy bodies in the post mortem

brains of patients with Parkinson's and other related parkinsonian syndromes. Additional data have also indicated that other proteins in addition to α-synuclein are nitrated in Lewy bodies in the brains of parkinsonian patients (*35–37*). Similar to TH, exposing HEK 293 cells, stably transfected with either wild type or mutant α-synuclein, to nitric oxide-and superoxide-generating compounds result in α-synuclein nitration (*38*).

In addition to nitration of proteins, reactive nitrogen species could also nitrate the free amino acid tyrosine resulting in free 3-nitrotyrosine. Interestingly, when free nitrotyrosine is injected into the striatum of rats at concentrations 50% lower than that of 6-hydroxydopamine, a neurotoxin to dopaminergic neurons, some nigrostriatal damage is observed (*39*). Collectively, these findings strongly support the notion that formation of reactive nitrogen is involved in the MPTP-related cascade of deleterious events and possibly in the pathogenesis of PD.

SOURCES OF REACTIVE NITROGEN SPECIES IN MPTP TOXICITY MODEL

Formation of reactive nitrogen species can be catalyzed by peroxidases such as myeloperoxidase (MPO), which by utilizing hydrogen peroxide, can oxidize nitrite to nitrogen dioxide (*40*). Normally, MPO produces hypochlorous acid, which among other reactions could chlorinate tyrosine residues (*41*). Administration of MPTP also activates myeloperoxidase and chlorotyrosine has been detected after MPTP injection (Choi et al., submitted).

Another source of reactive nitrogen species is the formation of peroxynitrite, which would require sources of superoxide. Under physiological conditions, various biological processes within cells constantly produce superoxide and its intracellular concentration is maintained at extremely low levels by the abundance of superoxide dismutases. Conversely, nitric oxide is present in abundance, both within cells and in the extracellular space surrounding dopaminergic neurons, produced by either nNOS or iNOS. Therefore, since under physiological situations, the level of superoxide is low, the basal level of peroxynitrite is also low and hence the level of oxidative damage inflicted by peroxynitrite is minimal. In contrast, in PD as modelled by the MPTP neurotoxin, intracellular levels of superoxide increase significantly, presumably via the inhibition of mitochondrial respiration and other mechanisms (*42*). In addition to intraneuronal formation of superoxide, neurons could also be exposed to extracellularly formed superoxide in the MPTP toxicity. Following administration of MPTP, there is activation of microglia in addition to a host of pro-inflammatory cytokines such as interleukin-1β (*21*). Microglia activation involves the expression of iNOS and the production of nitric oxide as well the activation of NADPH-oxidase (*43*). NADPH-oxidase is a multimeric enzyme, which resides in microglia and its activation reduces oxygen to form superoxide. The contribution of intracellular superoxide to the MPTP neurotoxicity is evaluated in transgenic mice that express two to three times more cytosolic form of superoxide dismutase (copper/zinc-SOD, SOD1) in the brain (*44*). These transgenic animals have minimal damage to the dopaminergic neurons after the typical regimen of MPTP (*45*). Similar results are also observed in transgenic mice expressing mitochondrial manganese-SOD (SOD2), another SOD isoform (*46*). The significant involvement of extracellular superoxide to the MPTP toxicity is demonstrated when stereotaxic injection of the membrane-impermeant SOD1, which remains in the extracellular compartment, to striatum shows protection of striatal TH-positive fibers on the infused side as compared to the non-infused side (*43*). Consistent with this protection, mice defective in NADPH-oxidase and thus having reduced levels of extracellular superoxide show less dopaminergic neuronal loss and protein oxidation than their wildtype litermates after MPTP injections (*43*). Together, these findings indicate that the levels of superoxide both intracellular and extracellular appear to be critial components in the MPTP neurotoxic process. Furthermore, the employment of iNOS-knockout mice, different NOS antagonists (*23, 47, 48*) and minocycline to inhibit microglial activation (*49, 50*) has also demonstrated that blockade of NOS, which reduces the production of nitric oxide, attenuates significantly MPTP-induced neurotoxicity. Collectively, these studies indicate that both superoxide and nitric oxide are critical components of the deleterious biochemical reactions involved in the MPTP-mediated demise of the nigrostriatal dopaminergic pathway (Fig. 1).

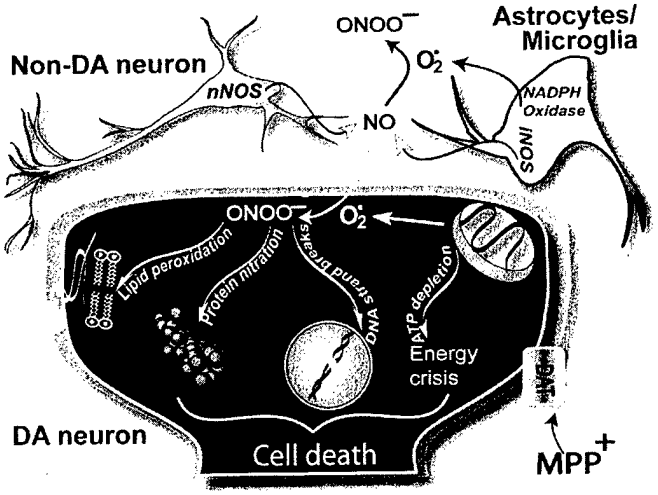

Figure 1. Proposed scenario of MPTP neurotoxic process and pathogenesis of Parkinson's disease.

CONCLUSION

In light of the outlined data, we can propose a working model for the contribution of nitric oxide-derived reactive intermediates in the pathogenic mechanisms of PD, based on the observations gathered in the MPTP model (Fig. 1). The first step of the working hypothesis relies on the premise that the initiating factor of the deleterious cascade is a molecule that shares similarities with MPTP's active metabolite 1-methyl-4-phenylperydinium (MPP^+). As such, this putative molecule has to enter dopaminergic neurons via the plasma membrane dopamine transporter (DAT). Once inside dopaminergic neurons, MPP^+ acts on mitochondria, where it blocks mitochondrial respiration. This has two immediate consequences: (i) blockade of ATP production and, therefore, an ensuing energy crisis; and (ii) increased production of superoxide. As indicated above, other mechanisms such as activation of microglia and NADPH-oxidase may also increase the production of superoxide following MPTP administration. At the same time, neighbouring neurons that contain nNOS produce nitric oxide, while later, when gliosis develops, activated glial cells that contain iNOS also contribute to the production of nitric oxide. Nitric oxide, a lipophillic diatomic molecule, can travel several micrometers away from its site of production and can freely cross the plasma membrane to reach the intracellular space of dopaminergic neurons. However, the concomitant production of superoxide in the intracellular and extracellular space will convert nitric oxice to peroxynitride, which in turn, will inflict oxidative damage to lipids, DNA, and proteins such as the oxidation and nitration of tyrosine residues. Possibly at the same time, activation of MPO and other proteins with peroxidase-like activity as well as increased hydrogen peroxide production, could also oxidize nitrite to nitrogen dioxide resulting in oxidative damage to lipids, DNA, and protein. The increased frequency of biological oxidations and the energy crisis lead to cellular dysfunction, which subsequently and eventually reach a magnitude that results in cell death (Fig. 1). Although this working model is primarily relevant to MPTP neurotoxicity, it is highly probable that a similar sequence of events may well underlie the neurodegenerative process in PD.

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Review

Parkinson's Disease: Mechanisms and Models

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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular events that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction and oxidative stress, may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models (particularly MPTP) have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

Introduction

In his classic 1817 monograph "Essay on the Shaking Palsy," James Parkinson described the core clinical features of the second most common age-related neurodegenerative disease after Alzheimer's disease (AD). Although more than a century passed before the central pathological feature of Parkinson's disease (PD) was found to be the loss of neurons in the substantia nigra pars compacta (SNpc), the pace of discovery accelerated following Arvid Carlsson's 1958 discovery of dopamine (DA) in the mammalian brain. SNpc neurons were then found to form the nigrostriatal dopaminergic pathway, and this line of research culminated with two key discoveries. First, loss of SNpc neurons leads to striatal DA deficiency, which is responsible for the major symptoms of PD. Second, replenishment of striatal DA through the oral administration of the DA precursor levodopa (L-3,4-dihydroxyphenylalanine) alleviates most of these symptoms.

Although the discovery of levodopa revolutionized the treatment of PD, we soon learned that after several years of treatment most patients develop involuntary movements, termed "dyskinesias," which are difficult to control and significantly impair the quality of life. Current research is directed toward prevention of dopaminergic neuron degeneration. Nevertheless, despite advances toward this goal, all current treatments are symptomatic; none halt or retard dopaminergic neuron degeneration.

The main obstacle in the development of neuroprotective drugs is ignorance of the specific molecular events that provoke neurodegeneration in PD. Prior to the last 5 years, most of the current hypotheses about the etiology and pathogenesis of PD derived from postmortem tissue or neurotoxic animal models, most notably, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration. Exposure of humans to MPTP causes a syndrome that mimics the core neurological symptoms and relatively selective dopaminergic neurodegeneration of PD, and MPTP toxicity in mice is the most commonly studied animal model of PD. These studies have focused on three types of cellular dysfunction that may be important in the pathogenesis of PD: oxidative stress, mitochondrial respiration defect, and abnormal protein aggregation. In addition, the MPTP monkey model has yielded valuable information regarding the functional alterations in basal ganglia circuits that occur subsequent to striatal DA depletion, and this model remains the gold standard for the preclinical evaluation of new therapies aimed at alleviating the symptoms of PD. While many findings from MPTP studies have been confirmed in human PD brains, there is intense debate about the relationship between MPTP and PD neurodegeneration.

This situation changed in 1997 with the discovery that mutations in the gene for α-synuclein cause an inherited form of PD. In just 5 years since this breakthrough, three additional PD-causing genes have been identified, and linkage has been reported for three more. As in AD, these rare PD genes appear to operate through a common molecular pathway, and their discovery may lead to the creation of novel animal models for the study of PD pathogenesis. It will also be important to determine whether these pathogenic proteins participate in the molecular events leading to neurodegeneration in existing animal models of PD, in order to evaluate how closely these models mimic the pathogenic events of the human disease.

