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PRINCIPAL INVESTIGATOR: Serge E. Przedborski, M.D., Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032

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13. ABSTRACT (Maximum 200 Words) MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is the tool of choice for investigations into the cascade of events that lead to Parkinson's Disease (PD). Herein, we investigated the role of inflammation in PD using the MPTP mouse model of PD. We found that MPTP causes a robust microglial activation which is accompanied by the up-regulation of inducible nitric oxide synthase (iNOS) and of cell surface markers such as macrophage activating complex-1 (MAC-1) as well as the production of cytotoxic molecules such as reactive oxygen species (ROS) nitric oxide (NO) and a host of proinflammatory cytokines like interleukin-1 β , cyclooxygenase-2 and the prostaglandin PGE ₂ . We also noted that NADPH oxidase, a multimeric enzyme that is a major source of the superoxide radical, is up-regulated in ventral midbrains of MPTP-treated mice and in tissues from PD brains. Although both the COX-2 enzyme and PGE ₂ were also up-regulated in MPTP-treated mouse ventral midbrains, only COX-2 was up-regulated in PD brains. Minocycline, a tetracycline antibiotic that has anti-inflammatory action independent of its antimicrobial action, attenuated most of the microglial-related events caused by MPTP. Thus, inflammation may push the progressive nature of PD through microglial activation and may be an ideal point for therapeutic intervention in PD.					
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Introduction.

Parkinson's Disease (PD) is a common neurodegenerative disorder characterized by tremor, rigidity, akinesia and postural instability, all attributed to a severe loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) which results in a dramatic reduction in the neurotransmitter dopamine (DA) due to the degeneration of the dopaminergic terminals that project to the striatum (1). This disorder affects about 1 million people in North America alone with about 50,000 newly diagnosed cases each year (2). Symptoms of PD can be alleviated with the use of levodopa and other dopamine agonists, but, these do not stop the progression of the disease (3). Many hypotheses abound as to the etiology of PD, however, it is the oxidative stress theory that seems to take precedence over the others. In our investigations of PD and the oxidative stress theory of PD, we have used the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to demonstrate that both the superoxide radical and nitric oxide are pertinent to the neurodegenerative process in the SNpc (4, 5, 6). From our work and the work of other investigators, we noted that the two can interact to produce the tissue-damaging peroxynitrite which can inflict damage to lipids, proteins and amino acids (7). We also noticed that there is an inflammatory component to the neurodegenerative process which was not only seen in the MPTP-treated mouse model of PD (8) but also in PD patients who developed their PD as a result of the injection MPTP during drug-abuse behavior (9). In this inflammatory component, there is a massive activation of microglia (8, 10) in the SNpc of MPTP-treated mice and in PD patients. This activation of microglia was accompanied by the up-regulation of inducible nitric oxide synthase (iNOS) within the microglia and the formation of 3-nitrotyrosine (6), the premier fingerprint which indicates that nitrative damage to proteins, lipids and amino acids had occurred in brain extracts from the substantia nigra (SN) following MPTP treatment (11). What was extremely important here was the fact that this microglial activation occurred during the most active phase of neuronal death in the MPTP mouse model of PD (12). Furthermore, we were able to block both the microglial activation and the iNOS up-regulation using the second generation tetracycline antibiotic minocycline (13). Thus, we surmised that microglial activation must be germane to the neurodegenerative process and should be investigated further. Since other inflammatory agents such as interleukin-1-beta (IL-1 β), the prostaglandin PGE₂ and cyclooxygenase-1 and -2 (COX1 and COX-2) are all up-regulated during inflammation (13), it is of keen interest to demonstrate whether these compounds are also up-regulated during the inflammatory phase of the MPTP neurotoxic process and what are their contributions to the neurodegenerative process in the MPTP mouse model and in PD.

Body of the Research.

Our overall long-term goal is the study of the pathogenesis of PD based on the oxidative stress hypothesis of PD. The recent findings of microglial activation during the most active phase of neuronal degeneration along with the up-regulation of iNOS and with the additional knowledge that other factors are involved in the inflammatory process led us to outline a series of experiments that, hopefully, will help us to decipher, in a step by step fashion, the cascade of events that are germane to the up-regulation of microglia and to the progression of DA neurodegeneration, and along the way, lead to possible therapies

to improve the symptomology of or to stop the progression of PD. In continuing our line of reasoning about the oxidative stress theory of PD, our research plan should produce strong support to the involvement of inflammation in the neurotoxic process following MPTP administration. For instance, in **Specific Aim I**, we will determine the role of microglial activation in the MPTP neurotoxic process by administering different doses of MPTP to mice pretreated with different doses of minocycline, a drug known to block microglial activation and assessing neuroprotection in the SNpc using HPLC, immunostaining and Western blot analyses. We will also employ primary mixed neuronal/microglial cultures to study more definitively the role of activated microglia in MPTP-mediated DA neuronal death and the contribution of noxious factors here. We will also assess, by pharmacological intervention, the beneficial effects of inhibiting such noxious factors. In **Specific Aim II**, we will define the role of NADPH oxidase in the MPTP neurotoxic process at different time points in MPTP-treated mice that are deficient in NADPH oxidase. Neuroprotection and microglial activation will be assessed as in Specific Aim I. In **Specific Aim III**, the contribution of another inflammatory factor, IL-1 β will be assessed in different brain regions and at different time points following MPTP administration to mice deficient in IL-1 β and in IL-1R1 (interleukin 1 receptor1). Neuroprotection and microglial activation will be assessed as in Specific Aim I. Finally, **Specific Aim IV** will examine the contribution of the prostaglandin PGE₂ to MPTP neurotoxicity by assessing the roles of the PGE₂-synthesizing enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), two other inflammatory factors, in the MPTP neurotoxic process in different brain regions and at different time points using different doses of the toxin. For this, COX-1 and COX-2 knockout mice will be used. This comprehensive set of experiments should provide insight into the role and the mechanism of inflammation in the MPTP neurotoxic process and in PD as well as identify targets for intervention here.

Key Research Accomplishments

Core A.

Core A, the administrative arm of the Parkinson's Disease Research Program, provides the centralized scientific leadership necessary for this program to succeed. While its members have not yet met physically, they have been in constant contact with each other for continual reviews of the program and its progress.

Core B.

Core B is the centralized MPTP facility which is located on the 19th floor of the College of Physicians and Surgeons at Columbia University. Its role is to support the research activities of all three projects. There are four specific aims which constitute the work of Core B. The **first** of these is to maintain this facility in perfect order and to keep, a line of communication with the Animal Care Facility. The **second** is the performance of the needed injections for the necessary experiments of the Program Project. The **third** is the standardization of sample preparation and the **fourth** is the storing and shipment of the needed samples. Thus far, the MPTP facility has been maintained in proper order and its inspection record by members of the Institutional Animal Care and Use Committee here at Columbia University shows no violations of any sort. No viruses such as MHV have been detected in this facility.

Mice are housed by animal care technicians and are placed outside of the MPTP Facility. Whenever there is a delivery, Core B is informed and a member of the Core B staff puts the housed mice into the MPTP room. The delivery is logged in and the animals are left to acclimate for one week prior to injection. Experiments for tissues necessary for the Program Project are discussed, worked out and scheduled with Dr. V. Jackson-Lewis. Once this is done, injection schedules are set and injections are performed by Dr. Jackson-Lewis. Samples are then collected according to the scheduled experiment, are used here at Columbia or sent as per the scheduled experiments to the individual who needs them. No glitches in shipping samples have occurred thus far. To insure that samples are prepared properly, quality controls are run frequently with experimental samples on the HPLC. Research fellows here at Columbia are trained on how to handle MPTP samples by Dr. Jackson-Lewis or Dr. Vila. All research fellows who handle the MPTP-treated mice are also required to read our paper regarding the safe handling of MPTP mice and samples (14).

Specific Aim I.

Cell Culture Experiments.

These experiments have not yet begun. However, we have just finished setting up a temporary cell culture facility. This facility is temporary because our permanent laboratories are undergoing renovation at this time. The target date for completion of our part of the entire renovation is the middle of 2004.

In Vivo Experiments.

From previous experiments, we noted that aside from a dramatic loss of DA neurons in the SNpc following MPTP administration, there is significant microglial activation in this same brain region which seems to occur at relatively the same time as does DA neuronal death (8, 10, 11). Although gliosis can have beneficial effects in many situations, it can also have cytotoxic effects. For instance, microglia normally exist in a resting state in the brain, however, upon injury to specific areas, they have the ability to react promptly, quickly proliferate, become hypertrophic and increase the production of a variety of marker molecules (13). Marker molecules such as iNOS, macrophage antigen complex-1 (MAC-1), nitric oxide (NO) and a host of proinflammatory cytokines including IL-1B (15), COX-2 (16, 17) and the prostaglandin PGE₂ (18) have been linked to pathological and inflammatory events, thus, it can be surmised that these must also serve the same function in the brain. To address this issue and to test the effects of blocking microglial activation, we administered an acute regimen of MPTP (16-20mg/kg i.p. x 4 doses over 8 hours) to C57/bl6 mice from Charles River. Some of the mice also received minocycline (1.4-45 mg/kg i.p. x 2 daily for five days) (19).

Results (See Attached Publication for Figures)

Minocycline attenuates MPTP-induced dopaminergic neurodegeneration.

MPTP alone (18mg/kg in 4 doses over 8 hours) caused a significant reduction (55%) in the number of DA neurons in the SNpc of treated mice compared to saline-treated mice as evidenced by TH immunostaining). In MPTP-treated mice that received minocycline, the number of surviving SNpc TH-positive neurons was significantly increased (figure 1a-1g). Whereas minocycline at a dose of 1.4-5.6mg/kg twice daily had no neuroprotective effect on MPTP-induced DA neurodegeneration in the SNpc, doses of 11.25mg/kg and higher showed a significant neuroprotective effect (figure 1g). In mice given 16mg/kg of MPTP in four

doses over 8 hours, this lower regimen also caused significant (30%) though less damage to the SNpc compared to the 18mg/kg dose. Minocycline at 45mg/kg twice daily increased the survival of SNpc DA neurons to 90%. Since the sparing of DA neurons does not always translate to the sparing of DA terminals, we assessed the effects of the two doses of MPTP (18 and 16mg/kg x 4 doses) with and without minocycline (45 mg/kg twice daily) on DA terminals in the striatum of these mice. Both doses of MPTP reduced striatal TH immunoreactivity significantly (96% and 79%, respectively) compared to control (figure 2c, 2e). Whereas mice that received the 18 mg/kg x 4 doses of MPTP and minocycline (45 mg/kg twice daily) showed no neuroprotection of the DA terminals in the striatum (figure 2c, d), mice that received the 16 mg/kg x 4 doses of MPTP and minocycline (45mg/kg twice daily) exhibited a significant sparing of the striatal DA nerve terminals (figure 2e-2f). Figure 2g represents the optical densities of striatal DA fibres in the various groups of mice.

Minocycline decreases MPTP-mediated nitrotyrosine formation.

A significant part of the MPTP neurotoxic process is mediated by NO-related oxidative damage which can be assessed by measuring 3-nitrotyrosine (NT) formation. NT is the indicator that nitration of specific proteins has occurred (6, 20). In saline-injected and minocycline-injected mice, ventral midbrain and cerebellum (area unaffected by MPTP) NT levels were similar. Conversely, MPTP (18mg/kg in 4 doses over 8 hours) caused a significant elevation in ventral midbrain NT levels while not disturbing cerebellar NT levels (Table 1). In the MPTP-minocycline treated animals, levels of NT were almost, but not quite, normalized.

MPTP metabolism is unaffected by minocycline treatment

To make sure that the protective effect on SNpc DA neurons afforded by minocycline was not caused by any interference in the MPTP metabolic process, we assessed the uptake and metabolism of MPTP in striatal tissues in the presence and absence of minocycline (Table 2). We measured striatal levels of MPP⁺, the toxic metabolite of MPTP, 90 mins after a single injection of MPTP, 18mg/kg. Minocycline was administered pre- and post-MPTP injection. We used [³H]-MPP⁺ for the uptake studies and lactate measurements for mitochondrial function studies. Investigations here showed that minocycline given post-MPTP treatment did not interfere with the uptake of MPTP into the striatum nor with its metabolism.

Minocycline inhibits MPTP-induced microglial activation.

To demonstrate that the neuroprotective effect of minocycline is attributed to its inhibition of microglial activation, we examined the expression of MAC-1 (a specific marker for microglia) and GFAP (a specific marker for astrocytes) in MPTP-treated (18mg/kg x 4 doses over 8 hours) and in MPTP-minocycline (45 mg/kg x twice daily) mice using immunostaining techniques. In saline- and minocycline-treated mice, only a few faintly stained cells were observed on MAC-1 and GFAP immunostaining in both SNpc and striatum. In contrast, in MPTP-treated mice, a robust response to the MAC-1 antibody was noted as early as 24 hours after the last injection of MPTP in both SNpc and striatum (figure 3) that was effectively blocked by minocycline (45mg/kg) (figure 3). The GFAP response, though not nearly as strong as the microglial response at 24 hours after the last dose of MPTP, was also seen (figure 4) and was stronger at 7 days after MPTP than at 24 hours after MPTP. Minocycline did not block the astrocytic response to MPTP (figure 4). Similar to the responses demonstrated by immunostaining, administration of minocycline blocked the

MPTP microglial response but not the astrocytic response to MPTP (figure 5) on Western blot analyses.

Minocycline prevents the production of microglial-derived cytotoxic mediators.

Given the effect of minocycline on MPTP-induced microglial activation, we assessed the effects of minocycline on the production of known cytotoxic compounds such as NO, IL-1 β and ROS that are produced within microglia using Western blot techniques. Figure 6 shows that at 24 hours after MPTP treatment (18 mg/kg regimen), there was significant up-regulation of IL-1 β activity (figure 6a) in ventral midbrain extracts from MPTP-only mice that was effectively attenuated by minocycline administration (45mg/kg). Significant levels of NADPH oxidase (assessed by the translocation of its subunit p⁶⁷ from the cytosol to the plasma membrane) and iNOS, two enzymes prominent in the production of the superoxide radical and NO, respectively, were induced 24 hours after the last dose of MPTP in a manner similar to that of IL-1 β activity (figure 6b, 6c) and were also effectively suppressed by minocycline administration.

Minocycline confers resistance to MPTP beyond iNOS ablation.

Since we have demonstrated previously that iNOS ablation attenuates MPTP neurotoxicity (6), in the last experiment of this series of experiments, we tested whether the minocycline protective effect is due solely to its blockade of microglial activation in the MPTP-treated mouse by comparing the effect of MPTP treatment (16mg/kg x 4 doses over 8 hours) in iNOS deficient mice that received or did not receive minocycline (45 mg/kg). Figure 7 shows that MPTP caused a significant and equal reduction in TH-positive fibres in the striatum of both iNOS-deficient mice and their wild-type littermates which is consistent with our previous data showing that iNOS ablation protects SNpc DA neurons but not striatal DA fibres against MPTP. In contrast, iNOS deficient mice that received both MPTP and minocycline exhibited optical densities at least two-fold greater than that of the iNOS deficient mice that received MPTP only. There was, however, no difference in TH optical densities between the iNOS-deficient MPTP-minocycline-treated mice and their wild-type littermates.

Specific Aim II.

Epidemiological studies suggest that inflammation increases the risk for developing neurodegenerative condition such as Parkinson's Disease (21). Consistent with this is the fact that experimental models of PD demonstrate significant microglial activation (6, 8) which suggests that inflammatory factors either initiate or modulate SNpc DA neuronal death. Among the inflammatory mediators capable of promoting DA neurodegeneration are microglial-derived reactive oxygen species (ROS) which probably contribute to the oxidative stress that is reportedly part of the pathogenic hypothesis of PD (21). Now, a significant source of ROS during inflammation is NADPH oxidase. This multimeric enzyme is composed of 4 subunits (GP^{91phox}, p^{22phox}, p^{47phox} and p^{40phox}) and is inactive in resting microglia because p^{47phox}, p^{67phox} and p^{40phox} are all present in the cytosol as a complex and are separated from the transmembrane proteins, GP^{91phox} and p^{22phox} (22). When microglia become activated, p^{47phox} is phosphorylated and the entire complex translocates to the plasma membrane where it assembles with GP^{91phox} and p^{22phox} to form the NADPH oxidase complex, which is now capable of reducing oxygen to the superoxide radical (22). The superoxide radical, in turn, gives rise to secondary reactive oxygen species (22) Although NADPH oxidase is known to be instrumental in the killing of invading microorganisms in

infections through the sustained production of the superoxide (22), its role in the neurodegenerative process seen in PD is unknown. Thus, the purpose of Specific Aim II is to study the role of NADPH oxidase in inflammation induced by MPTP administration.

Animals and treatment

Eight week old male C57/BL6 mice (Charles River Laboratories), mice deficient in GP^{91phox} and their wild-type littermates were used here. Mice received MPTP (16 mg/kg x 4 doses over 8 hours); controls received saline only. Minocycline (45 mg/kg x 2) daily was given as described (13). Bovine erythrocyte superoxide dismutase 1 (SOD1, 20U/hour) was infused into the striatum via Alzet osmotic minipumps, starting 24 hours before MPTP treatment and continuing for 5 days after MPTP treatment. Mice were sacrificed from 1 to 7 days after MPTP treatments.

Results (See Attached Publication for Figures)

NADPH oxidase is induced in ventral midbrain of mice during MPTP treatment.

In MPTP-injected mice, ventral midbrain GP^{91phox} , p^{67phox} and MAC-1 mRNA all show, at around 2 days post MPTP injection, time dependent increases (Figure 1a-1d) whereas in saline-injected controls, these subunits are almost non-existent. In situ hybridization for GP^{91phox} reveals no specific nonradioactive labeling in the SNpc of saline-injected mice whereas in MPTP-treated mice, there is a considerable amount of labeling (Figure 1e-1f). These results demonstrate that NADPH oxidase is induced following MPTP injections and that this induction is time-dependent.

NADPH oxidase is expressed in activated microglia after MPTP injection.

To examine the location of NADPH oxidase induction, we assessed the immunoreactivity of GP^{91} in mice treated with MPTP. On Western blot analyses, ventral midbrain GP^{91} levels patterned those of NADPH oxidase (2a, b). In the immunostaining studies, saline-injected mice showed a mild immunoreactivity to the GP^{91} antibody in the SNpc, the area of the ventral midbrain that houses TH-positive neurons (figure 2c,d). Immunoreactivity was found in small cells with thin ramifications reminiscent of resting microglia (figure 2e, f). In MPTP-treated mice, robust GP^{91} immunoreactivity was noted in the SNpc in larger cells (figure 2g,h) with thick short ramifications reminiscent of activated microglia (figure 2j). Similar GP^{91} immunoreactivity alterations were noticed in the striatum of these same mice. On confocal examination of the MPTP-treated tissues, GP^{91} immunoreactivity seemed to co-localize with MAC-1 (figure 2k-2m) and not with GFAP (Figure 2n-2p) or TH immunoreactivity. These results demonstrate that NADPH oxidase is upregulated in microglia in response to MPTP administration.

GP^{91} expression is increased in PD midbrains.

Using both Western blot analyses (figure 3a, b) and GP^{91} immunostaining (figure 3c, d), we have observed increases in GP^{91} activity in post-mortem tissues from the SNpc of PD brains when compared to control brains. Results here were consistent with our MPTP results.

Lack of ROS production in GP^{91} -deficient mice after MPTP administration.

To demonstrate ROS production, particularly the production of superoxide radicals (23) or the lack thereof, we injected ethidium bromide into saline- and MPTP-treated mice and examined the ethidium fluorescence in the SNpc of these mice at 2 days after MPTP administration. Little to no ethidium fluorescence was seen in saline-injected mice (figure 4a) and there was no microglial activation on MAC-1 immunostaining in the same tissue

section as demonstrated in figure 4b. In contrast, a strong ethidium fluorescent response was observed in the SNpc of wild-type littermates at 2 days after MPTP administration (figure 4c) which was attributed to the robust microglial activation that was evident in the same tissue section (figure 4d) and which indicated NADPH oxidase up-regulation through the translocation of p^{67phox} from the cytosol to the plasma membrane. GP⁹¹-deficient mice, on the other hand, displayed no evidence of ethidium fluorescence in the SNpc in the face of a mild activation of microglia (figure 4e, f). Furthermore, wild-type MPTP-treated mice given minocycline also showed no SNpc evidence of ethidium fluorescence (figure 4g) even though a very mild activation of microglia (figure 4h) was present. These results indicate that without the presence of GP91, there is no production of superoxide radicals following MPTP treatment and that minocycline in blocking microglial activation, attenuates ROS production in MPTP-treated mice.

NADPH oxidase deficiency protects against MPTP-induced neurodegeneration.

We next asked if NADPH oxidase deficiency can protect SNpc DA neurons against the damaging effects of MPTP. Stereological counts of TH-positive neurons in the SNpc of saline-treated mice did not differ between wild-type and GP⁹¹-deficient mice (figure 5a). In contrast, in MPTP-treated mice, both wild-type and GP⁹¹-deficient SNpc showed a significant decrease in numbers of TH-positive neurons, however, the GP⁹¹-deficient SNpc exhibited higher numbers of TH-positive neurons when compared to their treated wild-type littermates (figure 5a). Furthermore, as in the SNpc, the density of the TH-positive fibres in the striatum was greater in GP⁹¹-deficient mice than in their wild-type littermates (figure 5b). Because of the neuroprotective effect observed in the SNpc of the GP⁹¹-deficient mice, we examined MPTP metabolism in these mice. No differences in MPTP uptake and metabolism were found in striatal and ventral midbrain tissues from MPTP-treated wild-type and GP⁹¹-deficient mice. Thus, the protective effect afforded by GP⁹¹-deficiency is not attributed to any interference in MPTP metabolism.

NADPH oxidase damages ventral midbrain proteins.

We next assessed the extent of NADPH oxidase-related oxidative damage to proteins in MPTP-treated mice by examining protein carbonyl content in ventral midbrain from MPTP-treated mice with a GP⁹¹ deficiency. GP⁹¹ deficient mice had carbonyl content levels that were not different from control values whereas their wild-type littermates show a significant elevation in carbonyl content which indicates that the presence of NADPH oxidase is necessary for protein oxidation to occur following MPTP injections (figure 6a).

MPTP-induced neurotoxicity is attenuated by scavenging extracellular superoxide.

In the final experiment of this series of experiments, we assessed the noxious role of extracellular ROS production in the MPTP mouse model of PD. Prior to MPTP treatment, the membrane-impermeant enzyme superoxide dismutase 1 (SOD1) was infused into the left striatum of mice via Alzet minipumps. Mice were then injected with MPTP (18 mg/kg x 4 doses over 8 hours) and the striatum and SNpc were examined at 7 days after the last MPTP injection. In the MPTP-treated mice, the infused side of the brain showed protection of both TH fibres in the striatum and DA neurons in the SNpc (6b, c). In contrast to these results, the non-infused side of the brain showed significant damage to TH fibres in the striatum and to TH-positive neurons in the SNpc. These results demonstrate that extracellular oxidative stress is necessary for SNpc damage to occur following MPTP administration.

Specific Aim III

In this Specific Aim, we are to assess the contribution of IL-1 β to MPTP neurotoxicity by examining the protein expression of IL-1 β mRNA and its receptor, IL-1R1 in different brain regions and at different time points using different concentrations of MPTP in normal and in IL-1 β deficient mice. We have not started these experiments yet. However, we have started to fine tune all of the morphological and Western blot analyses parameters necessary for this set of experiments. We will begin our breeding program for the mice for Specific Aim III shortly to acquire the numbers needed for this part of the project.

Specific Aim IV

The inflammatory response in the brain is thought to contribute to the neurodegenerative process typical of PD and Alzheimer's disease. (21). This response contains several different facets including microglial activation and proinflammatory and cytokine up-regulation (15, 16, 17, 18). Since we are investigating the contribution of inflammation to the neurodegenerative process, it is necessary to examine the components which contribute to this process. Two such components are the cyclooxygenase (COX) enzymes, COX-1 and COX-2. The COX enzymes convert arachidonic acid to prostaglandin PGH₂, the precursor of the prostaglandin PGE₂ and several other prostanoids (17). Whereas COX-1 is constitutively expressed in many eukaryotic cells and is relevant to the production of prostanoids in physiological processes (17), induction of COX-2 expression and elevation of PGE₂ have been implicated in degeneration in a variety of situations (17). Thus, it seems logical to investigate the role of COX-2 in the MPTP mouse model as it relates to the neurodegenerative process in PD.

Animals and treatment

COX-1 and COX-2 deficient mice and their respective wild-type littermates were obtained from Taconic Farms. Wild-type C57/BL/6 mice were obtained from Charles River Laboratories. Mice, 8-10 week old males, received MPTP, 20 mg/kg in 4 doses over 8 hours and were sacrificed at 0 to 7 days after the last dose of MPTP. Some of the mice received Refocoxib (a gift from Merck Frosst Labs; 12.5-50 mg/kg dissolved in methylcellulose) orally by gavage daily for 5 days before and after MPTP treatment. Control mice received saline and/or methylcellulose only.

Results. (See Attached Publication for Figures)

MPTP induces COX-2 expression and activity in mouse ventral midbrain.

To determine whether the expression of COX isoforms is affected during nigrostriatal neurodegeneration, we assessed the contents of COX-1 and COX-2 mRNA and protein in ventral midbrains of saline- and MPTP-treated mice at different time points. Cox-1 mRNA and protein were detected in saline-treated ventral midbrains and were not significantly altered by MPTP injection (figure 1A, B, D, E); although there were transient alterations in COX-1 mRNA but not in protein content at 2 and 4 days after MPTP, these alterations were not significant. In contrast, while COX-2 was not detected in saline-injected mice, it was evident by the 1st day, peaked by the 4th day and could still be detected at 7 days after MPTP injections (figure 1A, C, D, F). To determine whether the MPTP-related up-regulation of COX-2 paralleled an increase in COX-2 activity, we quantified ventral midbrain levels of PGE₂. Levels of PGE₂ were detected in saline-treated mice and showed increases at 2 and 4

days following MPTP administration coincidental to the changes in COX-2 expression (figure 1G). Thus, COX-2, but not COX-1, is up-regulated in the MPTP mouse model.

COX-2 is induced in SNpc dopaminergic neurons after MPTP administration.

Immunohistochemical analyses showed faint immunoreactivity for COX-2 in the SN of saline-treated mice (figure 2A, B). In the MPTP-treated mice, at 2 and 4 days after the last injection, ventral midbrain immunostaining in the neuropil was increased showing several COX-2 positive cells in the SNpc with a neuronal morphology (figure 2C, D). These COX-2 positive neurons showed immunoreactivity in both the cytoplasmic and nuclear areas, consistent with the known cellular distribution of COX-2. On double immunofluorescence analysis, it was demonstrated that the COX-2-positive cells were neuronal and almost all were dopaminergic (figure 2E-G). Furthermore, COX-2 immunoreactivity did not colocalize with MAC-1 (microglial marker) or with GFAP (astrocyte marker) (figure 2H-M). These data show that COX-2 is up-regulated primarily in midbrain dopaminergic neurons following MPTP treatment.

COX-2 up-regulation in postmortem midbrain samples from PD brains.

We next assessed whether the COX-2 changes seen in MPTP-treated mice were also present in midbrain samples from PD brains. Consistent with the MPTP findings, PD samples had significantly higher COX-2 protein and PGE₂ levels than normal brain samples (figure 3A, B). No changes were noted in PGE₂ levels in the striatum of the PD brain samples. Although COX-2 immunoreactivity was found in the normal control brain samples (figure 3C, D), it was in the cytosol and in the Lewy bodies of the neuromelanized neurons in the SNpc of the PD brains that COX-2 immunoreactivity was most prominent (figure 3E-G). The fact that results in the MPTP brains and in the PD brains were similar strengthens the value of the MPTP mouse model of PD.

Ablation of COX-2 mitigates MPTP-induced neurodegeneration.

We next asked whether COX-2 up-regulation plays a role in the nigrostriatal degeneration seen in PD and in the MPTP mouse model of PD. Thus we examined TH⁺ neuron numbers in the SNpc of COX-1- and COX-2-deficient mice and their wild-type littermates treated with MPTP. Whereas the lack of COX-1 did not decrease MPTP toxicity in the SNpc of COX-1-deficient mice, the lack of COX-2 increased the number of surviving DA neurons in the SNpc and surviving DA fibres in the striatum of the treated mice (figure 4 and Table 1). Thus, COX-2 but not COX-1 participates in the MPTP neurotoxic process affecting DA neurons in the SNpc and DA terminals in the striatum.

MPTP-induced toxicity requires COX-2 catalytic activity.