Here, after discussing clinical and neuropathological characteristics of PD, we review current concepts of the etiology and pathogenesis of PD. We then focus on animal models of PD, evaluating how both well-established toxin-induced models and newer genetic models have contributed to the understanding of PD.

Clinical Characteristics of PD

PD is a progressive disease with a mean age at onset of 55, and the incidence increases markedly with age, from 20/100,000 overall to 120/100,000 at age 70. In about 95% of PD cases, there is no apparent genetic linkage (referred to as "sporadic" PD), but in the remaining cases, the disease is inherited. Over time, symptoms worsen, and prior to the introduction of levodopa, the mortality rate among PD patients was three times that of the normal age-matched subjects. While levo-

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Table 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 disease, diffuse Lewy body disease, frontotemporal dementia
Lytico-Bodig (Guamanian Parkinsonism-dementia-ALS)
Multiple system atrophy syndromes: striatonigral degeneration, Shy-Drager syndrome, sporadic olivopontocerebellar degeneration (OPCA), motor neuron disease-parkinsonism
Progressive pallidal atrophy
Progressive supranuclear palsy
Familial Neurodegenerative Diseases
Hallervorden-Spatz disease
Huntington disease
Lubag (X-linked dystonia-parkinsonism)
Mitochondrial cytopathies with striatal necrosis
Neuroacanthocytosis
Wilson disease

MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ALS, amytrophic lateral sclerosis.

dopa has dramatically improved the quality of life for PD patients, population-based surveys suggest that these patients continue to display decreased longevity compared to the general population (Hely et al., 1989; Morgante et al., 2000; Levy et al., 2002). Furthermore, most PD patients suffer considerable motor disability after 5–10 years of disease, even when expertly treated with available symptomatic medications.

Clinically, any disease that includes striatal DA deficiency or direct striatal damage may lead to "parkinsonism," a syndrome characterized by tremor at rest, rigidity, slowness or absence of voluntary movement, postural instability, and freezing (Table 1). PD is the most common cause of parkinsonism, accounting for ~80% of cases.

PD tremor occurs at rest but decreases with voluntary movement, so typically does not impair activities of daily living. Rigidity refers to the increased resistance (stiffness) to passive movement of a patient's limbs. Bradykinesia (slowness of movement), hypokinesia (reduction in movement amplitude), and akinesia (absence of normal unconscious movements, such as arm swing in walking) manifest as a variety of symptoms, including paucity of normal facial expression (hypomimia), decreased voice volume (hypophonia), drooling (failure to swallow without thinking about it), decreased size (micrographia) and speed of handwriting, and decreased stride length during walking. Bradykinesia may significantly impair the quality of life because it takes much longer to perform everyday tasks such as dressing or eating. PD patients also typically develop a stooped posture and may lose normal postural reflexes, leading to falls and, sometimes, confinement to a wheelchair. Freezing, the inability to begin a voluntary movement such as walking (i.e., patients remain "stuck" to the ground as they attempt to begin moving), is a common symptom of parkinsonism. Abnormalities of affect and cognition also occur frequently; patients may become passive or withdrawn, with lack of initiative; they may sit quietly unless encouraged to participate in activities. Responses to questions are delayed, and cognitive processes are slowed ("bradyphrenia"). Depression is common, and dementia is significantly more frequent in PD, especially in older patients.

Neurochemical and Neuropathological Features of PD

The pathological hallmarks of PD are the loss of the nigrostriatal dopaminergic neurons and the presence of intraneuronal proteinacious cytoplasmic inclusions, termed "Lewy Bodies" (LBs) (Figure 1). The cell bodies of nigrostriatal neurons are in the SNpc, and they project primarily to the putamen. The loss of these neurons, which normally contain conspicuous amounts of neuromelanin (Marsden, 1983), produces the classic gross neuropathological finding of SNpc depigmentation (Figure 1B). The pattern of SNpc cell loss appears to parallel the level of expression of the DA transporter (DAT) mRNA (Uhl et al., 1994) and is consistent with the finding that depletion of DA is most pronounced in the dorsolateral putamen (Bernheimer et al., 1973), the main site of projection for these neurons. At the onset of symptoms, putamenal DA is depleted ~80%, and ~60% of SNpc dopaminergic neurons have already been lost. The mesolimbic dopaminergic neurons, the cell bodies of which reside adjacent to the SNpc in the ventral tegmental area (VTA), are much less affected in PD (Uhl et al., 1985). Consequently, there is significantly less depletion of DA in the caudate (Price et al., 1978), the main site of projection for these neurons.

Neuropathological studies of PD-related neurodegeneration suggest possible clues to the pathogenesis of the disease. First, PD-associated loss of dopaminergic neurons has a characteristic topology, distinct from the pattern seen in normal aging. In PD, cell loss is concentrated in ventrolateral and caudal portions of the SNpc, whereas during normal aging the dorsomedial aspect of SNpc is affected (Fearnley and Lees, 1991). Thus, even though age is an important risk factor for PD, the processes that produce age-related dopaminergic neuronal death are probably different from those in PD. Second, the degree of terminal loss in the striatum appears to be more pronounced than the magnitude of SNpc dopaminergic neuron loss (Bernheimer et al., 1973), suggesting that striatal dopaminergic nerve terminals are the primary target of the degenerative process and that neuronal death in PD may result from a "dying back" process. Experimental support for the concept of dying back includes the observations that in MPTP-treated monkeys the destruction of striatal terminals precedes that of SNpc cell bodies (Herkenham et al., 1991), and in MPTP-treated mice, protection of striatal terminals prevents the loss of SNpc dopaminergic neurons (Wu et al., 2003). Third, the mechanism of

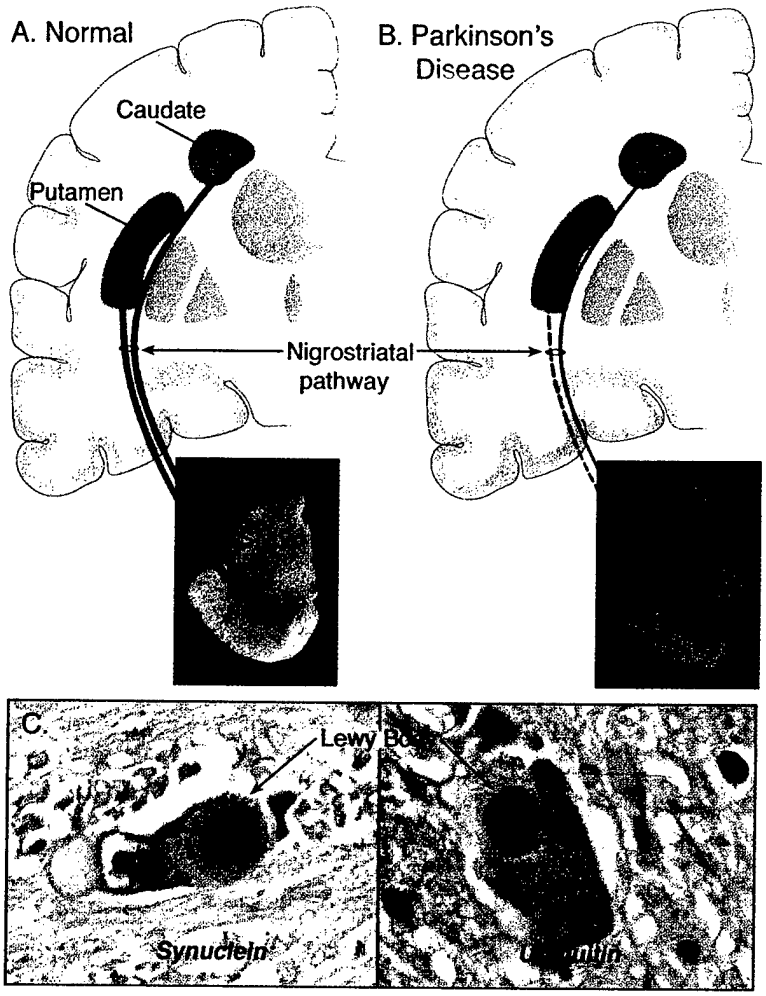

Figure 1. Neuropathology of Parkinson's Disease

(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's 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, termed Lewy bodies, in a SNpc dopaminergic neuron. Immunostaining with an antibody against α-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 yeilds more diffuse immunoreactivity within the Lewy body (right photograph).

synaptic DA clearance in the striatum seems to be more dependent on DAT than in the prefrontal cortex, where other monoaminergic transporters and the synaptic enzyme catechol-O-methyltransferase play a greater role in terminating the actions of DA (Giros et al., 1996; Gogos et al., 1998; Mundorf et al., 2001). The prefrontal cortex is a primary site of projection for VTA dopaminergic neurons, so this difference may be of importance in understanding the relative resistance of VTA neurons to PD-related degeneration. Differences in neuronal milieu have also been identified surrounding SNpc dopaminergic cell bodies. The neuropil of the substantia nigra, composed of axon projections from the striatum and globus pallidus, stains strongly for calbindin D_{28K}, and most dopaminergic cell bodies reside within this calbindin-rich neuropil (Damier et al., 1999a). However, the susceptible neurons in PD tend to be in calbindin-poor areas of the substantia nigra (Damier et al., 1999b).

Although it is commonly thought that the neuropathology of PD is characterized solely by dopaminergic neuron loss, the neurodegeneration extends well beyond dopaminergic neurons (reviewed by Hornykiewicz and Kish, 1987). Neurodegeneration and LB formation are found in noradrenergic (locus coeruleus), serotonergic (raphe), and cholinergic (nucleus basalis of Meynert, dorsal motor nucleus of vagus) systems, as well as in the cerebral cortex (especially cingulate and entorhinal cortices), olfactory bulb, and autonomic nervous system. Degeneration of hippocampal structures and cholinergic cortical inputs contribute to the high rate of dementia that accompanies PD, particularly in older patients. However, the clinical correlates of lesions to the serotonergic and noradrenergic pathways are not as clearly characterized as are lesions in the dopaminergic systems. Thus, while involvement of these neurochemical systems is generally thought to occur in more severe or late-stage disease, the temporal relationship of damage to specific neurochemical systems is not well established. For example, some patients develop depression months or years prior to the onset of PD motor symptoms, which could be due to early involvement of nondopaminergic pathways.