Since COX-2 can exert deleterious effects in transfected cells in the absence of its catalytic activity (24), we next asked whether a similar situation exists in the *in vivo* situation in relation to the death of DA neurons caused by MPTP. For these studies we examined the nigrostriatal DA pathway in mice that received MPTP in the absence of and in the presence of rofecoxib, a selective COX-2 inhibitor. Rofecoxib was given orally daily starting 5 days prior to and 5 days post MPTP treatment, was well tolerated and did not alter MPTP uptake and metabolism. Rofecoxib, a COX-2 inhibitor, at doses of 25 and 50 mg/kg orally, given daily for 5 days pre- and post-MPTP, completely blocked COX-2 derived PGE₂ production in ventral midbrain (Table 2). Mice injected with MPTP and receiving rofecoxib (25 or 50 mg/kg per day, orally), had about 74% and 88%, respectively, of their SNpc DA neurons survive the MPTP onslaught compared with 41% in the MPTP only group (5C-G). These same doses of this inhibitor also attenuated the loss of striatal TH-positive fibres (figure 5H)

in a dose-dependent manner. The effect of this COX-2 inhibitor was, however, less effective than COX-2 ablation (Table 2). Thus, COX-2 enzymatic function is necessary for neurotoxic effects to occur in SNpc DA neurons.

JNK activation controls COX-2 induction during MPTP-induced death.

The stress-activated protein kinase JNK can regulate COX-2 transcription in mammalian cells (25). Therefore, we asked whether MPTP-induced COX-2 up-regulation is a JNK-dependent event. In these experiments, mice received MPTP only or MPTP and the JNK inhibitor, CEP-11004. In the MPTP only mice, there was a robust activation of JNK in the ventral midbrain of these mice as evidenced by the phosphorylation of c-Jun (figure 4G) and a marked up-regulation of ventral midbrain COX-2 (figure 4H). Although CEP-11004 decreased the MPTP-induced SNpc DA neuronal death, it did not afford the same protection to striatal DA fibres (figure 4C, E and Table 1). Thus, our data shows that blocking JNK activation blocks c-Jun phosphorylation and COX-2 up-regulation (figure 4G, H) and this demonstrates that the JNK/c-Jun pathway is critical to the MPTP-mediated induction of COX-2.

COX-2 ablation and inhibition does not affect MPTP metabolism.

We have demonstrated neuroprotection against MPTP in COX-2 deficient mice as well as in mice treated with the COX-2 inhibitor rofecoxib or the JNK pathway inhibitor CEP11004. Thus, next we examined whether the ablation of COX-2 or inhibition of the COX-2 enzyme or the JNK pathway might affect MPTP uptake and metabolism by measuring MPP⁺ levels in striata 90 mins after the last injection of MPTP, striatal uptake of [3H]-MPP⁺ into synaptosomes and striatal MPP⁺-induced lactate production. Striatal MPP⁺ levels were not affected regardless of the presented treatment (Table 3) compared to MPTP only mice. Striatal uptake was also not affected regardless of the presented treatment. Furthermore, MPP⁺-induced lactate levels were also not affected by ablation or inhibition of the COX-2 enzyme.

COX-2 modulation does not alleviate MPTP-associated microglial activation.

We next asked whether the SNpc DA neuronal production of prostaglandins is involved in MPTP-induced microglial activation. As shown previously, MPTP administration to mice causes a robust microglial activation that is evidenced by elevated levels of MAC-1, iNOS, GP⁹¹ and ICE (interleukin converting enzyme) mRNAs in ventral midbrain (figure 6A-E) as well as by increases in the numbers of MAC-1-positive cells in both SNpc (figure 6G) and in striatum. Although both COX-2 ablation and inhibition attenuated SNpc DA neuron death in the MPTP-treated mouse, neither altered the MPTP-induced microglial activation nor the production of microglial-derived noxious factors (6F-H).

COX-2 mediates oxidative stress during MPTP-induced neurodegeneration.

Because COX-2 can damage intracellular protein-bound sulfhydryl groups by the oxidation of catechols such as DA (26), we asked whether this was the case following MPTP administration. Therefore, we measured levels of protein 5-cysteinyl DA, a stable modification of DA caused by the COX-2-mediated oxidation of DA, in ventral midbrains from MPTP-treated mice. Baseline levels of protein 5-cysteinyl DA were slightly lower in rofecoxib-treated than in saline-treated mice (figure 6I). However, in MPTP only-treated mice, levels of protein 5-cysteinyl DA were significantly higher (2 times) than levels of this compound in MPTP-treated mice that received rofecoxib (figure 6I).

Reportable Outcomes (See Attached Publications).

Core B

1). The MPTP Facility is up and running smoothly.

Specific Aim I: Determination of the role of microglial activation in MPTP neurotoxicity.

- 1) MPTP causes a significant and robust microglial activation in the SNpc that can be attenuated by minocycline, a second generation tetracycline antibiotic.
- 2) Minocycline decreases MPTP-induced nitrotyrosine formation in the SNpc of MPTP-treated mice.
- 3). Minocycline prevents the production of microglial-derived noxious mediators of inflammation such as iNOS, IL-1 β , ROS and NADPH oxidase upregulation.
- 4). Minocycline confers resistance to MPTP beyond iNOS ablation.

Specific Aim II: Determine the relationship of NADPH oxidase to MPTP neurotoxicity?

- 1). NADPH oxidase is induced in ventral midbrain (SNpc) during MPTP neurotoxicity.
- 2). NADPH oxidase is expressed in activated microglia after MPTP administration
- 3). GP^{91phox} expression is increased in PD brains.
- 4). Ablation of GP^{91phox} attenuates MPTP-induced ROS production as evidenced by ethidium bromide fluorescence.
- 5). MPTP-induced neurotoxicity is attenuated by scavenging extracellular superoxide.

Specific Aim IV: The contribution of PGE2 to MPTP-induced neurotoxicity.

- 1). MPTP induces COX-2 activity and expression in mouse ventral midbrain, thus it is COX-2 and not COX-1 that contributes to damaging SNpc DA neurons .
- 2). COX-2 is induced specifically in SNpc DA neurons after MPTP administration.
- 3). COX-2 is up-regulated in post-mortem samples from PD brains.
- 4). Ablation of COX-2 attenuates MPTP-induced neurodegeneration.
- 5). MPTP-induced neurotoxicity requires COX-2 catalytic activity.
- 6). JNK activation controls COX-2 induction during MPTP-induced neuronal death.
- 7). Although COX-2 ablation and inhibition abates MPTP-induced neuronal death, they do not attenuate the MPTP-induced microglial activation.
- 8). COX-2 mediates oxidative stress during MPTP-induced neurodegeneration.

Conclusions

Inflammation is a primary feature of both the MPTP-mouse model of PD (ref) and of PD itself (21). Thus, the overall goal of this project is to sort out how inflammation figures in MPTP-induced degeneration in the SNpc of the MPTP-treated mouse and in PD. The first finding here was that **MPTP causes a robust microglial activation in the SNpc of mice and men.** The found **microglial activation paralleled the degeneration of SNpc DA neurons (12)** following MPTP administration. **During this microglial activation, a number of cytotoxic elements were produced which seem to push forward DA neurodegeneration in the SNpc.** For instance, iNOS was up-regulated in the SNpc

following MPTP administration and in MPTP-induced PD (6). **The up-regulation of iNOS contributes to the increased presence of and the cytotoxic effects of NO.** Of course, NO, by itself is relatively non-toxic. But, coupled with the superoxide radical which is also produced following MPTP administration (4), these two can produce peroxynitrite, which can damage lipids, proteins and amino acids (7). **Not only was iNOS-positive cells found in the SNpc as previously (4), but these iNOS-positive cells were shown to be activated microglia.** Thus, we conclude that iNOS up-regulation is a vital part of microglial up-regulation.

Although we previously demonstrated that the superoxide radical was a necessary player in the neurotoxic effect of MPTP (4), we did not determine its source. **We demonstrated that NADPH oxidase was among the other noxious elements that are up-regulated in the SNpc following MPTP injections.** NADPH oxidase is a significant source of ROS during inflammation (22, 23). During microglial activation, $p^{47\text{phox}}$ becomes phosphorylated and the entire cytosolic complex translocates to the cell membrane where it assembles with $GP^{91\text{phox}}$ and $p^{22\text{phox}}$ forming a NADPH oxidase complex which is now capable of reducing oxygen to the superoxide radical and in turn generates other noxious oxidants. In the MPTP mouse model of PD, **we found induction of NADPH oxidase in ventral midbrain. This induction was noted to occur in activated microglia.** We also noted that **the lack of $GP^{91\text{phox}}$ lessens the production of ROS and that this defect protects SNpc DA neurons against MPTP-induced neurodegeneration since the presence of $GP^{91\text{phox}}$ contributes to the SNpc DA cell degeneration.** Furthermore, superoxide radicals, found in the cytosol of microglia in the SNpc and identified by ethidium bromide fluorescence, can be scavenged by infusing the SOD1 enzyme directly into the striatum of MPTP-treated mice. This represents another piece of evidence that free radicals, particularly the superoxide radical, are indeed involved in PD as **NADPH oxidase was also up-regulated in ventral midbrain samples from PD brains.** Moreover, because most of these noxious compounds are up-regulated following and not during MPTP injections, this leads us to believe that inflammation is not the cause of PD but rather a promoter of its progression.

Inflammation can increase the risk of developing a chronic neurodegenerative disease. Therefore, inflammatory processes associated with the increased expression of cytotoxic compounds such as cyclooxygenase-2 (COX-2) and PGE_2 have been implicated in the series of deleterious events that lead to neurodegeneration in a variety of settings. In our investigations in the MPTP mouse model, we found **up-regulation of COX-2, whose expression is found essentially in TH-positive neurons, but not of COX-1 in ventral midbrains of MPTP-treated mice along with a coincidental rise in PGE_2 contents.** Of note, is the fact that PGE_2 can also give rise to ROS (26). In this respect, **DA was modified oxidatively to protein 5-cysteinyl-DA** which can account, in part, for the overwhelming presence of the superoxide radical. Since the brain is essentially under stress following MPTP administration, stress-related factors such as JNK are likely to be up-regulated here (ref). Indeed, we found evidence that **there was some kind of interplay between JNK and COX-2, which when blocked, shut off the up-regulation of the COX-2 enzyme and the ensuing SNpc DA neuronal death.** However, neither the ablation COX-2 nor its inhibition or inhibition of the JNK pathway attenuated microglial activation that follows MPTP

injections. From this, we concluded that neuronal COX-2 up-regulation following MPTP injections was not part of the inflammatory process discussed here. The activation of microglia in the SNpc following MPTP administration as well as the baggage that accompanies it represents a possible target for intervention in the MPTP model, thus we attempted intervention using the second generation tetracycline antibiotic minocycline. Minocycline has emerged as a potent inhibitor of microglial activation (19, 27, 28) and as an effective neuroprotective agent in experimental brain ischaemia ((29, 30), in traumatic brain injury (31) and in the 6-hydroxydopamine model of PD (32)). Its anti-inflammatory property is independent of its antimicrobial action. In using minocycline in our MPTP mouse model of PD, **we found that minocycline blocks the MPTP-induced microglial activation. In doing so, it blocked iNOS and NADPH oxidase up-regulation, attenuated SNpc DA neurodegeneration and prevented nitrotyrosine formation.** Thus, minocycline may indeed be of interest as a therapeutic agent for preventing the progression of SNpc DA neuronal degeneration in PD. Furthermore, we have brought to light, using the MPTP mouse model of PD, more evidence to support the oxidative stress hypothesis of PD including several pieces of matching evidence in tissues from PD brains.

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Appendices

A. Personnel Listed/Funded under DAMD17-03-1-0002

Serge Przedborski, MD, PhD: Project Principal Investigator

Vernice Jackson-Lewis, PhD: Core B Co-Principal Investigator

Miguel Vila, MD, PhD: Core-B Co-Investigator (under this award until 30 September 2003 because Dr. Vila has obtained his own Department of Defense Award).

David Sulzer, PhD: Core A Co-Principal Investigator

Kim Tieu, PhD: Senior Research Fellow.

Delphine Prou, PhD: 1st Year Research Fellow.

Norma Romero: Medical Technician, Certified Animal Care Technician.

Julia Arias: Assistant Technician (under this award until 1 January 2003).

B. Publications/Abstracts (See Attached)

Full Papers

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi D-K, Ischiropoulos H, Przewdborski S. **Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's Disease.** J Neurosci 22: 1763-1771 (2002).

Wu, DC, Teismann P, Tieu K, Vila M, Jackson-Lewis V, Ischiropoulos H, Przedborski S. **NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's Disease.** PNAS (USA) 100: 6145-6150 (2003)

Teismann P, Tieu K, Choi D-K, Wu DC, Naini A, Hunot S, Vila M, Jackson-Lewis V, Przedborski S. **Cyclooxygenase-2 is instrumental in Parkinson's Disease neurodegeneration.** PNAS (USA) 100: 5473-5478. (2003)

Reviews

Przedborski S, Vila M. **The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model : a tool to explore the pathogenesis of Parkinson's Disease.** Ann NY Acad Sci: 991: 189-198 (2003)

Teismann P, Vila M, Choi D-K, Tieu K, Wu DC, Jackson-Lewis V, Przedborski S. **COX-2 and neurodegeneration in Parkinson's Disease.** Ann NY Acad Sci: 991: 272-277 (2003)

Przedborski S, Jackson-Lewis V, Vila M, Wu DC, Teismann P, Tieu K, Choi DK, Cohen O.
Free radical and nitric oxide toxicity in Parkinson's Disease. Adv Neurol 91: 83-94.

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

Du Chu Wu,¹ Vernice Jackson-Lewis,¹ Miquel Vila,¹ Kim Tieu,¹ Peter Teismann,¹ Caryn Vadseth,³ Dong-Kug Choi,¹ Harry Ischiropoulos,³ and Serge Przedborski^{1,2}

Departments of ¹Neurology and ²Pathology, Columbia University, New York, New York 10032, and ³Stokes Research Institute, Department of Pediatrics, Children's Hospital of Philadelphia, and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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-1 β 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-1 β ; 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 up-regulation 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-1 β (IL-1 β) (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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Correspondence should be addressed to Dr. Serge Przedborski, Departments of Neurology and Pathology, BB-307, Columbia University, 650 West 168th Street, New York, NY 10032. E-mail: SP30@columbia.edu.

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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-1 β 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 μ g of protein extracts were loaded onto the 0.2 μ m 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 pre-conjugated 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 substrate (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 GAPDH. 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 PhosphorImager 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'-diaminobenzidine (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 [³H]arginine to [³H]citrulline as described previously (Liberatore et al., 1999).

Mature IL-1 β measurement. Ventral midbrain content of mature murine IL-1 β was done as described using an enzyme-linked immunosorbent 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 [³H]1-methyl-4-phenylpyridinium (MPP⁺) was assessed in the absence and presence of minocycline (concentration ranging from 1 to 330 μ M). The assay was repeated three times, each time using duplicate samples.