In life, the diagnosis of PD is made on clinical grounds, but definite diagnosis requires the identification of both LB and SNpc dopaminergic neuron loss. LBs are not specific for PD, however, and are also found in AD, in a condition called "dementia with LB disease," and as an incidental pathologic finding in people of advanced age at a greater frequency than the prevalence of PD (Gibb and Lees, 1988). The role of LB in neuronal cell death is controversial, as are the reasons for their increased frequency in AD and the relationship of inciden-

tal LB to the occurrence of PD. LBs are spherical eosinophilic cytoplasmic protein aggregates composed of numerous proteins (Figure 1C), including α-synuclein, parkin, ubiquitin, and neurofilaments, and they are found in all affected brain regions (Forno, 1996; Spillantini et al., 1998). LBs are more than 15 μm in diameter and have an organized structure containing a dense hyaline core surrounded by a clear halo. Electron microscopy reveals a dense granulovesicular core surrounded by a ring of radiating 8–10 nm fibrils (Duffy and Tennyson, 1965; Pappolla, 1986).

Etiology of PD

The cause of sporadic PD is unknown, with uncertainty about the role of environmental toxins and genetic factors. The environmental toxin hypothesis was dominant for much of the 20th century, especially because of the example of postencephalitic PD (as described in the Oliver Sacks' book *Awakenings*) and the discovery of MPTP-induced parkinsonism. However, the discovery of PD genes (reviewed in "Gene-Based Models" section below) has renewed interest in hereditary susceptibility factors. Both probably play a role.

The environmental hypothesis posits that PD-related neurodegeneration results from exposure to a dopaminergic neurotoxin. Theoretically, the progressive neurodegeneration of PD could be produced by chronic neurotoxin exposure or by limited exposure initiating a self-perpetuating cascade of deleterious events. The finding that people intoxicated with MPTP develop a syndrome nearly identical to PD (Langston et al., 1983) is a prototypic example of how an exogenous toxin can mimic the clinical and pathological features of PD. Paraquat is structurally similar to 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of MPTP, and has been used as herbicide. Like MPP+, rotenone is also a mitochondrial poison present in the environment, and it is used as an insecticide and to kill unwanted lake fish. Human epidemiological studies have implicated residence in a rural environment and related exposure to herbicides and pesticides with an elevated risk of PD (Tanner, 1992). Yet, there are no convincing data to implicate any specific toxin as a cause of sporadic PD, and chronic environmental exposure to MPP+ or rotenone is unlikely to cause PD. MPP+'s quaternary ammonium cation prevents its passage across the blood-brain barrier, and rotenone is so unstable in solution that it only lasts a few days in lakes (Hisata, 2002). Still, cigarette smoking and coffee drinking are inversely associated with the risk for development of PD (Hernan et al., 2002), reinforcing the concept that some environmental factors do modify PD susceptibility.

Another possibility, which does not fit neatly into a genetic or environmental category, is that an endogenous toxin may be responsible for PD neurodegeneration. Distortions of normal metabolism might create toxic substances because of environmental exposures or inherited differences in metabolic pathways. One source of endogenous toxins may be the normal metabolism of DA, which generates harmful reactive oxygen species (ROS) (Cohen, 1984). Consistent with the endogenous toxin hypothesis is the report that patients harboring specific polymorphisms in the gene encoding the xenobiotic detoxifying enzyme cytochrome P450 may be at greater risk of developing young-onset PD (Sandy et al., 1996). Further, isoquinoline derivatives toxic to dopaminergic neurons have been recovered from PD brains (Nagatsu, 1997).

Pathogenesis of PD

Whatever insult initially provokes neurodegeneration, studies of toxic PD models and the functions of genes implicated in inherited forms of PD suggest two major hypotheses regarding the pathogenesis of the disease. One hypothesis posits that misfolding and aggregation of proteins are instrumental in the death of SNpc dopaminergic neurons, while the other proposes that the culprit is mitochondrial dysfunction and the consequent oxidative stress, including toxic oxidized DA species.

The pathogenic factors cited above are not mutually exclusive, and one of the key aims of current PD research is to elucidate the sequence in which they act and whether points of interaction between these pathways are key to the demise of SNpc dopaminergic neurons. Potential points of interaction are diagrammed in Figure 2. The finding that oxidative damage to α-synuclein can enhance its ability to misfold and aggregate is one example of such an interaction (Giasson et al., 2000). Another uncertain issue is whether the multiple cell death-related molecular pathways activated during PD neurodegeneration ultimately engage common downstream machinery, such as apoptosis, or remain highly divergent. Clearly, this issue is of great consequence in deciding about possible therapeutic strategies for PD.

Misfolding and Aggregation of Proteins

The abnormal deposition of protein in brain tissue is a feature of several age-related neurodegenerative diseases, including PD. Although the composition and location (i.e., intra- or extracellular) of protein aggregates differ from disease to disease, this common feature suggests that protein deposition per se, or some related event, is toxic to neurons.

Aggregated or soluble misfolded proteins could be neurotoxic through a variety of mechanisms. Protein aggregates could directly cause damage, perhaps by deforming the cell or interfering with intracellular trafficking in neurons. Protein inclusions might also sequester proteins that are important for cell survival. If so, there should be a direct correlation between inclusion formation and neurodegeneration. However, a growing body of evidence, particularly from studies of Huntington disease (HD) and other polyglutamine diseases (Saudou et al., 1998; Cummings et al., 1999), suggests that there is no correlation between inclusion formation and cell death. Cytoplasmic protein inclusions may not result simply from precipitated misfolded protein but rather from an active process meant to sequester soluble misfolded proteins from the cellular milieu (reviewed by Kopito, 2000). Accordingly, inclusion formation, while possibly indicative of a cell under attack, may be a defensive measure aimed at removing toxic soluble misfolded proteins (Cummings et al., 1999; Warrick et al., 1999; Cummings et al., 2001; Auluck et al., 2002). The ability of chaperones such as Hsp-70 to protect against neurodegeneration provoked by disease-related proteins (including α-synuclein-mediated dopaminergic

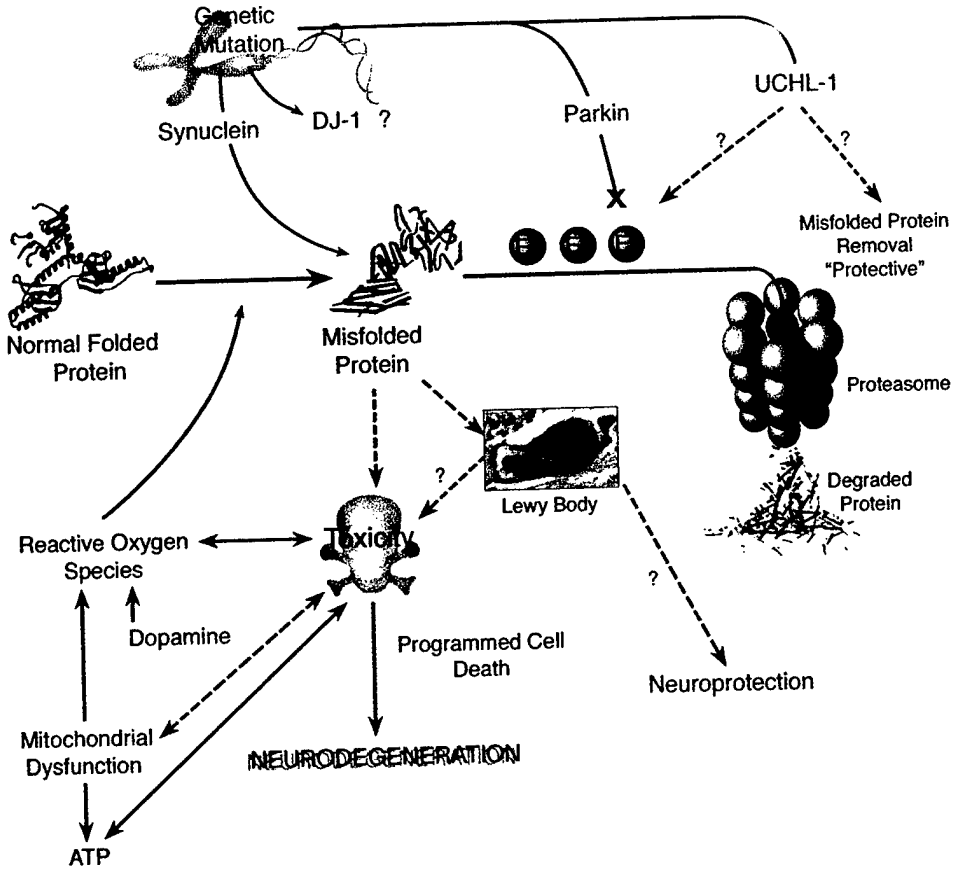

Figure 2. Mechanisms of Neurodegeneration

A growing body of evidence, detailed in this review, suggests that the accumulation of misfolded proteins is likely to be a key event in PD neurodegeneration. Pathogenic mutations may directly induce abnormal protein conformations (as believed to be the case with α-synuclein) or damage the ability of the cellular machinery to detect and degrade misfolded proteins (Parkin, UCH-L1); the role of DJ-1 remains to be identified. Oxidative damage, linked to mitochondrial dysfunction and abnormal dopamine metabolism, may also promote misfolded protein conformations. It remains unclear whether misfolded proteins directly cause toxicity or damage cells via the formation of protein aggregates (Lewy body). Controversy exists regarding whether Lewy bodies promote toxicity or protect a cell from harmful effects of misfolded proteins by sequestering them in an insoluble compartment away from cellular elements. Oxidative stress, energy crisis (i.e., ATP depletion) and the activation of the programmed cell death machinery are also believed to be factors that trigger the death of dopaminergic neurons in Parkinson's disease.

neuron loss) is consistent with the view that soluble misfolded proteins are neurotoxic (reviewed by Muchowski, 2002; Auluck et al., 2002).