Mouse tissue slices and lactate measurement. Striatal slices (300 μ m) were prepared and processed as described by Kindt et al. (1987) using 50 μ M MPP⁺ and varying concentrations of minocycline (0–333 μ M). 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 \pm 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 1G, 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. 1C,G). In MPTP-treated mice, minocycline increased significantly the number of surviving SNpc TH-positive neurons in a dose-dependent manner (Fig. 1D,G). Minocycline at a dose of 1.4 mg/kg twice daily had no effect on MPTP neuro-

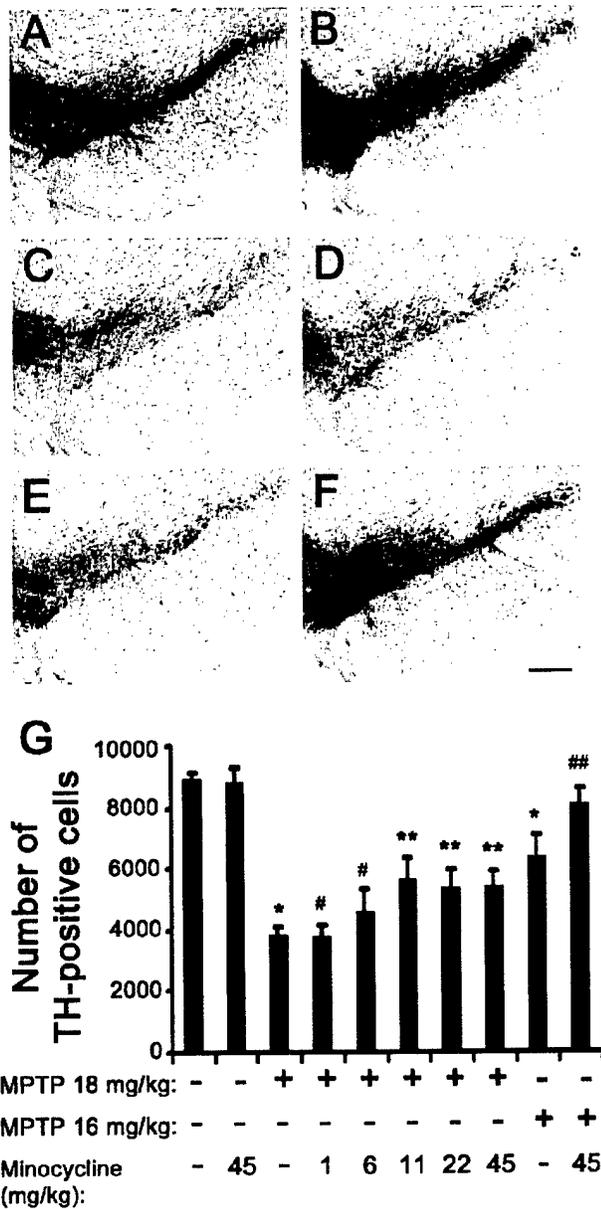


Figure 1. Effect of minocycline on MPTP-induced SNpc dopaminergic neuronal death. In saline-injected control mice treated without (A) or with (B; 45 mg/kg twice daily) minocycline, there are numerous SNpc TH-positive neurons (brown; A, B). MPTP (18 mg/kg for 4 injections) reduces the number of SNpc TH-positive 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 μ m. 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 \pm SEM ($n = 6-8$ per group).

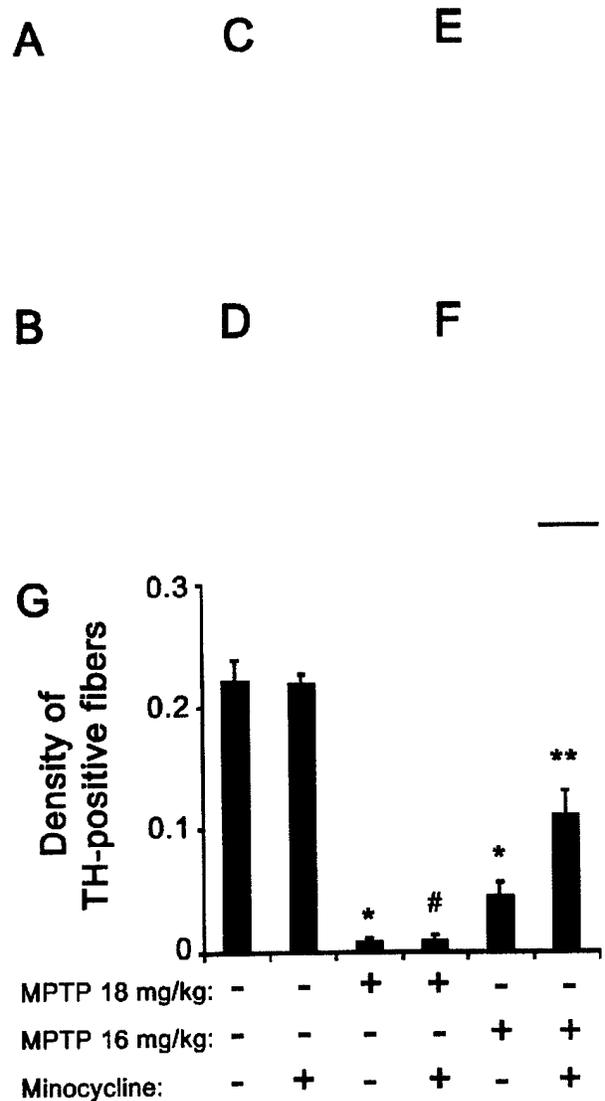


Figure 2. Effect of minocycline on MPTP-induced striatal dopaminergic fiber loss. In saline-injected control mice treated without (A) or with (B; 45 mg/kg twice daily) minocycline, there are a high density of striatal TH-positive fibers. MPTP (18 mg/kg for 4 injections) reduces the density of striatal 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 \pm SEM ($n = 6-8$ per group).

toxicity, whereas at doses of 11.25 mg/kg twice daily and higher, there was significant neuroprotection (Fig. 1G). 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	MPTP only	MPTP–minocycline pretreatment	MPTP–minocycline post-treatment
Treatment μg/gm tissue	6.42 ± 0.92	5.21 ± 0.66	6.52 ± 0.59
[³ H]MPP ⁺ uptake			
Minocycline (μM)	0	10	100
% of control	100	98 ± 3	96 ± 3
MPP ⁺ -induced lactate			
Minocycline (μM)	0	10	100
μM/100 mg protein	74 ± 4	71 ± 6	70 ± 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. 1E,G). Minocycline at 45 mg/kg twice daily produced >90% protection against MPTP at 16 mg/kg for four injections (Fig. 1F,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. 2C,E,G). Mice that received minocycline (45 mg/kg twice daily) and four injections of 18 mg/kg MPTP (Fig. 2D,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. 2F,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

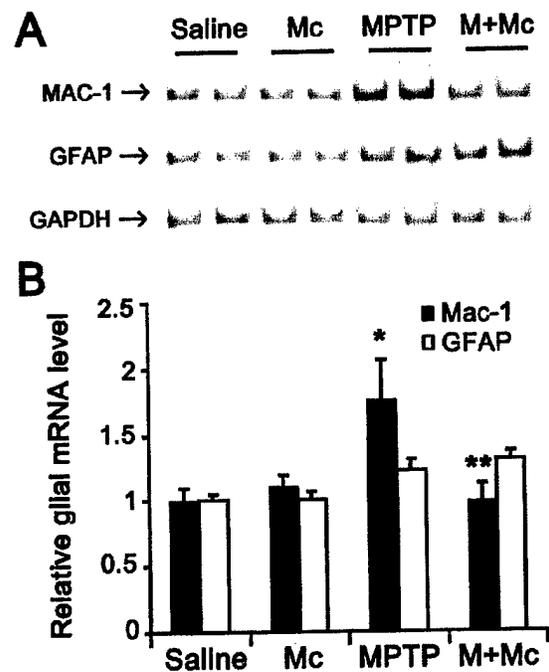


Figure 3. Minocycline prevents MPTP-induced MAC-1 transcription. *A, B*, Ventral midbrain MAC-1 mRNA levels but not GFAP mRNA levels are increased by 24 hr after MPTP injection compared with those of saline- or minocycline-injected mice. Minocycline prevents MPTP-induced MAC-1 mRNA increases. MAC-1 and 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.

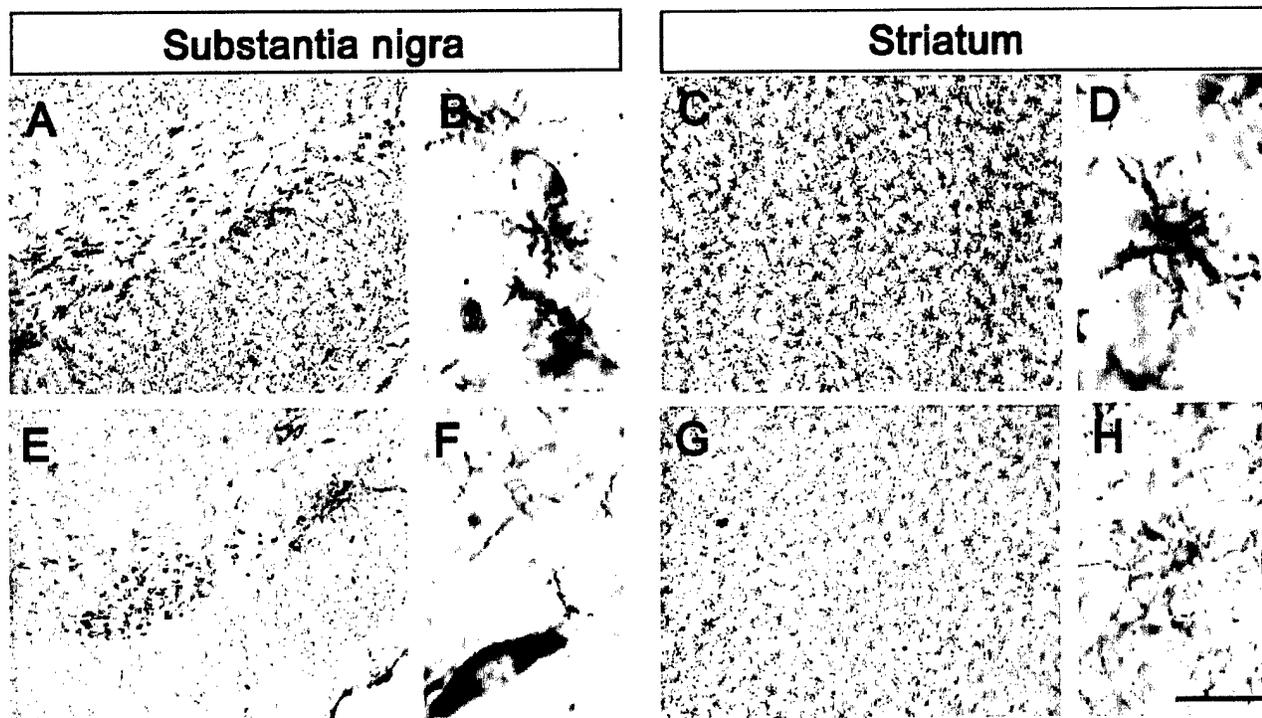


Figure 4. Minocycline prevents MPTP-induced microglia reaction. Microglia cells (brown) and TH-positive neurons (gray blue) are seen in both SNpc and striatum of all mice. One day after the last MPTP injection, numerous activated microglia (larger cell body, poorly ramified short and thick processes) 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 μ m.

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 [3H]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 μ M (maximal solubilizing concentration) did not affect striatal uptake of [3H]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 exam-

ined 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 not 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).

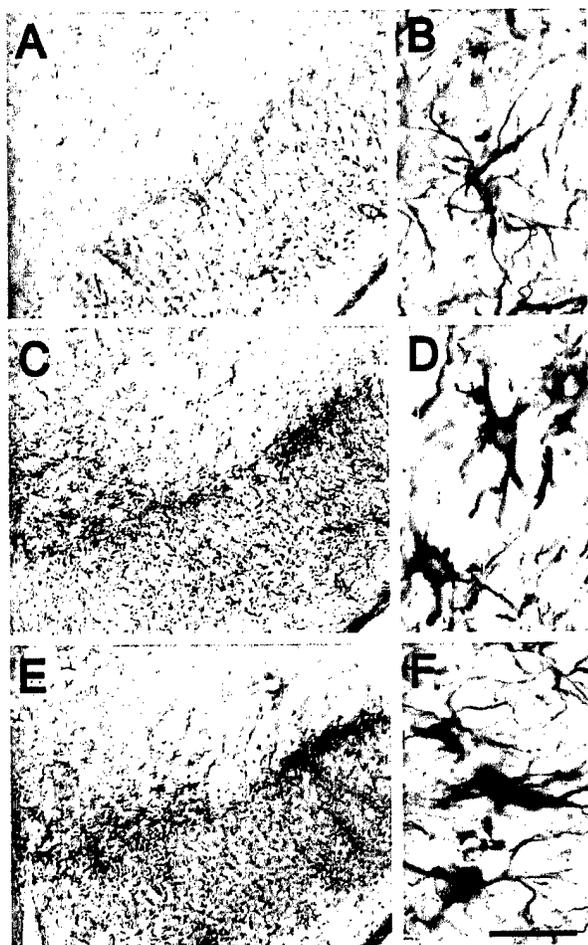


Figure 5. Minocycline does not affect MPTP-induced astrocytic reaction. One day after the last injection of MPTP, there is a mild astrocytic response (*A, B*), but 7 d after the last injection of MPTP, it becomes conspicuous (*C, D*). Minocycline does not affect the astrocytic response (*E, F*) 7 d after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

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-1 β , ROS, and NO will also be inhibited by minocycline (Fig. 6). The levels of ventral midbrain IL-1 β differed significantly among the four groups of mice ($F_{(3,21)} = 7.946$; $p < 0.001$) (Fig. 6*A*). Ventral midbrain levels of the proinflammatory cytokine IL-1 β in MPTP-injected mice (18 mg/kg for four injections) were significantly increased (Fig. 6*A*). However, MPTP produced significantly smaller increases in IL-1 β 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-1 β 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