In patients with *inherited* PD, pathogenic mutations are thought to cause disease directly by inducing abnormal and possibly toxic protein conformations (e.g., Bussell and Eliezer, 2001) or indirectly by interfering with the processes that normally recognize or process misfolded proteins (the function of genes identified in inherited PD is reviewed in the "Gene-Based Models" section below). In *sporadic* PD, there is a similar focus both on direct protein-damaging modifications and on dysfunction of chaperones or the proteasome that may indirectly contribute to the accumulation of misfolded proteins. The triggers for dysfunctional protein metabolism in sporadic PD are only just beginning to be elucidated. One trigger may be oxidative stress, long thought to play a key role in the pathogenesis of PD through damage caused by ROS (reviewed by Przedborski and Jackson-Lewis, 2000). The tissue content of abnormally oxidized proteins (which may misfold) increases with age (reviewed by Beckman and Ames, 1998), and neurons may be particularly susceptible because they are postmitotic. In PD, LBs contain oxidatively modified α-synuclein, which in vitro exhibits a greater propensity to aggregate than unmodified α-synuclein (Giasson et al., 2000). Several herbicides and pesticides induce misfolding or aggregation of α-synuclein (Uversky et al., 2001; Manning-Bog et al., 2002; Lee et al., 2002a). There also appears to be an age-related decline in the ability of cells to handle misfolded proteins (reviewed by Sherman and Goldberg, 2001). 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 as is the activity of the proteasome. Proteasomal dysfunction and the consequent accumulation of misfolded proteins may provoke a vicious cycle, with excess misfolded proteins further inhibiting an already compromised proteasome. Thus, factors that have been previously implicated in the pathogenesis of PD, including aging and oxidative stress, may converge

to generate a proteotoxic insult to cells. The discoveries regarding the genetics of inherited PD are consistent with this scenario (see below).

Mitochondrial Dysfunction and Oxidative Stress

The possibility that an oxidative phosphorylation defect plays a role in the pathogenesis of PD was fueled by the discovery that MPTP blocks the mitochondrial electron transport chain by inhibiting complex I (Nicklas et al., 1987). Subsequent studies identified abnormalities in complex I activity in PD (reviewed by Greenamyre et al., 2001). In vitro studies indicate that such a complex I defect may subject cells to oxidative stress and energy failure. The abnormality of oxidative phosphorylation identified in PD is not confined to the brain (Schapira et al., 1990), as reduced complex I activity has been found in platelets from PD patients (Parker et al., 1989) and in cybrid cells (cells lines engineered to contain mitochondria derived from platelets of PD patients [Swerdlow et al., 1996]). This latter finding suggests either that the observed complex I deficit is inherited from the mitochondrial genome or that some systemic toxicity leads to mutations in mitochondrial DNA. However, mitochondrial DNA mutations have not yet been identified in PD patients.

Nearly 100% of molecular oxygen is consumed by the mitochondrial respiration, and powerful oxidants are normally produced as byproducts, including hydrogen peroxide and superoxide radicals. Inhibition of complex I increases the production of the ROS superoxide, which may form toxic hydroxyl radicals or react with nitric oxide to form peroxynitrite. These molecules may cause cellular damage by reacting with nucleic acids, proteins, and lipids. One target of these reactive species may be the electron transport chain itself (Cohen, 2000), leading to mitochondrial damage and further production of ROS. Several biological markers of oxidative damage are elevated in the SNpc of PD brains (reviewed by Przedborski and Jackson-Lewis, 2000). Also, the content of the antioxidant glutathione is reduced in the SNpc of PD brains (Sian et al., 1994), consistent with increased ROS, although this could also indicate a primary reduction of protective mechanisms against ROS.

The presence of ROS would increase the amount of misfolded proteins, increasing the demand on the ubiquitin-proteasome system to remove them. Dopaminergic neurons may be a particularly fertile environment for the generation of ROS, as the metabolism of DA produces hydrogen peroxide and superoxide radicals, and autooxidation of DA produces DA-quinone (Graham, 1978), a molecule that damages proteins by reacting with cysteine residues. Mitochondria-related energy failure may disrupt vesicular storage of DA, causing the free cytosolic concentration of DA to rise and allowing harmful DA-mediated reactions to damage cellular macromolecules. Thus, DA may be pivotal in rendering SNpc dopaminergic neurons particularly susceptible to oxidative attack. Nevertheless, despite the literature documenting mitochondrial dysfunction and indices of oxidative damage in tissue from PD patients, all of these observations are correlative in nature, and the supportive data from postmortem studies of PD patients suffers from the fact that such specimens primarily consist of glial cells and nondopaminergic neurons, as most dopaminergic neurons die long before these specimens become available. There are no data that convincingly link a *primary* abnormality of oxidative phosphorylation or ROS generation with PD. Furthermore, parkinsonism is rare in many diseases known to result from mutations directly affecting oxidative phosphorylation ("mitochondrial cytopathies"). When parkinsonism is encountered in these diseases, it is generally accompanied by other symptoms not typical of PD. Therefore, many of the oxidative phosphorylation and ROS abnormalities documented in PD tissues could be nonspecific features of dying cells.

Mode of Cell Death

How do cells ultimately die in PD? Does a common downstream pathway mediate all PD-related cell loss, or is there significant heterogeneity in the pathways activated in different sick neurons in a single patient, or among different patients with PD? The answers to these questions are important for the rational development of therapeutic strategies for PD. In programmed cell death (PCD), intracellular signaling pathways are activated to cause cell demise. Although physiological PCD is crucial during normal development and as a homeostatic mechanism in some systems (e.g., immune system), dysregulation of this pathway in the brain may contribute 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. These morphological studies have yielded conflicting results (reviewed by Vila and Przedborski, 2003). Complicating matters, if apoptosis does occur in PD, it may be difficult to detect by morphological criteria because the rate of neuronal loss may be low (McGeer et al., (Raff et al., 1993). In addition, there may be nonapoptotic forms of PCD (Clarke, 1999; Sperandio et al., 2000). For these reasons, some studies of PCD in PD have measured molecular components of PCD instead of relying on morphological criteria. For example, investigations of the PCD molecule Bax demonstrate an increased number of Bax-positive SNpc dopaminergic neurons in PD (Hartmann et al., 2001a), and compared to controls, there is increased neuronal expression of Bax in PD, suggesting that these cells are undergoing PCD (Tatton, 2000). 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 (Hartmann et al., 2000, 2002). Other molecular markers of PCD are altered in PD, including the activation of caspase-8 (Hartmann et al., 2001b) and caspase-9 (Viswanath et al., 2001). Taken together, these studies suggest that the PCD machinery is activated in postmortem PD tissue. Nevertheless, because these studies are single time point-descriptive assessments of patient tissue they cannot address whether the findings reflect a primary abnormality of PCD regulation or an appropriate "suicide" decision by injured cells damaged by one of the processes reviewed above.

Modeling PD in Animals

While recent genetic discoveries have led to significant insight into molecular pathways of likely importance in

PD pathogenesis, these discoveries have not contributed to an understanding of other important aspects of the disease. Why is there a relatively selective loss of dopaminergic neurons in PD? Is the toxicity provoked by these disease alleles a cell-autonomous effect in dopaminergic neurons? What is the role of aging in both sporadic and inherited PD, or posed differently, why does it take many decades even for inherited PD to develop? Does pharmacological or genetic manipulation of the ubiquitin-proteasome pathway prevent (or provoke) dopaminergic neurodegeneration? Do the different genetic forms of PD display unique responses to cell-based (e.g., stem cell) or pharmacological therapies? What is the relationship between the neurodegeneration provoked by disease allele-related pathways and that occurring in sporadic PD? Although aspects of these questions can be assessed in PD patients, postmortem tissue, and in vitro systems, it is clear that these and related questions will be addressed most powerfully in animal models.

The crucial requirement for a disease gene-based model of PD (also referred to as an "etiologic model") is the adult onset of relatively specific and progressive dopaminergic neuron degeneration. A behavioral correlate of the nigrostriatal dopaminergic pathway degeneration is also desirable but, in rodents, will not likely parallel the motor deficits of PD because rodents do not develop typical parkinsonism. Alternatively, behaviors that involve striatal function, such as habituation to a novel environment or the ability to learn a stimulus-response paradigm, may be useful in assessing the striatal dopaminergic function. Because motor system organization differs in rodents and humans, the value of a particular behavioral phenotype depends upon its relationship to striatal dopaminergic function rather than apparent similarity to a symptom of PD. Specifically, behaviors claimed to result from striatal DA deficiency should improve with DA replacement. The formation of LBs is also a desirable but not essential feature. While LBs are characteristic of PD, they are not specific, are not found in a minority of clinically defined PD cases, and are not seen in parkin-related PD.

Other valuable approaches to modeling PD in animals do not depend on disease-related genes. These "pathologic models" use toxins or non-PD-related genetic mutations (Kostic et al., 1997) to mimic the selective degeneration of dopaminergic neurons or exploit the loss of dopaminergic neurons that normally occurs in rodents during early postnatal development (Macaya et al., 1994; Jackson-Lewis et al., 2000). These strategies are based on the premise that dopaminergic neurons have a stereotyped death cascade that can be activated by a range of insults or developmental signals. Clearly defining this cascade of events may lead to the identification of new molecules of potential relevance to PD pathogenesis or treatment. Most notable is the MPTP model, partially because of the striking similarity between PD and individuals intoxicated with MPTP. Finally, "symptomatic" or "pathophysiologic" models recapitulate the motor symptoms of PD and are used to develop symptomatic therapies or to study circuit-related questions. Only nonhuman primates accurately mimic the motor symptoms of PD and are therefore the only suitable animal for such studies.

The remainder of this review will focus on pathologic and genetic animal models of PD. We will first review toxin-induced models, with an emphasis on the MPTP model, to date the best characterized of this class. We will then focus on PD genes and review early attempts to exploit them to better model the disease.

Toxin-Based Models

Among the neurotoxins used to induce dopaminergic neurodegeneration, 6-hydroxydopamine (6-OHDA), MPTP, and more recently paraquate and rotenone have received the most attention. Presumably, all of these toxins provoke the formation of ROS. Rotenone and MPTP are similar in their ability to potently inhibit complex I, though they display significant differences, including, importantly, their ease of use in animals. Only MPTP is clearly linked to a form of human parkinsonism, and it is thus the most widely studied model.

6-Hydroxydopamine

6-hydroxydopamine, the first animal model of PD associated with SNpc dopaminergic neuronal death, was introduced more than 30 years ago (Ungerstedt, 1968). Although 6-OHDA-induced pathology differs from PD, it is still extensively used. 6-OHDA-induced toxicity is relatively selective for monoaminergic neurons, resulting from preferential uptake by DA and noradrenergic transporters (Luthman et al., 1989). Reminiscent of PD, there is a range of sensitivity to 6-OHDA between the ventral midbrain dopaminergic neuronal groups; greatest loss is observed in the SNpc, while tuberoinfundibular neurons are almost completely resistant (reviewed by Jonsson, 1980). Inside neurons, 6-OHDA accumulates in the cytosol, generating ROS and inactivating biological macromolecules by generating quinones that attack nucleophilic groups (reviewed by Cohen and Werner, 1994).

Because 6-OHDA cannot cross the blood-brain barrier, it must be administered by local stereotaxic injection into the substantia nigra, median forebrain bundle (MFB; which carries ascending dopaminergic and serotonergic projections to the forebrain), or striatum to target the nigrostriatal dopaminergic pathway (Javoy et al., 1976; Jonsson, 1983). After 6-OHDA injections into substantia nigra or the MFB, dopaminergic neurons start degenerating within 24 hr and die without apoptotic morphology (Jeon et al., 1995). When injected into the striatum, however, 6-OHDA produces a more protracted retrograde degeneration of nigrostriatal neurons, which lasts for 1–3 weeks (Sauer and Oertel, 1994; Przedborski et al., 1995). So far, however, none of the modes of 6-OHDA intoxication have led to the formation of LB-like inclusions. For striatal stereotaxic lesions, 6-OHDA is injected unilaterally, with the contralateral side serving as control (Ungerstedt, 1971). These injections produce an asymmetric circling behavior in the animals, the magnitude of which depends on the degree of the nigrostriatal lesion (Ungerstedt and Arbuthnott, 1970; Hefti et al., 1980; Przedborski et al., 1995). The unilateral lesion can be quantitatively assayed; thus, a notable advantage of this model is the ability to assess the anti-PD properties of new drugs (Jiang et al., 1993) and the benefit of transplantation or gene therapy to repair the damaged pathways (Bjorklund et al., 2002). However, it is not clear whether the mechanism by which 6-OHDA kills dopa-

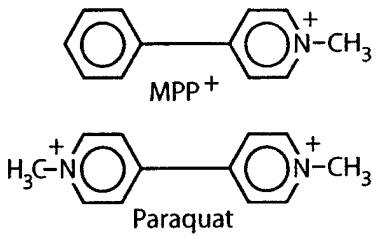

Figure 3. Structural Similarity between Paraquat and MPP+

The only difference between these two compounds is the second *N*-methyl-pyridinium group that paraquat has instead of the phenyl group as seen in MPP+.

minergic neurons shares key molecular features with PD.

Paraquat

The herbicide paraquat (N,N'-dimethyl-4-4'-bipiridinium) also induces a toxic model of PD. As noted above, paraquat shows structural similarity to MPP+ (Figure 3) and is present in the environment. Exposure to paraquat may confer an increased risk for PD (Liou et al., 1997). However, paraquat does not easily penetrate the blood brain barrier (Shimizu et al., 2001), and its CNS distribution does not parallel any known enzymatic or neuroanatomic distribution (Widdowson et al., 1996a, 1996b). The toxicity of paraquat appears to be mediated by the formation of superoxide radicals (Day et al., 1999). Systemic administration of paraquat to mice leads to SNpc dopaminergic neuron degeneration accompanied by α-synuclein containing inclusions, as well as increases in α-synuclein immunostaining in frontal cortex (Manning-Bog et al., 2002; McCormack et al., 2002). This study was the first to include stereologic cell counts to assess neurodegeneration, which may explain why the investigators found clear evidence of cell loss, compared to earlier inconsistent reports (Brooks et al., 1999; Thiruchelvam et al., 2000a, 2000b). It remains to be seen whether the dopaminergic toxicity is selective or whether other cell types are similarly affected. Regardless of the outcome of those investigations, the ability to induce dopaminergic neuronal loss and α-synuclein-positive inclusions in a reliable fashion may prove valuable for studies of the role of α-synuclein in neurodegeneration.

Rotenone

Rotenone is the most potent member of the rotenoids, a family of natural cytotoxic compounds extracted from tropical plants; it is widely used as an insecticide and fish poison. Rotenone is highly lipophilic and readily gains access to all organs (Talpade et al., 2000). Rotenone binds (at the same site as MPP+) to and inhibits mitochondrial complex I.

As discussed in the section on the etiology of PD, epidemiological studies suggest that exposure to pesticides may be a risk factor. Greenamyre and colleagues reported that the administration of low-dose intravenous rotenone to rats produces selective degeneration of nigrostriatal dopaminergic neurons accompanied by α-synuclein-positive LB-like inclusions (Betarbet et al., 2000). Because rotenone may freely enter all cells, this study suggested that dopaminergic neurons are preferentially sensitive to complex I inhibition. Rotenone-intoxicated animals developed abnormal postures and slowness of movement, but it is unknown whether these features improved with levodopa administration. Nevertheless, this model was the first to link an environmental toxin of possible relevance to PD to the pathologic hallmark of α-synuclein aggregation, an association also seen in cell culture studies (Uversky et al., 2001; Sherer et al., 2002; Lee et al., 2002a).

In contrast to the findings of Betarabet and colleagues, acute intoxication with rotenone seems to spare dopaminergic neurons (Ferrante et al., 1997). Furthermore, a subsequent study of rats chronically infused with rotenone demonstrated significant reductions in striatal DARPP-32-positive, cholinergic, and NADPH diaphorase-positive neurons (Hoglinger et al., 2003). These results suggest that rotenone exerts a more widespread neurotoxicity than originally proposed, challenging the concept that dopaminergic neurons display preferential sensitivity to complex I inhibition (Betarbet et al., 2000). In addition, the use of rotenone in rodents is technically challenging (Betarbet et al., 2000). Nevertheless, the characteristic of LB-associated dopaminergic neurodegeneration in this model should enable investigators to perform a novel series of experiments exploring the relationship between aggregate formation and neuronal death.

MPTP: False Narcotic, Real Parkinsonian Toxin

In 1982, young drug users developed a rapidly progressive parkinsonian syndrome traced to intravenous use of a street preparation of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), an analog of the narcotic meperidine (Demerol) (Langston et al., 1983). MPTP was the responsible neurotoxic contaminant, inadvertently produced during the illicit synthesis of MPPP in a basement laboratory. In humans and monkeys, MPTP produces an irreversible and severe parkinsonian syndrome characterized by all of the features of PD, including tremor, rigidity, slowness of movement, postural instability, and freezing. In MPTP-intoxicated humans and nonhuman primates, the beneficial response to levodopa and development of long-term motor complications to medical therapy are virtually identical to that seen in PD patients. Also similar to PD, the susceptibility to MPTP increases with age in both monkeys and mice (Rose et al., 1993; Irwin et al., 1993; Ovadia et al., 1995).

The data regarding the comparison between PD- and MPTP-related neuropathology derive largely from MPTP studies in monkeys (Forno et al., 1993), because only four human MPTP cases have come to autopsy (Davis et al., 1979; Langston et al., 1999). These studies show that, as in PD, monkeys treated with low-dose MPTP exhibit preferential degeneration of putamenal versus caudate dopaminergic nerve terminals (Moratalla et al., 1992). Similarly, MPTP damages the dopaminergic pathways in a pattern similar to that seen in PD, including relatively greater cell loss in the SNpc than the VTA and a preferential loss of neurons in the ventral and lateral segments of the SNpc (Sirinathsinghji et al., 1992; Varastet et al., 1994); this regional pattern is also found in MPTP-treated mice (Seniuk et al., 1990; Muthane et al., 1994). Also reminiscent of PD (Hirsch et al., 1988), dopaminergic neurons that contain neuromelanin are more susceptible to MPTP-induced degeneration (Herrero et al., 1993). Neuromelanin may contribute neurodegenera-

tion in PD and MPTP-treated monkeys by catalyzing ROS formation through an interaction with iron selectively in pigmented neurons (Zecca et al., 2001). A variety of organic molecules interact with neuromelanin, including pesticides, MPTP, and MPP+ (D'Amato et al., 1986), so it may contribute to toxicity of pigmented neurons by acting as a depot for toxic compounds.

The monkey MPTP model does not include two characteristic features of PD. First, neurons are not consistently lost from other monaminergic nuclei, such as the locus coeruleus, a typical feature of PD (Forno et al., 1986, 1993). Second, although intraneuronal inclusions resembling LBs have been described (Forno et al., 1986), classical LBs have not been demonstrated convincingly in the brains of MPTP-intoxicated patients or monkeys (Forno et al., 1993). These cases were exposed to acute regimens of MPTP, so the lack of LB-like formation in MPTP-intoxicated humans and monkeys may reflect the fact that in these cases dopaminergic neurons were rapidly injured. Chronic infusion of rotenone does produce intraneuronal α-synuclein-containing proteinacious aggregates (Betarbet et al., 2000), consistent with the possibility that the speed of intoxication may influence the subsequent neuropathologic features.

Despite these neuropathologic shortcomings, the monkey MPTP model is the gold standard for the assessment of novel strategies and agents for the treatment of PD symptoms. For example, electrophysiologic studies of MPTP monkeys revealed that hyperactivity of the subthalamic nucleus is a key factor in the genesis of PD motor dysfunction (Bergman et al., 1990). 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 (Limousin et al., 1998). In addition, MPTP-treated monkeys (Gash et al., 1996; Kordower et al., 2000) were used to demonstrate that the delivery of glial-derived neurotrophic factor (GDNF) both significantly limits MPTP-induced nigrostriatal dopaminergic neurodegeneration and can lead to behavioral recovery when given to previously lesioned animals (Kordower et al., 2000). These studies form the basis for current attempts to use GDNF in PD patients (Gill et al., 2003). 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.