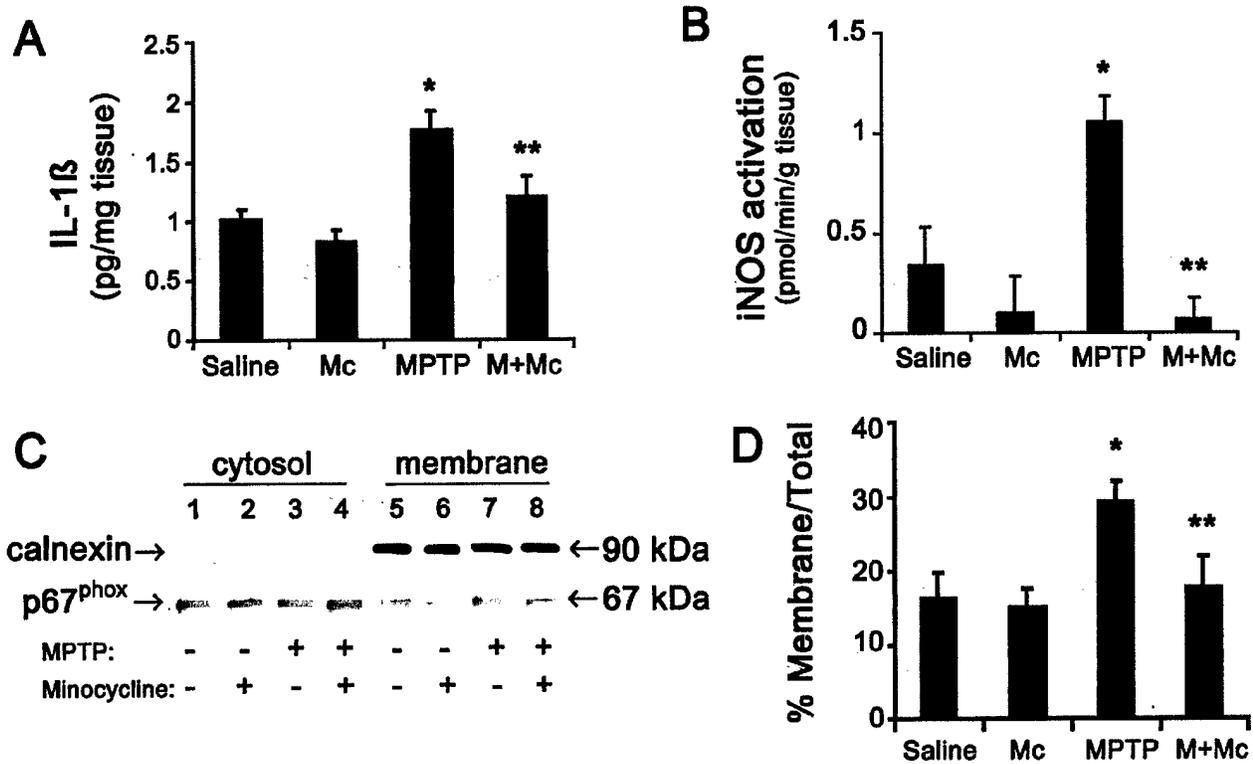


Figure 6. Effects of MPTP and minocycline on microglial-derived deleterious factors IL-1 β (A), iNOS (B), and NADPH-oxidase (C, D). MPTP (18 mg/kg for 4 injections) increases ventral midbrain mature IL-1 β formation, iNOS catalytic activity, and NADPH-oxidase activation, as evidenced by the translocation 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-1 β , 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 \pm 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 μ M (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

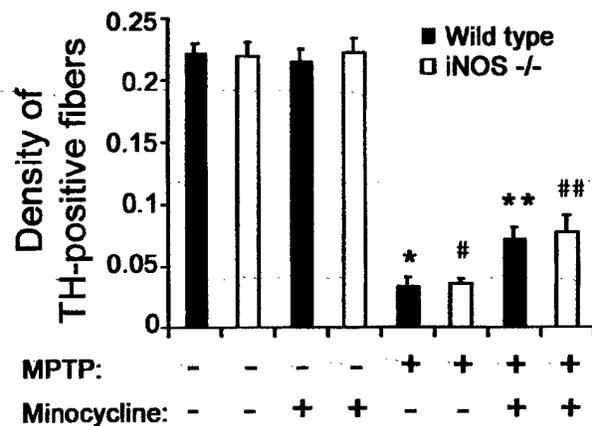


Figure 7. Minocycline attenuates MPTP-induced striatal damage by inhibiting microglia but not just by inhibiting iNOS. The optical density of striatal TH-positive fibers varied significantly among the various groups ($F_{(7,47)} = 83.576; p < 0.001$). Minocycline, Mice injected with minocycline 45 mg/kg 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-1 β 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 prostanooids, either directly or indirectly via the blockade of IL-1 β formation (Yrjanheikki et al., 1999). Little is known about the actual role of IL-1 β in either MPTP or PD neurodegenerative process, except that IL-1 β 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-1 β , 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 cel-

lular 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, possibly, 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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NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease

Du-Chu Wu*, Peter Teismann*, Kim Tieu*, Miquel Vila*, Vernice Jackson-Lewis*, Harry Ischiropoulos†, and Serge Przedborski**§1

Departments of *Neurology and †Pathology and ‡Center for Neurobiology and Behavior, Columbia University, New York, NY 10032; and †Stokes Research Institute, Department of Pediatrics, Children's Hospital of Philadelphia, and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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Parkinson's disease (PD) is a neurodegenerative disorder of uncertain pathogenesis characterized by a loss of substantia nigra pars compacta (SNpc) dopaminergic (DA) neurons, and can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Both inflammatory processes and oxidative stress may contribute to MPTP- and PD-related neurodegeneration. However, whether inflammation may cause oxidative damage in MPTP and PD is unknown. Here we show that NADPH-oxidase, the main reactive oxygen species (ROS)-producing enzyme during inflammation, is up-regulated in SNpc of human PD and MPTP mice. These changes coincide with the local production of ROS, microglial activation, and DA neuronal loss seen after MPTP injections. Mutant mice defective in NADPH-oxidase exhibit less SNpc DA neuronal loss and protein oxidation than their WT littermates after MPTP injections. We show that extracellular ROS are a main determinant in inflammation-mediated DA neurotoxicity in the MPTP model of PD. This study supports a critical role for NADPH-oxidase in the pathogenesis of PD and suggests that targeting this enzyme or enhancing extracellular antioxidants may provide novel 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 (DA)-containing neurons, whose cell bodies are in the substantia nigra pars compacta (SNpc) and nerve terminals are 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 PD (3). Consistent with this view, experimental models of PD show that inflammatory factors may trigger or modulate SNpc DA neuronal death (4–6). Among inflammatory mediators capable of promoting neurodegeneration are microglial-derived reactive oxygen species (ROS). These may deserve particular attention, because oxidative stress is a leading pathogenic hypothesis of PD (7).

A significant source of ROS during inflammation is NADPH-oxidase, which is a multimeric enzyme composed of gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, and p40^{phox} subunits (8). In resting microglia, NADPH-oxidase is inactive because p47^{phox}, p67^{phox}, and p40^{phox}, which are present in the cytosol as a complex, are separated from gp91^{phox} and p22^{phox}, which are transmembrane proteins. Upon microglial activation, p47^{phox} becomes phosphorylated and the entire cytosolic complex translocates to the membrane, where it assembles with gp91^{phox} and p22^{phox}, thus forming a NADPH-oxidase entity now capable of reducing oxygen to superoxide radical (O₂⁻), which in turn gives rise to the production of other secondary reactive oxidants (8).

Although NADPH-oxidase is critical to the killing of invading microorganisms in infections through its abundant and sustained

production of O₂⁻ (8), its role in noninfectious chronic neurodegenerative processes, such as PD, is not known. In the present study, we show not only that the NADPH-oxidase main subunit gp91^{phox} is up-regulated in the SNpc of PD and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice, but also that NADPH-oxidase inactivation attenuates MPTP neurotoxicity by mitigating inflammation-mediated oxidative attack on SNpc neurons. These findings indicate that NADPH-oxidase-induced oxidative stress is instrumental in SNpc DA neurodegeneration caused by MPTP, and suggest that NADPH-oxidase is a valuable therapeutic target for the development of neuroprotective strategies for PD.

Materials and Methods

Animals and Treatment. Eight-week-old male C57BL/6 mice (Charles River Breeding Laboratories), gp91^{phox}-deficient mice (B6.129S6-Cybb^{tm1din}, The Jackson Laboratory), and their WT littermates were used. Mice received four i.p. injections of MPTP-HCl (16 mg/kg free base; Sigma) dissolved in saline at 2-h intervals, and were killed 0–14 days after the last injection. Control mice received saline only. MPTP handling and safety measures were in accordance with our published guidelines (9). Minocycline (2 × 45 mg/kg per day; Sigma) was given to MPTP mice as described (5). Bovine erythrocyte superoxide dismutase 1 (SOD1; 20 units/h; Sigma) was infused into the left striatum with an osmotic minipump (Alzet, Palo Alto, CA) starting 1 day before and stopping 6 days after the MPTP injections. This protocol was in accordance with the National Institutes of Health guidelines for use of live animals and was approved by the Institutional Animal Care and Use Committee of Columbia University (New York). Striatal 1-methyl-4-phenylpyridinium levels were determined by HPLC as described (5).

RNA Extraction and RT-PCR. Total RNA was extracted as described (5). The primer mouse sequences were as follows: gp91^{phox}, 5'-CAGGAGTTCCAAGATGCCTG-3' (forward) and 5'-GATTGGCCTGAGATTTCATCC-3' (reverse); p67^{phox}, 5'-CAGCCAGCTTCGGAACATG-3' (forward) and 5'-GACAGTACCAGGATTACATC-3' (reverse); macrophage antigen complex 1 (Mac-1), 5'-TTCTCATGGTCACCTCCTGC-3' (forward) and 5'-GGTCTGACCATCTGAACCTG-3' (reverse); GAPDH, 5'-GTTTCTTACTCCTTGGAGGCCAT-3' (forward) and 5'-TGATGACATCAAGAAGTGGTGAA-3' (reverse). PCR was carried out for 29 cycles for gp91^{phox}, 27 cycles for p67^{phox} and Mac-1, and 20 cycles for GAPDH. Each cycle

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Abbreviations: Mac-1, macrophage antigen complex 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; DA, dopamine/dopaminergic; SOD1, superoxide dismutase 1.

†To whom correspondence should be addressed. E-mail: sp30@columbia.edu.

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consisted of 30 s of denaturation at 94°C, 40 s of annealing at 55°C, and 60 s of extension at 72°C, followed by a final 7-min extension at 72°C. Products were quantified by a phosphor imager (Bio-Rad).

Digoxigenin-Labeled cRNA Probe and *in Situ* Hybridization. Antisense and sense RNA probes were prepared by *in vitro* transcription from a mouse gp91^{phox} cDNA fragment (nucleotides 1020–1493; GenBank accession no. U43384) by using SP6/T7 RNA polymerase in the presence of digoxigenin-linked UTP (Roche Molecular Biochemicals) according to the supplier's instructions. Frozen midbrain sections (14 μm thick) were incubated with the antisense or sense (for control) digoxigenin-labeled probes. Hybridization signal was detected by 5-bromo-4-chloro-indolyl-phosphatase and nitroblue tetrazolium.

Immunohistochemistry and Quantitative Morphology. Mouse brains were fixed and processed for immunostaining as described (5). Primary Abs were as follows: for mouse sections, monoclonal anti-mouse gp91^{phox} (1:1,000; Transduction Laboratories, Lexington, KY), rat anti-MAC-1 (1:200; Serotec), monoclonal anti-tyrosine hydroxylase (TH; 1:1,000; Chemicon), and polyclonal anti-TH (1:1,000; Calbiochem, San Diego); for human sections, monoclonal anti-human gp91^{phox} (gift from Genentech) and monoclonal anti-human CD68 (DAKO). Immunolabeling was visualized by using 3,3'-diaminobenzidine (brown), VECTOR SG (blue/gray), 3-amino-9-ethylcarbazole (red), or fluorescein and Texas red (all from Vector Laboratories).

Total numbers of TH-positive SNpc neurons were counted by stereology by using the optical fractionator method described previously (6). Striatal density of TH immunoreactivity was determined as described (5).

Western Blots. Total, cytosolic, and plasma membrane proteins were prepared as described (5). Primary Abs were as follows: for mouse proteins, monoclonal anti-mouse p67^{phox} (1:1,000; Transduction Laboratories), polyclonal anti-gp91^{phox} (1:5,000, gift from M. C. Dinauer, Indiana University, Indianapolis), and polyclonal anti-calnexin (1:5,000; Stressgen Biotechnologies, Victoria, Canada); for human proteins, monoclonal anti-human gp91^{phox} (1:500, Genentech). A monoclonal anti-β-actin (1:5,000; Sigma) was used for both mouse and human proteins. Bound primary Ab was detected by using a horseradish peroxidase-conjugated secondary Ab against IgG and a chemiluminescent substrate (SuperSignal Ultra, Pierce). Films were quantified by using the NIH IMAGE analysis system.

***In Situ* Visualization of O₂⁻ and O₂⁻-Derived Oxidant Production.** *In situ* visualization of O₂⁻ and O₂⁻-derived oxidant production was assessed by hydroethidine histochemistry (10). At selected time points after MPTP administration, mice were injected i.p. with 200 μl of PBS containing 1 μg/μl hydroethidine (Molecular Probes) and 1% DMSO. Brains were harvested 15 min later and frozen on dry ice. Midbrain sections (14 μm thick) mounted onto gelatin-coated glass slides were examined for hydroethidine oxidation product, ethidium accumulation, by fluorescence microscopy (excitation, 510 nm; emission, 580 nm). The same tissue sections were used for Mac-1 immunohistochemistry.

Protein carbonyls were detected after derivatization of brain homogenates with 2,4-dinitrophenylhydrazine by using a modification of the method described by Levine *et al.* (11). The concentration of protein carbonyls was calculated from the difference in absorbance at 360 nm between the underivatized and 2,4-dinitrophenylhydrazine-derivatized samples normalized to the protein concentration. The extinction coefficient of 21 mM⁻¹·cm⁻¹ was applied to calculate the concentration of protein carbonyls.

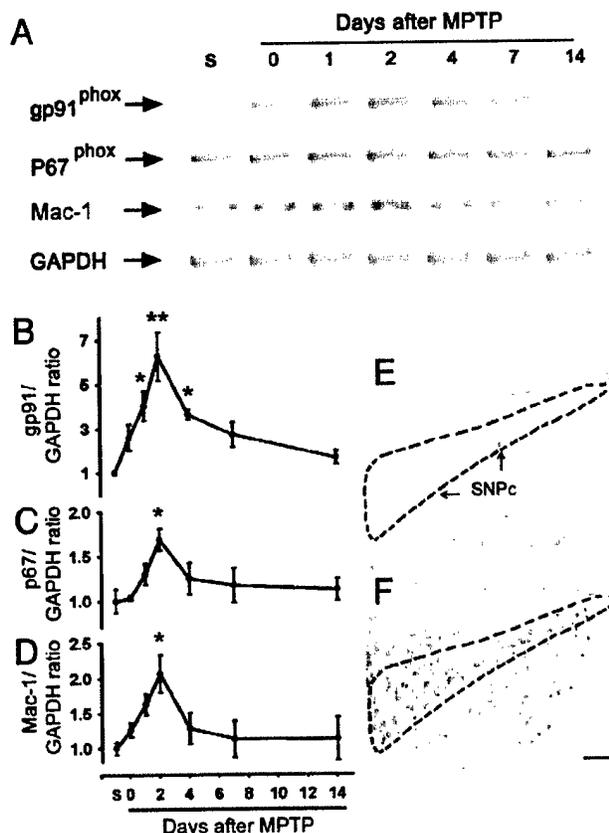


Fig. 1. (A–D) RT-PCR shows ventral midbrain gp91^{phox}, p67^{phox}, and Mac-1 mRNA levels in saline-injected (S) and MPTP-injected mice from 0 to 14 days after injections. SNpc gp91^{phox} mRNA labeling is negligible in saline-injected mice (E), whereas it is copious in MPTP-injected mice at 2 days (F). *, $P < 0.05$; **, $P < 0.001$, more than saline-treated mice ($n = 4–6$ per time point). (Scale, 2.5 mm.)

Human Samples. Age at death and interval from death to tissue processing (mean ± SEM) were as follows: for the control group ($n = 3$), 72.2 ± 8.8 y and 13.0 ± 3.5 h, respectively; for the PD group ($n = 6$), 77.2 ± 2.3 y and 10.1 ± 2.4 h, respectively. For the PD patients, the mean duration of disease was 16.8 ± 2.3 y.

Statistical Analysis. Values represent means ± SEM. Differences among means were analyzed by using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were analyzed by Newman–Keuls post hoc testing. The null hypothesis was consistently rejected at the 0.05 level.

Results

NADPH-Oxidase Is Induced in Mouse Ventral Midbrain During MPTP Neurotoxicity.

To define the temporal relationship between NADPH-oxidase expression and MPTP neurotoxicity, contents of ventral midbrain (brain region containing SNpc) membrane-bound subunit gp91^{phox} and cytosolic subunit p67^{phox} mRNA were assessed throughout the time course of MPTP-induced SNpc DA neurodegeneration (12). In saline-injected mice, ventral midbrain gp91^{phox}, p67^{phox}, and Mac-1 (microglial marker) mRNAs were low (Fig. 1 A–D). In contrast, in MPTP-injected mice, ventral midbrain gp91^{phox}, p67^{phox}, and Mac-1 mRNAs

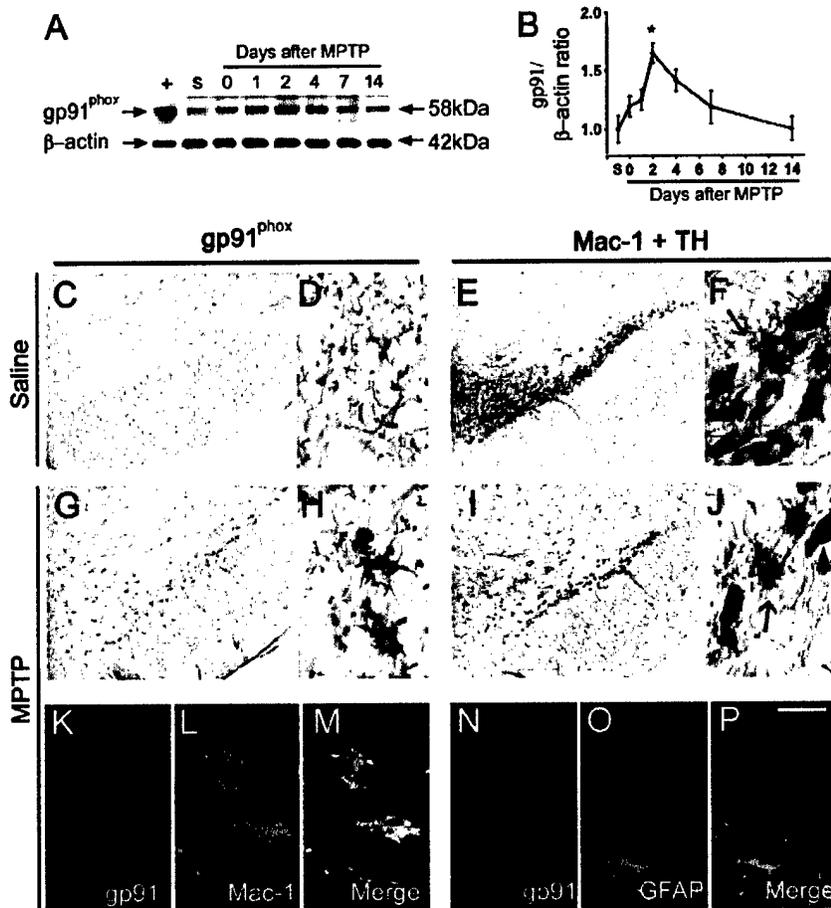


Fig. 2. Western blot shows the time-dependent induction of gp91^{phox} in mouse ventral midbrain after MPTP injections. +, Mouse macrophage lysate; s, saline. In saline-injected mice, gp91^{phox} immunoreactivity (C and D, brown) is mild and localized in resting microglia, which are not abundant in the SNpc, as shown (E and F) by Mac-1 labeling (brown, arrow) and are intermingled with TH-positive neurons (gray-blue). Two days after MPTP injections, numerous gp91^{phox}-positive cells are seen in the SNpc (G and H). These cells resemble activated microglial cells (H vs. J, arrow). At this point there are many fewer TH-positive neurons (I and J, arrowhead). Confocal microscopy shows that all gp91^{phox}-positive cells are Mac-1-positive, thus confirming their microglial origin (K and L). Conversely, no gp91^{phox}-positive cells are glial fibrillary acidic protein-positive cells, thus excluding their astrocytic origin (N–P). *, $P < 0.05$, more than saline-treated mice ($n = 6$ per time point). [Scale bar, 2.5 mm (C, E, G, and I); 0.25 mm (D, F, H, and J); and 0.2 mm (K–P).]

increased in a time-dependent manner after MPTP injections (Fig. 1A–D).

In saline-injected mice, nonradioactive *in situ* hybridization for gp91^{phox}, which is one of the main functional subunits of NADPH-oxidase, revealed no specific labeling in ventral midbrain (Fig. 1E), whereas in MPTP-injected mice there was conspicuous specific labeling over the SNpc at 2 days after MPTP injections (Fig. 1F). Thus, these results indicate that NADPH-oxidase is induced after MPTP administration specifically in the area where the demise of DA neurons arises in this model of PD.

NADPH-Oxidase Is Expressed in Activated Microglia After MPTP Injection. Consistent with the mRNA data, ventral midbrain gp91^{phox} protein contents rose in a time-dependent manner after MPTP injections (Fig. 2A and B). In cell cultures, NADPH-oxidase has been identified in different cell types, including neurons (13). In saline-injected mice, mild gp91^{phox} immunoreactivity was seen throughout the substantia nigra (Fig. 2C) without greater gp91^{phox} immunolabeling in the SNpc, which hosts the TH-positive neurons (Fig. 2E and F). Immunoreactivity of gp91^{phox} was in small cells with thin ramifications (Fig. 2D) reminiscent

of resting microglia (Fig. 2F). In MPTP-injected mice, robust gp91^{phox} immunoreactivity was seen specifically in the SNpc (Fig. 2G) in larger cells with thick, shorter ramifications (Fig. 2H) reminiscent of activated microglia (Fig. 2J). Similar immunohistochemical gp91^{phox} alterations were seen in the striatum, which contains the nerve terminals of the projecting SNpc DA neurons, between the saline- and MPTP-injected mice (data not shown). By confocal microscopy, gp91^{phox} immunoreactivity appeared to colocalize with Mac-1 (Fig. 2K–M). Conversely, gp91^{phox} immunoreactivity did not colocalize either with the astrocytic marker glial fibrillary acidic protein (Fig. 2N–P) or with TH (data not shown). Thus, these results demonstrate that, after MPTP administration, SNpc microglia become activated at the site of NADPH-oxidase induction.

Expression of gp91^{phox} Is Increased in PD Midbrain. Consistent with the finding in the MPTP mice, postmortem SNpc samples from sporadic PD patients had higher gp91^{phox} protein contents than controls (Fig. 3A and B). In these autopsy specimens, cellular gp91^{phox} immunoreactivity was barely identified in controls (Fig. 3C) but was strong in PD midbrain sections, where it was identified in microglial cells (Fig. 3D). The similarity of the

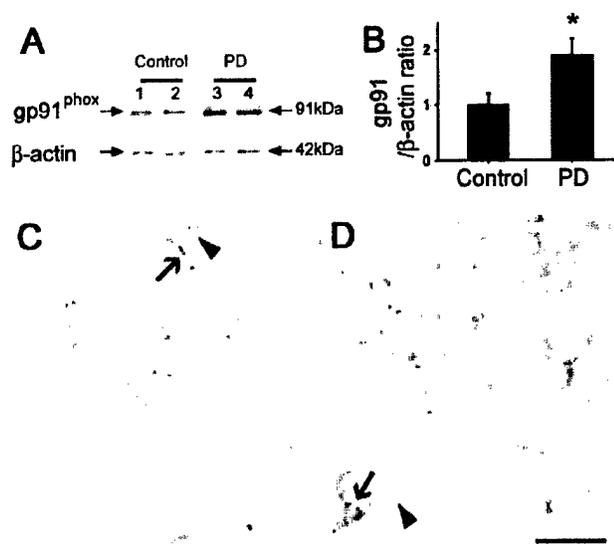


Fig. 3. (A) Representative Western blots illustrating the increase in ventral midbrain gp91^{phox} protein content in two PD and two controls. (B) Bar graph showing mean Western blot gp91^{phox}/β-actin ratios ± SEM for six PD and three control ventral midbrain samples. (C and D) Representative gp91^{phox} immunostaining that shows positive cells in PD samples (arrowhead, gray-blue, membrane labeling) colocalizing with the microglial marker CD68 (arrow, red, cytosol labeling), but not with neuromelanin (brown pigment). *, $P < 0.05$, higher than controls. (Scale bar, 0.5 mm.)

gp91^{phox} alterations between the MPTP mice and the PD postmortem specimens validates the use of the MPTP experimental model to study the role of NADPH-oxidase in the PD neurodegenerative process.

The Lack of gp91^{phox} Abates MPTP-Associated ROS Production. In saline-injected mice, ventral midbrain O₂⁻ and O₂⁻-derived oxidant production, evidenced by ethidium fluorescence, was minimal (Fig. 4A). In contrast, in MPTP-treated mice, ventral midbrain production of O₂⁻ or O₂⁻-derived oxidants shown by ethidium fluorescence was increased by 12 h (data not shown), was maximal by 2 days (Fig. 4C), and remained elevated at 7 days after MPTP (data not shown). SNpc ethidium fluorescence coincided with the location and the time course of microglial activation seen after MPTP administration (Fig. 4C and D).

In mutant mice lacking the gp91^{phox} subunit, no translocation of the cytosolic p67^{phox} subunit to the plasma membrane was seen after MPTP injections (Fig. 4I and J), which is mandatory for NADPH-oxidase to become catalytically competent (8). Unlike WT littermates (Fig. 4C and D), mutant mice with defective NADPH-oxidase failed to show any increase in SNpc ethidium fluorescence (Fig. 4E), despite normal microglial activation after MPTP administration (Fig. 4F). WT mice treated with minocycline (i.e., antibiotic that blocks microglial activation) showed no increase in SNpc ethidium fluorescence (Fig. 4G) and no microglial activation after MPTP administration (Fig. 4E). Thus, these findings demonstrate that during the MPTP neurotoxic process there is an increased production of ROS in the SNpc that originates from activated microglial cells and is mediated by NADPH-oxidase.

NADPH-Oxidase Defect Protects Against MPTP Neurodegeneration. In the ventral midbrain of saline-injected mice, the stereological counts of SNpc TH-positive neurons did not differ between gp91^{phox}-deficient mice and their WT littermates (Fig. 5A). In MPTP-injected mice, the numbers of SNpc TH-positive neurons

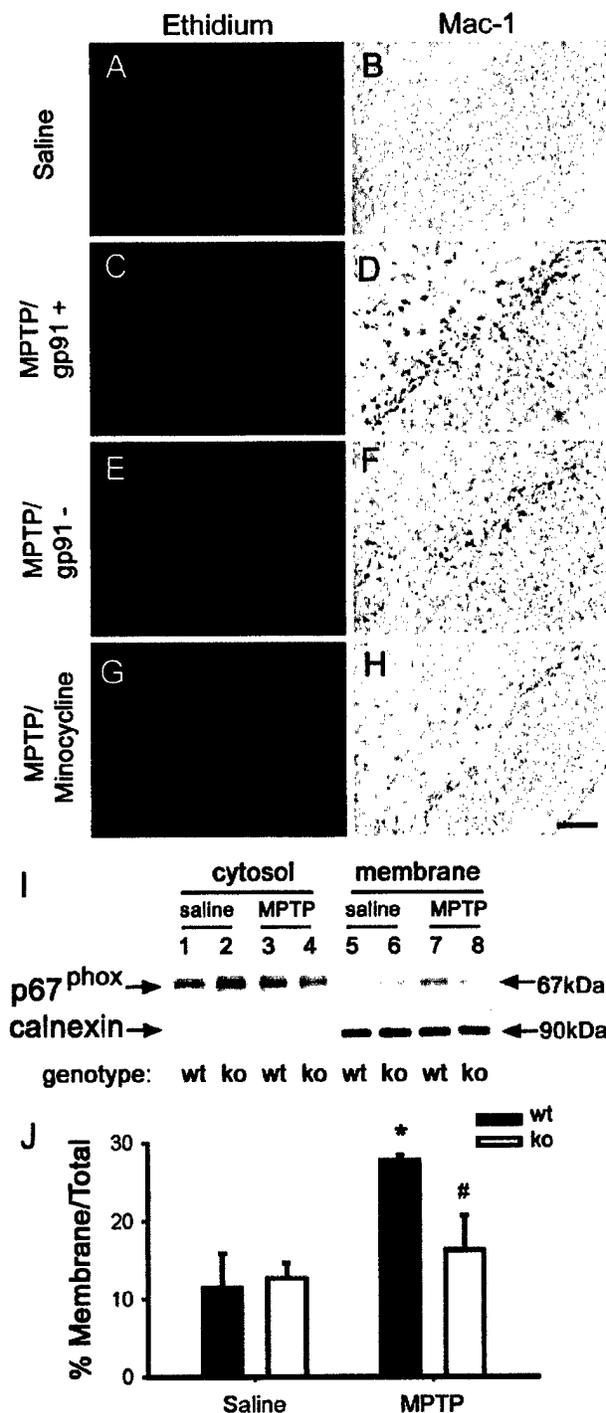


Fig. 4. Ethidium fluorescence (A) and Mac-1 immunostaining (B) are minimal in the saline-treated mice. By 2 days after MPTP injections, SNpc ethidium fluorescence is increased in WT mice (C) and is absent in gp91^{phox}-deficient mice (E) and minocycline-treated WT mice (G). Microglial activation is prevented by minocycline (H) but is normal in gp91^{phox}-deficient mice (E). MPTP stimulates NADPH-oxidase activation, as evidenced by p67^{phox} translocation from the cytosol to the plasma membrane in WT mice (wt), but not in gp91^{phox}-deficient mice (ko) (I and J); the membrane protein calnexin is used to normalize the data. Data are means ± SEM for four to six samples per group. *, $P < 0.05$, higher than controls; #, $P < 0.05$, less than MPTP-injected WT mice, but not different from both saline-injected groups.