MPTP Metabolism and PD Neurodegeneration Selectivity. Since the initial discovery of MPTP-induced parkinsonism, much has been learned about the molecular pathway used by this toxin, as illustrated in Figure 4. Importantly, this knowledge enables investigators to use MPTP as a biological probe to explore the functions of PD genes and dissect the molecular events that occur during neurodegeneration of dopaminergic neurons. For example, mice mutant for PD genes (or other genes of possible relevance to dopaminergic neuronal death) can be injected with MPTP, and if these mice display markedly enhanced or suppressed dopaminergic neuronal death, one can then investigate which of the known molecular targets of MPTP are altered.

After systemic administration, MPTP, which is highly lipophilic, crosses the blood-brain barrier within minutes (Markey et al., 1984). Once in the brain, the pro-toxin MPTP is oxidized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) by monoamine oxidase B (MAO-B) in glia and serotonergic neurons, the only cells that contain this enzyme. It is then converted to MPP+ (probably by spontaneous oxidation), the active toxic molecule, and released by an unknown mechanism into the extracellular space. Since MPP+ is a polar molecule, it depends on the plasma membrane carriers to enter cells. MPP+ is a high-affinity substrate for the DAT, as well as for norepinephrine and serotonin transporters (Javitch et al., 1985; Mayer et al., 1986). Pharmacological inhibition or genetic deletion of DAT prevents MPTP-induced dopaminergic damage (Javitch et al., 1985; Bezard et al., 1999), demonstrating the obligatory character of this step in MPTP neurotoxicity. However, uptake by DAT does not entirely explain the selectivity of the nigrostriatal dopaminergic lesion caused by MPTP. While there are quantitative differences in DAT expression between more susceptible SNpc neurons and less susceptible VTA neurons in monkeys (Haber et al., 1995), differences in DA uptake activity of comparable magnitudes between rats and mice and among mouse strains do not correlate with differences in MPTP sensitivity (Giovanni et al., 1991, 1994). Furthermore, while MPP+ is concen-

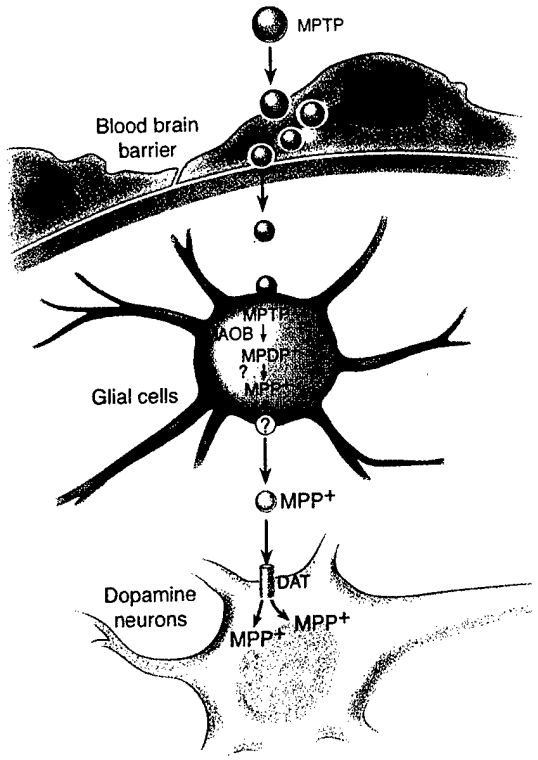

Figure 4. Schematic Representation of MPTP Metabolism

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).

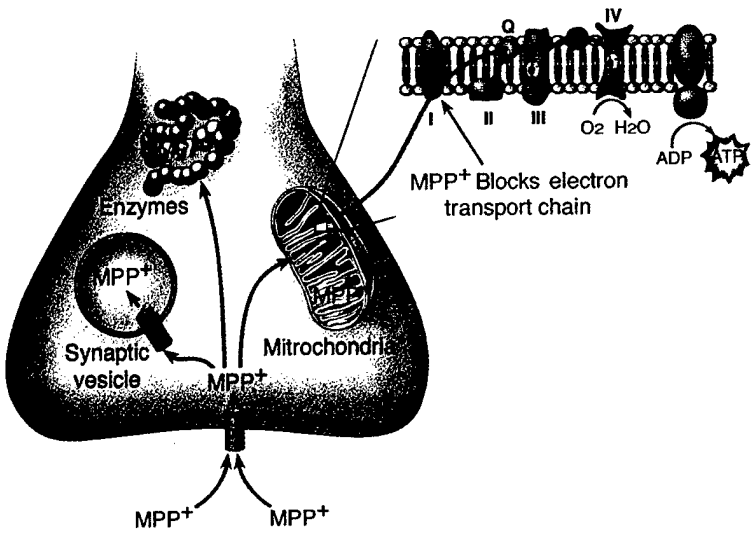

Figure 5. Schematic Representation of MPP+ Intracellular Pathways

Inside dopaminergic neurons, MPP+ can follow one of three routes: (1) concentration into mitochondria through an active process (toxic); (2) interaction with cytosolic enzymes (toxic); (3) sequestration into synaptic vesicles via the vesicular monoamine transporters (VMAT; protective). Within 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.

trated in (Speciale et al., 1998) and produces biochemical alterations in all monoaminergic neurons (Burns et al., 1983; Hallman et al., 1984; Wallace et al., 1984; Rose et al., 1993; Ovadia et al., 1995), degeneration is most prominent in dopaminergic neurons. In this regard, it is particularly striking that the highest levels of MPP+ are found in the adrenal medulla without causing the loss of chromaffin cells (Reinhard et al., 1987).

Inside neurons (Figure 5), MPP+ can follow at least three routes: (1) it can bind to the vesicular monoamine transporter-2 (VMAT2), which translocates MPP+ into synaptosomal vesicles (Liu et al., 1992); (2) it can be concentrated within the mitochondria by a mechanism that relies on the mitochondrial transmembrane potential (Ramsay and Singer, 1986); and (3) it can remain in the cytosol to interact with cytosolic enzymes, especially those carrying negative charges (Klaidman et al., 1993). Vesicular sequestration of MPP+ appears to protect cells from MPTP-induced neurodegeneration by sequestering the toxin and preventing it from accessing mitochondria, its likely site of action (see below). The importance of vesicular sequestration has been established by a number of experiments, including those showing that cells transfected to express greater density of VMAT2 are converted from MPP+-sensitive to MPP+-resistant cells (Liu et al., 1992) and that heterozygous VMAT2 null mice display enhanced sensitivity to MPTP-induced neurodegeneration (Takahashi et al., 1997). It appears that the ratio of DAT to VMAT2 expression predicts the likelihood of neuronal degeneration both in PD and the MPTP model. For instance, the putamenal dopaminergic terminals, which are most severely affected by both MPTP and PD, have a higher DAT/VMAT2 ratio than those in the caudate, which are less affected (Miller et al., 1999).

Mechanisms of Nigrostriatal Neurodegeneration: Hints from MPTP. Once inside the mitochondria, MPP+ impairs oxidative phosphorylation by inhibiting the multienzyme complex I of the mitochondrial electron transport chain (Nicklas et al., 1985). This blockade rapidly leads to decreases in tissue ATP content, particularly in the striatum and ventral midbrain (Chan et al., 1991; Fabre et al., 1999), the brain regions the most sensitive to MPTP. In vitro experiments in mitochondria isolated from *whole brain* demonstrate that complex I activity must be inhibited by ~70% to significantly impair ATP production (Davey and Clark, 1996), but data from PD postmortem tissues demonstrate only a ~40% inhibition of complex I activity (Schapira et al., 1990). Interestingly, in vitro experiments with *synaptic-derived* mitochondria demonstrate that significant ATP depletion results from as little as ~25% inhibition of complex I (Davey et al., 1998), indicating a much tighter functional relationship between complex I activity and ATP production in synaptic than in somatic mitochondria. Thus, mitochondria from phenotypically distinct neuronal populations may be differentially affected in PD, and the current approach of assessing mitochondrial function in specimens from whole tissue may not depict accurately abnormalities present in only a minority of cells. Furthermore, even the small alterations in complex I activity observed in PD may be particularly harmful to dopaminergic nerve terminals, which are rich in synaptic mitochondria.

Another early effector of complex I inhibition due to MPP+ may be oxidative stress. Indeed, by hampering the flow of electrons through complex I, MPP+ can stimulate the production of ROS, especially superoxide (Hasegawa et al., 1990, 1997). MPP+ effects on mitochondria can also indirectly stimulate the production of ROS by triggering DA leakage from synaptic vesicles to the cytosol, likely due to the inability of VMAT2 to maintain concentration gradients in the face of the ATP depletion (reviewed by Johnson, 1988). Findings from in vivo studies provide support for the importance of ROS in MPTP-induced neurodegeneration. Mice transgenic for superoxide dismutase-1 (SOD1), a key ROS scavenging enzyme, are resistant to MPTP-induced dopaminergic neuron degeneration (Przedborski et al., 1992), and other studies in mice imply a key role for reactive species, including NO, as critical effectors in MPTP toxicity (reviewed by Przedborski and Vila, 2003; Przedborski et al., 2003).

Alterations in energy metabolism and generation of ROS peak within hours of MPTP administration, days

before overt neuronal death has occurred (Jackson-Lewis et al., 1995). Therefore, these initial events are not likely to directly kill most cells but rather set into play downstream cellular events that ultimately kill most dopaminergic neurons (Mandir et al., 1999; Saporito et al., 2000; Vila et al., 2001).

Prolonged administration of low to moderate doses of MPTP to mice leads to morphologically defined apoptosis of SNpc dopaminergic neurons (Tatton and Kish, 1997). Under this regimen of MPTP intoxication, Bax, a potent PCD agonist and member of the Bcl-2 family, is upregulated in SNpc dopaminergic neurons (Vila et al., 2001). Bax upregulation coincides with its translocation to mitochondria, mitochondrial release of cytochrome c (an electron carrier and a mediator of PCD), and activation of caspases 9 and 3 (Viswanath et al., 2001). At the same time, PCD antagonists such as Bcl-2 are downregulated in the SNpc (Vila et al., 2001). Consistent with these observations, Bax null and Bcl-2 transgenic mice are both resistant to MPTP neurotoxicity (Yang et al., 1998; Offen et al., 1998; Vila et al., 2001).

How MPTP provokes these changes in Bcl-2 family members remains to be elucidated. MPTP causes oxidative damage to DNA (Mandir et al., 1999; Mandavilli et al., 2000), which may be important in inducing Bax via p53 activation. The tumor suppressor protein p53 is one of the few molecules known to regulate Bax expression and is activated by DNA damage. Furthermore, pharmacological inhibition of p53 attenuates MPTP-induced Bax upregulation and the subsequent SNpc dopaminergic neuron death (Duan et al., 2002), and p53 null mice are resistant to MPTP-induced neurodegeneration (Trimmer et al., 1996).

Activation of the JNK pathway following DNA damage is required in vitro for Bax mitochondrial translocation and the ensuing recruitment of the mitochondrial apoptotic pathway (Ghahremani et al., 2002; Lei et al., 2002). Activation of the JNK pathway follows MPTP administration (Saporito et al., 2000; Xia et al., 2001), and pharmacological blockade of JNK (Saporito et al., 1999) or adenoviral-directed expression of the JNK binding domain of JNK-interacting protein-1 (Xia et al., 2001) results in marked attenuation of MPTP-induced SNpc dopaminergic cell death.

Approaches aimed at inhibiting PCD at a more downstream level, such as by interfering with activation of caspases, have yielded inconsistent results. Adenoviral gene transfer of X chromosome-linked inhibitor of apoptosis (XIAP), a protein caspase inhibitor, prevents MPTP-induced SNpc dopaminergic neuron death, although it does not prevent the loss of striatal dopaminergic terminals (Eberhardt et al., 2000). In contrast, transgenic neuronal expression of the general caspase inhibitor protein baculoviral p35 specifically attenuates both MPTP-induced neuronal death and DA depletion (Viswanath et al., 2001). As with XIAP, some in vitro studies suggest that resistance to PCD can be induced selectively in the cell body. The broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone and peptide inhibitors of caspases 2, 3, and 9 prevent the loss of dopaminergic cell bodies of cultured ventral midbrain neurons exposed to MPP+, but the neurites are not spared (Bilsland et al., 2002); the molecular pathways governing neuronal death may differ from those governing axonal destruction (Raff et al., 2002).

MPTP administration also leads to the accumulation and nitration of α-synuclein in the cytosol of SNpc dopaminergic neurons (Vila et al., 2000; Przedborski et al., 2001), and ablation of α-synuclein in mutant mice prevents MPTP-induced dopaminergic neurodegeneration (Dauer et al., 2002). While it is not clear whether α-synuclein plays any direct role in regulating PCD, the expression of mutant α-synuclein in cell cultures may promote apoptosis (Xu et al., 2002), and cytochrome c has been reported to stimulate in vitro aggregation of α-synuclein (Hashimoto et al., 1999). Collectively, these data demonstrate that the activation of PCD is instrumental in MPTP toxicity. They also suggest that PCD alterations in PD postmortem samples are of pathological significance and that targeting specific PCD molecules may be a valuable neuroprotective strategy for the treatment of PD (Vila and Przedborski, 2003).

Gene-Based Models

As discussed above, uncertainty remains regarding which of the molecular events provoked by toxins relate to human PD. The discovery of PD genes is particularly exciting because theoretically it will allow the generation of novel models of definite significance to specific forms of the human disease, and evidence is emerging to link these genetic forms to idiopathic PD. Here, we will briefly review the current state of knowledge of PD genes and then discuss early attempts to exploit these discoveries to generate novel PD models.

The rationale for studying rare genetic forms of a common sporadic illness is the expectation that the phenotypic similarity between the genetic and sporadic forms of the disease indicates that they share important pathogenic mechanisms and, consequently, that genetic information will help focus research on a key biochemical pathway (Figure 2). Indeed, all of the PD genes that have been identified and studied in some detail—α-synuclein, parkin, and ubiquitin C-terminal hydrolase L1 (UCHL-1)—appear to participate in the ubiquitin-proteasome pathway, a particularly compelling finding considering the LB protein aggregates that characterize PD neuropathology. Although PD-causing mutations in the gene DJ-1 have only recently been identified, this protein also appears to have a potential link to the ubiquitin-proteasome pathway (Takahashi et al., 2001). Much of the current research in PD is focused on the normal role and functional interaction between these PD proteins and how these functions are disrupted by pathogenic mutations. Polymorphisms at the parkin and synuclein loci may also contribute to the risk of idiopathic PD (Farrer et al., 2001), and parkin mutations are found in patients without a family history of PD, especially with symptom onset before the age of 30 (Lucking et al., 2000). A number of epidemiological studies suggest that single-nucleotide polymorphisms at different loci may be associated with PD susceptibility (Martin et al., 2001; Li et al., 2002; Zareparsi et al., 2002), but the lack of concordance for PD in monozygotic twins argues against a *strong* genetic contribution in sporadic PD (Tanner et al., 1999).

Synuclein

Two missense mutations [$Ala^{53} \rightarrow$ Thr (A53T) and $Ala^{30} \rightarrow$ Pro (A30P)] in α-synuclein cause dominantly inherited PD (Polymeropoulos et al., 1997; Kruger et al., 1998). Clinical and pathological features typical of PD have been found in brains from patients with either mutation, although some atypical features have also been noted (Kruger et al., 1998; Spira et al., 2001). Mutations in α-synuclein have not been found in sporadic PD (Lynch et al., 1997; Munoz et al., 1997; Chan et al., 1998), so the concept that α-synuclein-mutant and sporadic PD share common pathogenic mechanisms rests predominantly on the observation that α-synuclein is a major component of LBs in sporadic PD (Spillantini et al., 1998).

The normal physiological role of α-synuclein is just beginning to be elucidated, and this prevalent presynaptic protein may modulate synaptic vesicle function (reviewed by Kahle et al., 2002). α-Synuclein is widely expressed in the nervous system, where it is found in presynaptic nerve terminals in close association with synaptic vesicles (Maroteaux et al., 1988; George et al., 1995). It binds reversibly to brain vesicles and components of the vesicular trafficking machinery (Jensen et al., 1998, 1999, 2000). In striatal dopaminergic terminals, α-synuclein participates in the modulation of synaptic function, possibly by regulating the rate of cycling of the readily releasable pool (Abeliovich et al., 2000). Downregulation of this protein by antisense oligonucleotide in hippocampal cell culture is reported to decrease the distal pool of synaptic vesicles and alters the expression of vesicular-associated proteins in cultured hippocampal glutamatergic neurons (Murphy et al., 2000). However, no abnormalities were identified in an extensive quantitative analysis of synaptic-related proteins from either whole-brain homogenates (Schluter et al., 2003) or hippocampal cultures (Cabin et al., 2002) from synuclein null mice. While the ultrastructure of striatal synapses appears normal in brain sections from mice that lack synuclein (Abeliovich et al., 2000), there may be fewer "non-docked" distal synaptic vesicles in hippocampal brain sections from synuclein null mice (Cabin et al., 2002). Nevertheless, since quantitative EM studies are challenging to perform, this finding awaits confirmation. Unfortunately, none of the studies of synuclein null mice specifically assessed dopaminergic nerve terminal synaptic protein expression and morphology; this remains a significant gap in the characterization of these animals.

Biochemical and biophysical evidence is also consistent with a role for α-synuclein in cellular membrane dynamics. As seen with synaptic vesicles, α-synuclein binds to lipid membranes, and this binding changes the conformation of the previously unfolded N terminus of the protein to a stable α-helical secondary structure (Davidson et al., 1998; Eliezer et al., 2001), suggesting that membrane binding elicits a functionally important alteration in the protein. Additional observations support the view that the cellular membrane is a key site of α-synuclein action (Pronin et al., 2000; Ahn et al., 2002). One membrane-related function of α-synuclein may be trafficking proteins to the plasma membrane, as suggested by the demonstration that α-synuclein could be involved in the membrane localization of DAT (Lee et al., 2001).

The fact that α-synuclein is abundant in LBs suggests that its propensity to misfold and form amyloid fibrils may be responsible for its neurotoxicity in pathological situation such as PD and that pathogenic mutations endow it with a toxic gain of function. A growing literature supports this notion and links the pathogenesis of PD to other neurodegenerative diseases that involve protein aggregation (reviewed by Goedert, 2001). Misfolding of α-synuclein may interfere with its normal functions, but it is unlikely that loss of function plays a major role in α-synuclein-related neurodegeneration (Abeliovich et al., 2000; Dauer et al., 2002).

Both wild-type and mutant α-synuclein form amyloid fibrils resembling those seen in LBs (Conway et al., 1998; Giasson et al., 1999) as well as nonfibrillary oligomers (Conway et al., 1998), termed "protofibrils." Since the two known pathogenic α-synuclein mutations promote the formation of protofibrils (Conway et al., 2000), they may be the toxic species of α-synuclein. Consistent with this view and the association of α-synuclein with synaptic vesicles, protofibrils may cause toxicity by permeabilizing synaptic vesicles (Volles et al., 2001; Lashuel et al., 2002), allowing DA to leak into the cytoplasm and participate in reactions that generate oxidative stress (reviewed above). Furthermore, the selective vulnerability of dopaminergic neurons in PD may derive from the ability of DA itself to stabilize these noxious α-synuclein protofibrils (Conway et al., 2001). Nevertheless, protofibrils have only been observed and studied in vitro, so further work will need to explore whether they form in neurons and if their formation correlates with neurotoxicity.

Parkin

Loss-of-function mutations in the gene encoding parkin cause recessively inherited parkinsonism (Kitada et al., 1998). Although this form of parkinsonism was originally termed autosomal recessive juvenile parkinsonism, the clinical phenotype is now known to include older-onset patients (Lincoln et al., 2003). 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 (Mizuno et al., 2001). Clinically, parkin mutant patients display the classical 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 PD (Hilker et al., 2001; Hedrich et al., 2002). Pathologically, parkin-related PD is characterized by loss of SNpc dopaminergic neurons, but it is not typically associated with LBs (Mizuno et al., 2001).