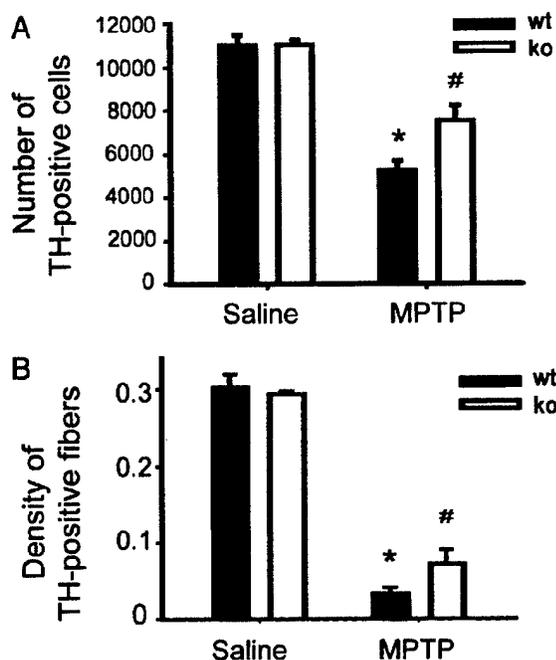


Fig. 5. Stereological counts of TH-positive neurons in the SNpc (A) and optical density of striatal DA fibers (B) are higher in gp91^{phox}-deficient mice (ko) compared with their WT littermates (wt) 7 days after MPTP injections ($n = 4-8$ samples per group). *, $P < 0.05$, less than saline-injected mice; #, $P < 0.05$, higher than MPTP-injected WT mice.

were reduced in the two groups of animals (Fig. 5A). However, the loss was smaller in gp91^{phox}-deficient mice compared with their WT counterparts (Fig. 5A). In the striatum of saline-injected mice, the density of TH-positive nerve fibers was similar between gp91^{phox}-deficient mice and their WT littermates (Fig. 5B). Like for the number of SNpc TH-positive neurons, in MPTP-injected mice the density of striatal TH-positive nerve fibers was less reduced in the gp91^{phox}-deficient mice than in their WT counterparts (Fig. 5A). Because MPTP neurotoxic potency on the nigrostriatal pathway correlates linearly with 1-methyl-4-phenylpyridinium levels in the striatum (14), the content of this active metabolite of MPTP between the two genotypes was evaluated. There were no differences in striatal levels of 1-methyl-4-phenylpyridinium between the gp91^{phox}-deficient mice ($17.8 \pm 1.4 \mu\text{g/g}$ striatum; $n = 5$) and WT littermates ($17.7 \pm 2.0 \mu\text{g/g}$ striatum; $n = 5$; $P > 0.05$). These results show that NADPH-oxidase participates in the MPTP neurotoxic process affecting DA cell bodies in the SNpc and nerve fibers in the striatum by a mechanism unrelated to an alteration in MPTP toxicokinetics.

NADPH-Oxidase Damages Ventral Midbrain Proteins. To assess the extent of NADPH-oxidase-related oxidative damage, protein carbonyl levels were determined in ventral midbrain of gp91^{phox}-deficient and WT mice after saline or MPTP administration. In saline-injected mice, the levels of ventral midbrain protein carbonyls were similar between the two groups of animals (Fig. 6A). In MPTP-injected WT mice, levels of ventral midbrain protein carbonyls were increased (Fig. 6A), but in gp91^{phox}-deficient mice they were not different from controls (Fig. 6A).

MPTP-Induced Neurotoxicity Is Attenuated by Scavenging Extracellular Superoxide. To test the noxious role of extracellular ROS, the membrane-impermeant enzyme SOD1 was infused into the left

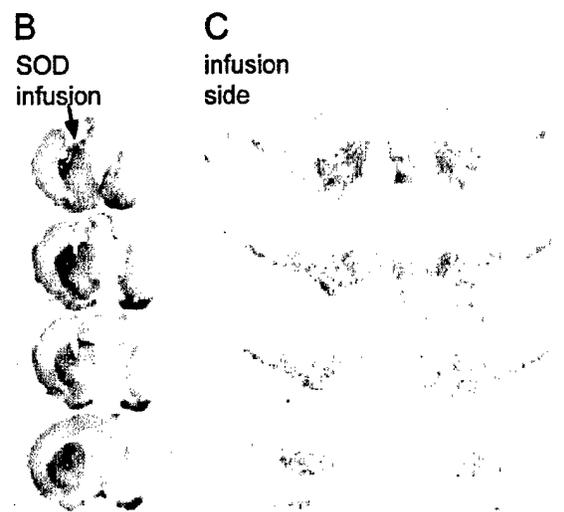
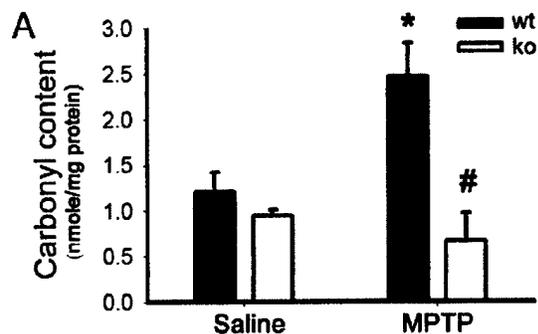


Fig. 6. (A) Ventral midbrain carbonyl content, used as a marker of protein oxidative damage, is increased at 2 days after MPTP injections in WT mice (wt), but not in gp91^{phox}-deficient mice (ko). Infusion of SOD1 into the left striatum attenuates the striatal (B) and the SNpc lesion on the infused side, but not on the contralateral, noninfused side (C) after a systemic injection of MPTP. *, $P < 0.05$, higher than controls; #, $P < 0.05$, less than MPTP-injected WT mice, but not different from the two saline-injected groups.

striatum. In the MPTP-injected mice, there was a protection of striatal TH-positive fibers on the infused side compared with the noninfused side (Fig. 6B). There was also a preservation of SNpc TH-positive cell bodies ipsilateral to the infused side compared with the contralateral noninfused side (Fig. 6C). These findings demonstrate the importance of the oxidative stress that emanates from the extracellular space on the demise of neighboring DA neurons.

Discussion

This study shows that the microglial activation in MPTP and PD SNpc specimens is associated with an induction of NADPH-oxidase. This up-regulation correlates topographically and temporally with the DA neurodegenerative changes seen in MPTP mouse and human PD brains. It also parallels the production of ROS seen in the SNpc by 2 days after MPTP injections. The use of minocycline and mutant mice deficient in gp91^{phox} demonstrates collectively that ROS production originates from activated microglia and, within these cells, from NADPH-oxidase.

In the MPTP model, ROS can emanate from both cytosol and mitochondria of DA neurons (15-18). Rise of markers reflecting oxidative damage in the nigrostriatal DA pathway culminates during the first 24 h after MPTP injections (19, 20). In contrast, SNpc NADPH-oxidase-mediated ROS attack becomes signifi-

cant by 2 days after MPTP injections. Therefore, nigrostriatal DA neurons may be subjected first to an intracellular oxidative insult, and then to an extracellular oxidative insult mediated by activated microglia.

Mutant mice deficient in gp91^{phox} exhibited less ventral midbrain protein carbonyl contents and more SNpc DA neurons than their WT littermates after MPTP injections. These results prove that NADPH-oxidase is instrumental in the MPTP neurotoxic process. Activated microglia can also exert deleterious effects unrelated to ROS. Relevant to this notion, mutant mice deficient in gp91^{phox}, despite being defective in NADPH-oxidase, showed no evidence of impaired activation of microglial cells in response to MPTP. Lack of gp91^{phox} expression was also not associated with alteration in the formation of 1-methyl-4-phenylpyridinium, which is the most significant modulator of MPTP potency (14). Therefore, the resistance of gp91^{phox}-deficient mice to MPTP results from the defect of NADPH-oxidase and the consequent reduction of O₂⁻ formation, and not from either an impaired microglial effector function or an altered MPTP metabolism.

Activated NADPH-oxidase produces O₂⁻ inward into intracellular vesicles and outward into the extracellular space (8). Neurons located in the vicinity of activated microglial cells may thus have their plasma membrane proteins and lipids exposed to NADPH-oxidase-derived O₂⁻ and other secondary oxidants, such as hydrogen peroxide. Infusion of SOD1 in the striatum attenuates MPTP-induced loss of striatal DA fibers and SNpc DA neurons; the latter effect may result from a reduction of MPTP-mediated retrograde degeneration (21). This finding indicates that extracellular hydrogen peroxide may not play a great neurotoxic role in the MPTP model, because its formation should have been greatly increased by the combination of infused SOD1 and increased steady-state levels of O₂⁻ (22) derived from activated NADPH-oxidase. Instead, an increase in the steady-state levels of extracellular O₂⁻ appears to be pivotal to the killing of SNpc DA neurons.

Among the main isoforms that catalyze NO synthesis, inducible NO synthase is the most closely linked to inflammation. In keeping with this, inducible NO synthase is up-regulated in activated microglial cells both in PD and in the MPTP model (6,

23, 24). In mutant mice deficient in inducible NO synthase, MPTP causes less death of SNpc DA neurons and smaller increases in ventral midbrain nitrotyrosine levels compared with their WT counterparts (6, 24). These findings suggest that a critical part of activated microglial cytotoxicity in the MPTP model and perhaps in PD is also fulfilled by inducible NO synthase-derived NO. Given this, extracellular O₂⁻ toxicity in the MPTP model could derive from peroxynitrite that is formed by the diffusion-limited reaction of O₂⁻ with NO (25). Consistent with peroxynitrite involvement in MPTP and PD neurodegenerative processes are the demonstrations that ventral midbrain nitrotyrosine levels are increased after MPTP injections (26, 27), with overexpression of SOD1 preventing the nitration of several important proteins, such as TH (19). α -Synuclein, a presynaptic protein with critical relevance to PD etiopathogenesis, is also nitrated both in the MPTP model and in PD (28, 29).

Activated microglial cells, by generating an extracellular oxidative stress, would likely injure all cells and not solely DA neurons. One way to reconcile the anticipated nonselectivity of the injury with the selectivity of the lesions is to consider that SNpc DA neurons may be particularly vulnerable to extracellular ROS attack compared with the other cells. It is also possible that in the MPTP model and in PD, the magnitude of microglial activation and resulting oxidative stress is mild and only inflicts sublethal lesions. This would succeed in killing only neurons already compromised, as DA neurons probably are in PD and after MPTP injections.

We thank Eric Swanberg and Charles Rohrbach for their expert assistance in quantification of carbonyl contents, Shi-Xuan Wang for assistance with *in situ* hybridization, and the New York Brain Bank at Columbia University for providing the human postmortem samples. This study was supported by National Institutes of Health/National Institute of Neurological Disorders and Stroke Grants NS37345, NS38586, NS42269, NS38370, and NS11766-27A1; National Institutes of Health/National Institute on Aging Grant AG13966; U.S. Department of Defense Grants DAMD 17-99-1-9471 and DAMD 17-03-1; the Lowenstein Foundation; the Lillian Goldman Charitable Trust; and the Parkinson's Disease Foundation. P.T. is the recipient of German Research Foundation Grant TE 343/1-1.

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Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration

Peter Teismann*, Kim Tieu*, Dong-Kug Choi*, Du-Chu Wu*, Ali Naini*, Stéphane Hunot†, Miquel Vila*, Vernice Jackson-Lewis*, and Serge Przedborski**§¶

*Neuroscience Research Laboratories of the Movement Disorder Division, Department of Neurology, †Department of Pathology, and ‡Center for Neurobiology and Behavior, Columbia University, New York, NY 10032; and †Institut National de la Santé et de la Recherche Médicale U289, Hôpital de la Salpêtrière, 75013 Paris, France

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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 E₂ have been implicated in neurodegeneration in several pathological settings. Here we show that COX-2, the rate-limiting enzyme in prostaglandin E₂ 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 E₂ (PGE₂) 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 PGH₂, the precursor of PGE₂ 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 PGH₂, 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-tetrahydropyridine (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 PGE₂ 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-1 β -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-1 β converting enzyme; iNOS, inducible NO synthase.

¶To whom correspondence should be addressed at: Departments of Neurology and Pathology, BB-307, Columbia University, 650 West 168th Street, New York, NY 10032. E-mail: sp30@columbia.edu.

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 Na₃VO₄. 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).

PGE₂ Tissue Content. PGE₂ 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-Cysteinyldopamine. Quantification of protein-bound 5-cysteinyldopamine 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 [³H]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 \pm 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-

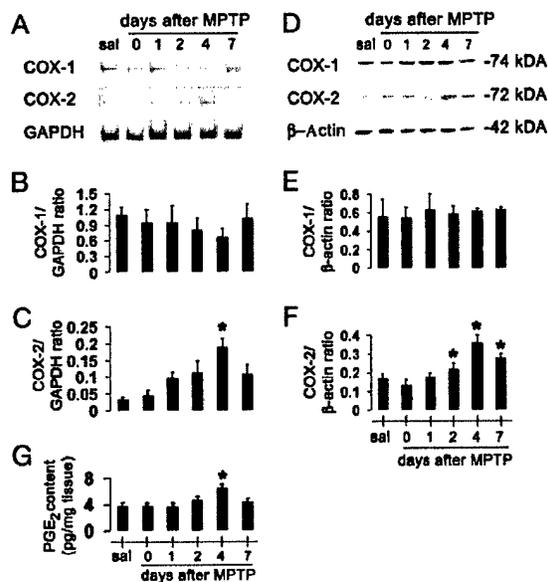


Fig. 1. Ventral midbrain COX-1 and COX-2 mRNA and protein expression after MPTP. COX-2 mRNA levels are increased by 4 days after MPTP injection (A) compared with controls (C), and almost return to basal levels by 7 days. COX-2 protein contents are minimal in saline-injected mice (sal) (D) but rise in a time-dependent manner after MPTP injection (F). COX-1 expression is not altered by MPTP (A, B, D, and E). Ventral midbrain PGE₂ levels are also increased 4 days after MPTP (G). Data are mean \pm SEM for four to six mice per group. *, $P < 0.05$, compared with saline (Newman-Keuls post hoc test).