It is uncertain how loss of parkin function leads to dopaminergic neuron degeneration, but clues are emerging from the identification of its normal function. Parkin, a 465 amino acid protein, contains two RING finger domains separated by an in-between RING (IBR) finger domain at the C terminus and an ubiquitin-like homology domain at the N terminus. The presence of an IBR led to the finding that parkin is an E3 ubiquitin ligase (Zhang et al., 2000; Shimura et al., 2000), a component of the ubiquitin-proteasome system that identifies and targets misfolded proteins to the proteasome for degradation (reviewed by Sherman and Goldberg, 2001). The upstream ubiquitin ligases (E1 and E2) cooperate nonspecifically to tag misfolded proteins with a single

ubiquitin, while E3 ligases confer target specificity by binding to specific molecules or classes of molecules facilitating the polyubiquitination necessary for targeting to the proteasome. Many parkin mutations abolish this E3 ligase activity, suggesting that the accumulation of misfolded parkin substrates could be responsible for the demise of SNpc dopaminergic neurons in PD.

A number of parkin substrates have been identified (Zhang et al., 2000; Shimura et al., 2001; Chung et al., 2001; Imai et al., 2001; Staropoli et al., 2003). Some of these substrates appear to link parkin and synuclein function, and one—cyclin E—links parkin function to a molecule previously implicated in neuronal apoptosis. Three reports suggest a relationship between parkin and synuclein function (Shimura et al., 2001; Petrucelli et al., 2002) or aggregation (Chung et al., 2001). Notably, the E3 ligase activity of parkin modulates the sensitivity of cells to both proteasome inhibitor- and mutant synuclein-dependent cell death (Petrucelli et al., 2002). A number of observations suggest that the functional interaction between synuclein and parkin may involve the proteasome: synuclein interacts with and may be degraded by the proteasome (Ghee et al., 2000; Snyder et al., 2003), overexpression of synuclein inhibits the proteasome (Stefanis et al., 2001), and mutant synuclein increases the sensitivity of cells to proteasome inhibition (Tanaka et al., 2001; Petrucelli et al., 2002). Parkin has also been found to function in a multiprotein ubiquitin ligase complex that ubiquitinates cyclin E (Staropoli et al., 2003). Importantly, these investigators also demonstrated that there is an accumulation of cyclin E in midbrain extracts from parkin mutant as well as idiopathic PD and that in excitotoxin-treated cultured postmitotic neurons parkin overexpression attenuates cyclin E accumulation and promotes survival. Thus, a number of findings are beginning to strengthen the functional links between parkin, synuclein, and proteasome function as well as to highlight parkin substrates that might play a key role in cell death. However, none of the identified parkin substrates normally display a pattern of selective or enriched expression in dopaminergic neurons. Thus, these data have yet to suggest a molecular explanation for the relative specificity of dopaminergic neuron degeneration in PD.

Ubiquitin C-Terminal Hydrolase-L1

A dominant mutation (I93M) in UCH-L1 was identified in one family with inherited PD (Leroy et al., 1998), but no pathological data were included in this report. This enzyme catalyzes the hydrolysis of C-terminal ubiquityl esters and is thought to play a role in recycling ubiquitin ligated to misfolded proteins after their degradation by the proteasome (reviewed by Wilkinson, 2000). Although the I93M mutation decreases the activity of this deubiquitinating enzyme, mice null for UCH-L1 do not display dopaminergic neurodegeneration (Saigoh et al., 1999). Rather, they develop an axonopathy affecting primary sensory axons in the gracile nucleus of the medulla, whose cell bodies reside in the dorsal root ganglia (Saigoh et al., 1999). Additionally, a polymorphism (S18Y) of UCH-L1 appears to be protective for the development of PD (Maraganore et al., 1999; Levecque et al., 2001; Satoh and Kuroda, 2001). Aside from its deubiquitinating function, UCH-L1 exerts a previously unrecognized ubiquitin ligase activity upon dimerization (Liu et al., 2002). Both the I93M mutation and the S18Y polymorphism alter UCH-L1 ligase activity in a manner consistent with the hypothesis that impaired activity of the ubiquitin proteasome system is critical in PD pathogenesis: UCH-L1 ligase activity is decreased by the pathogenic I93M mutation and increased by the protective S18Y polymorphism (Liu et al., 2002).

DJ-1

DJ-1 mutations were identified in two consanguineous pedigrees with autosomal recessive PD (Bonifati et al., 2002). One family carried a deletion predicted to abolish protein function, while the other harbored a missense mutation that results in the insertion of a proline into an α-helical region. Expression of this proline mutant form of DJ-1 appears to lead to its accumulation in mitochondria (Bonifati et al., 2002), and DJ-1 has been implicated as a cellular monitor of oxidative stress (Mitsumoto and Nakagawa, 2001; Mitsumoto et al., 2001).

Synuclein-Based Models

All published genetic models of PD have been based on α-synuclein, primarily the transgenic overexpression of mutant or wild-type forms in mice or flies (Masliah et al., 2000; van der Putten et al., 2000; Feany and Bender, 2000; Matsuoka et al., 2001; Giasson et al., 2002; Lee et al., 2002b). In general, these studies demonstrate that transgenic overexpression of α-synuclein causes neurotoxicity but that α-synuclein ablation is not associated with neuropathological changes, supporting the notion that PD-causing mutations operate via a toxic gain-of-function mechanism. However, a striking disappointment of the α-synuclein transgenic mice has been a complete failure to model dopaminergic neurodegeneration (i.e., actual cell death). Instead, these mice display a variety of neuropathologic changes, including neuronal atrophy, dystrophic neurites, and astrocytosis accompanied by α-synuclein-positive LB-like inclusions. Indeed, compared to other neuronal populations, murine dopaminergic neurons appear inexplicably resistant to α-synuclein-induced neurotoxicity, even in the face of marked accumulations of the protein (Matsuoka et al., 2001; Giasson et al., 2002; Lee et al., 2002b), significantly limiting the utility of these models.

In contrast to the transgenic mouse studies, two groups have demonstrated that the injection of human α-synuclein expressing viral vectors into the substantia nigra of adult rats causes the selective death of dopaminergic neurons accompanied by synuclein-containing inclusions and other pathologic changes reminiscent of those observed in PD (Kirik et al., 2002; Lo Bianco et al., 2002). The reasons for the discrepancy between the rat and mouse studies are not clear. Significantly higher levels of α-synuclein expression may be achieved with the viral vectors, or it may be important that, in contrast to transgenic mice, in these models α-synuclein is suddenly overexpressed during adulthood. It is also possible that a species-dependent difference in susceptibility to α-synuclein toxicity exists between mice and rats. While the viral vector approach will be useful for certain studies, it has significant limitations. Most importantly, because the investigator must generate each individual animal, it is technically challenging to produce large cohorts of rats that express similar amounts of protein in

a consistent anatomic pattern. Thus, unlike the situation with heritable transgenes, each rat is in effect an independent experiment. Furthermore, this approach does not allow investigators to take advantage of the large number of mouse mutants or genetic strategies available in mice that would greatly facilitate the further assessment of the molecular mechanisms of this synuclein-dependent dopaminergic neurodegeneration.

Overexpression of either wild-type or mutant α-synuclein in *Drosophila* leads to LB-like synuclein-containing inclusions and loss of dopaminergic neurons, as well as a behavioral abnormality that appears to be corrected by levodopa or DA agonists (Feany and Bender, 2000; Pendleton et al., 2002). This model should be particularly useful for genetic screens to identify novel genes involved in α-synuclein-mediated neurodegeneration.

While these transgenic studies suggest that a component of cellular toxicity may derive from α-synuclein aggregates, the relationship between aggregate formation and neurodegeneration is not straightforward. Although all reports of α-synuclein-related toxicity feature α-synuclein-containing aggregates, there exist clear examples of dissociation between aggregate formation and neurodegeneration. For example, in *Drosophila*, transgenic coexpression of the chaperone Hsp-70 prevented the dopaminergic neuronal loss caused by α-synuclein but did not affect the number of α-synuclein-containing aggregates (Auluck et al., 2002), arguing against a direct role for inclusions. Similarly, lentiviral-mediated expression of wild-type rat α-synuclein in rats led to aggregates but no cell loss (Lo Bianco et al., 2002). Thus, it is possible that a soluble misfolded species of α-synuclein or an increase in normal α-synuclein function rather than or in addition to inclusion formation contributes to the cellular toxicity observed in some of these studies.

Conclusions and Future Directions

In the past 20 years, two discoveries have profoundly influenced our understanding of PD pathogenesis, provided a conceptual framework for novel therapies, and spawned an accelerating research effort. First, the discovery of MPTP-induced PD and subsequent research exploring the molecular basis of MPTP-induced neurodegeneration established relationships between mitochondrial function, oxidative stress, and neurodegeneration. Second, the discovery of genetic causes of PD and the demonstration that dysfunction of these genes probably plays a role in sporadic PD has highlighted the importance of protein misfolding-related toxicity as a fundamental insult in neurodegeneration. The identification of PD-causing genes has also demonstrated how dysfunction of the ubiquitin-proteasome system can provoke neurodegeneration, presumably by leading to an excess of misfolded proteins.

Looking forward, a number of goals clearly emerge from the discovery of multiple PD-related genes. Future work must search for links between the molecular pathways modified by these disease-associated genes. A related goal will be to understand the relationship between previously identified factors in PD neurodegeneration (e.g., mitochondrial dysfunction, ROS) and the molecular events provoked by disease alleles. A specific aspect of this work should be to clarify primary initiating events from those that may be a nonspecific consequence of neuronal demise. While the identification of PD genes has also allowed the generation of etiologic-specific PD animal models, none of these models manifests the crucial feature of the disease: relatively selective degeneration of dopaminergic neurons. This is a vital future goal, as it would enable investigators to explore the unique features of dopaminergic neurons that make them preferentially susceptible to neurodegeneration in PD as well as to test novel therapies.

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