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. 1A, 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. 1A, C, D, and F), but were detected in MPTP-treated mice at 24 h after injections and thereafter (Fig. 1A, 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 PGE₂. Ventral midbrain PGE₂ 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 PGE₂ contents rose during MPTP neurotoxicity, coincidentally to the changes in COX-2 expression (Fig. 1G). Although whole-tissue PGE₂ deriving from COX-2 almost doubles after MPTP, $\approx 65\%$ 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.

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. 2A 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. 2C and D). COX-2-positive neurons showed immunoreactivity over the cytoplasmic and nuclear areas (Fig. 2D), which is consistent with the known subcellular localization of this

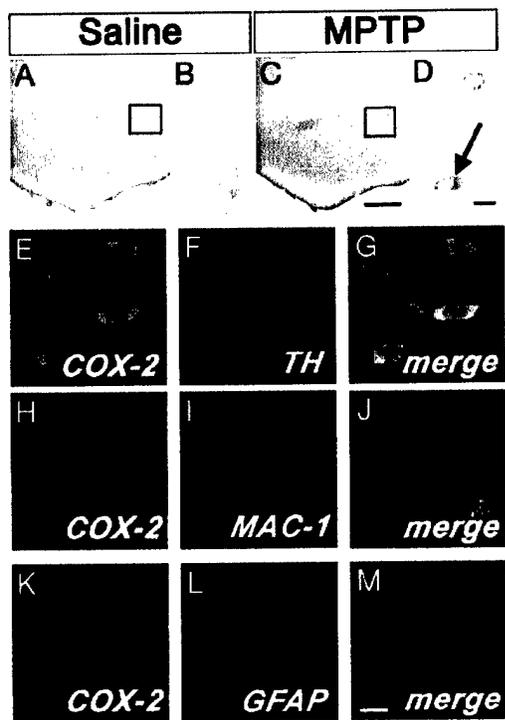


Fig. 2. Ventral midbrain illustration of COX-2 immunolocalization. No COX-2-positive cells are seen in saline-injected mice (A and enlarged *Inset* from A in B). Conversely, COX-2-positive cells are abundant after MPTP (C and enlarged *Inset* from C in D, arrow). Double immunofluorescence confirms that 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 μ m (A and C), 10 μ m (B and D–G), and 20 μ m (H–M).]

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 PGE₂ contents in postmortem SNpc samples. Consistent with the MPTP findings, PD samples had significantly higher contents of COX-2 protein and PGE₂ than normal controls (Fig. 3 A and B). As in the mice, no significant change in PGE₂ 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 proteinaceous inclusions, Lewy bodies (Fig. 3G). 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,

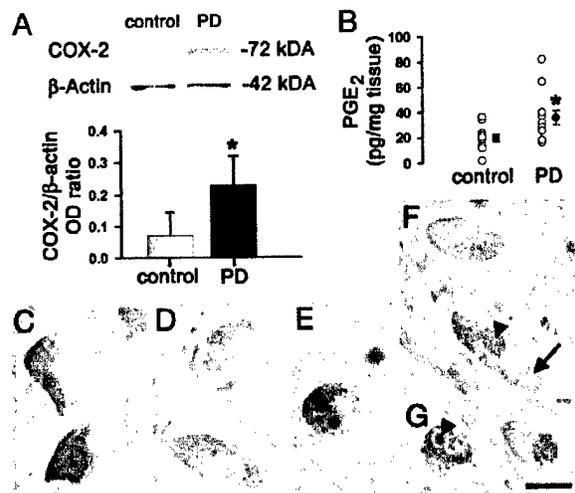


Fig. 3. Ventral midbrain COX-2 expression is minimal in normal human specimens but is increased 3-fold in PD samples (A). Ventral midbrain PGE₂ levels are also increased in PD (B). COX-2 (blue) is not detected in neuromelanized (brown) dopaminergic neurons in controls (C and D) but is well detected in 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 \pm SEM for 3–6 samples for COX-2 protein and 11 samples for PGE₂ assessment. *, $P < 0.05$, compared with normal controls (Newman–Keuls posthoc test). (Scale bar, 25 μ m.)

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. 4C 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 \pm 725, MPTP = 4,247 \pm 554 (mean \pm SEM for three to eight mice per group)] and *Ptgs1*^{+/+} littermates (saline = 8,577 \pm 334, MPTP = 5,274 \pm 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 PGE₂ production (see Table 2). In mice injected with MPTP that received either 25 or 50 mg/kg rofecoxib, \approx 74% and 88%,

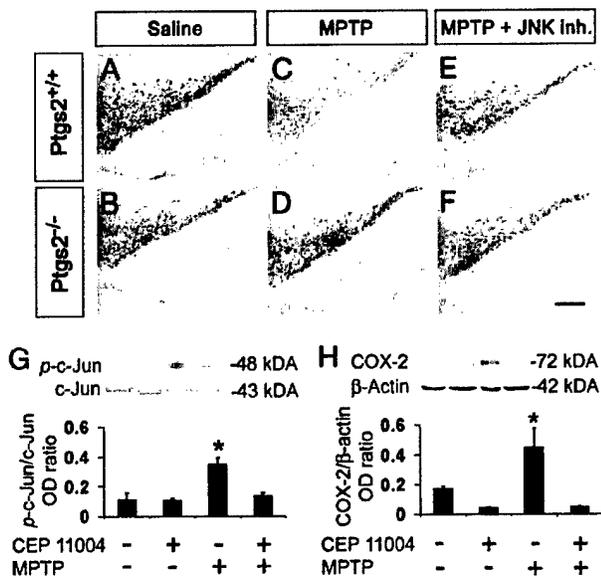


Fig. 4. Effect of COX-2 ablation and JNK pathway inhibition on MPTP-induced neuronal loss. TH-positive neuronal counts are shown in Table 1 and appear comparable between saline-injected *Ptgs2^{-/-}* and *Ptgs2^{+/+}* mice (A and B and Table 1). SNpc TH-positive neurons are more resistant to MPTP in *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 \pm SEM for three to six mice per group. *, $P < 0.05$, compared with the other three groups (Newman-Keuls posthoc test). (Scale bar, 250 μ m.)

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. 5H) 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. 4G) and, as shown above, a marked up-regulation of COX-2 (Fig. 4H). 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

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

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

Values are mean \pm 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.

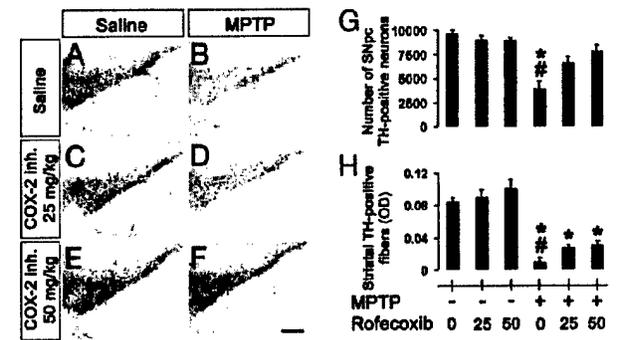


Fig. 5. TH-positive neurons and striatal fibers are more resistant to MPTP in mice treated with rofecoxib (25 or 50 mg/kg p.o.; D and F) than in mice receiving vehicle (B), 7 days after MPTP injection (SNpc neuronal counts are shown in G and striatal fiber optical density is shown in H). Rofecoxib by itself has no effect on TH-positive neurons (A, C, and E). Data are mean \pm 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 μ m.)

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 [³H]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 μ M did not affect MPP⁺-induced lactate production (lactate in μ M/100 mg of protein: *Ptgs2^{+/+}* = 56.1 \pm 1.9, *Ptgs2^{-/-}* = 58.6 \pm 5.2; regular mice/vehicle = 60.8 \pm 3.8, regular mice/rofecoxib = 53.8 \pm 7.3;

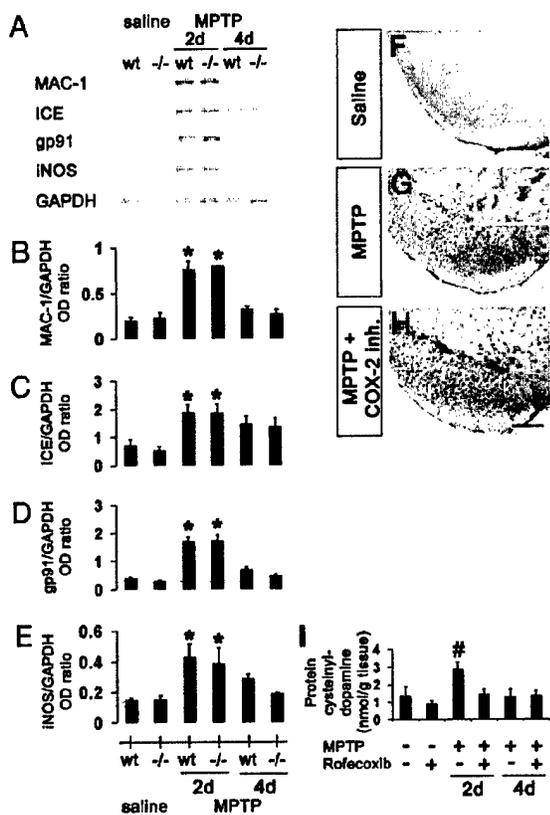


Fig. 6. Expression of inflammatory and oxidative stress markers after MPTP. Two days after MPTP injection, mRNA expression of MAC-1 (A and B), ICE (A and C), gp91 (A and D), and iNOS (A and E) are increased in the ventral midbrain and none is attenuated by COX-2 ablation. MAC-1 immunoreactivity is minimal 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 \pm 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 μ m.)

mean \pm SEM for six mice per group). Striatal uptake of [3 H]MPP⁺ was not impaired in *Ptgs2*^{-/-} mice, compared with *Ptgs2*^{+/+} mice, with an IC₅₀ of 226.3 \pm 21 nM for *Ptgs2*^{-/-} mice and 195.3 \pm 6.38 nM for their wild-type littermates (mean \pm 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 PGE₂, 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. 6A–E), as well as by increased numbers of MAC-1-positive cells in both SNpc (Fig. 6G) 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. 6A–E, data not shown for COX-2 inhibition; immunostaining for MAC-1: Fig. 6F–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. 6I). 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. 6I). 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. 6I).

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 PGE₂ tissue content, thus indicating that the increased COX-2 is catalytically active. However, we found that ventral midbrain PGE₂ 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 PGE₂, 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 PGE₂ 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 PGG₂ conversion to PGH₂ (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 cysteinyl-dopamine 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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The 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model

A Tool to Explore the Pathogenesis of Parkinson's Disease

SERGE PRZEDBORSKI^{a,b,c} AND MIQUEL VILA^a

Neuroscience Research Laboratories of the Movement Disorder Division, Departments of ^aNeurology and ^bPathology and the ^cCenter for Neurobiology and Behavior, Columbia University, New York, New York 10032, USA

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-Methyl-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-

Address for correspondence: Dr. Serge Przedborski, BB-307, Columbia University, 650 West 168th Street, New York, New York 10032. Voice: 212-305-1540; fax: 212-305-5450. SP30@columbia.edu

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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 lipophilic, 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

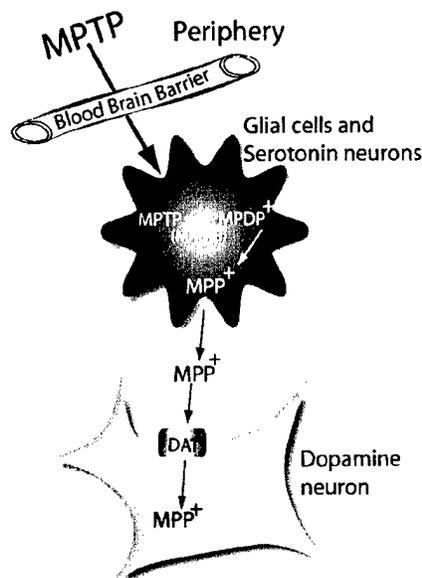


FIGURE 1. Schematic diagram of MPTP metabolism. After its systemic administration, MPTP crosses the blood-brain barrier. Once in the brain, MPTP is converted to MPDP⁺ by monoamine oxidase B within nondopaminergic cells, and then to MPP⁺ by an unknown mechanism. Thereafter, MPP⁺ is released, again by 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 mazindol²⁰ or ablation of the DAT gene in mutant mice²¹ 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, mitochondrial carriers, and cytosolic enzymes. The importance of the vesicular sequestration of

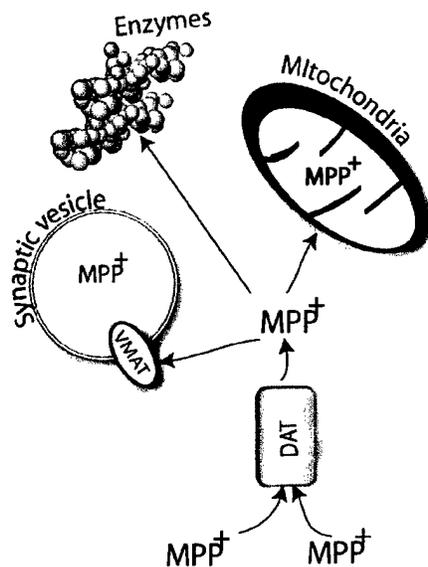


FIGURE 2. Schematic diagram of MPP⁺ intracellular pathways. Inside dopaminergic neurons, MPP⁺ can bind to the vesicular monoamine transporters, be translocated into synaptic vesicles, be concentrated by an active process within the mitochondria, and remain in the cytosol and interact with different cytosolic 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 mitochondria³¹ 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 demonstration³⁶ 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 Vila¹ 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 toxin⁵⁹ suggests an ongoing degenerative process and thus challenges the notion that MPTP produces a "hit and run" kind of damage. Therefore, this important observation⁵⁹ 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 E₂, or PGE₂), 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 PGE₂ 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-1⁷³ and the genetic ablation of Cox-2 attenuates MPTP neurotoxicity.⁷⁴

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COX-2 and Neurodegeneration in Parkinson's Disease

P. TEISMANN,^a M. VILA,^a D.-K. CHOI,^a K. TIEU,^a D. C. WU,^a
V. JACKSON-LEWIS,^a AND S. PRZEDBORSKI^{a,b,c}

*Neuroscience Research Laboratories of the Movement Disorder Division,
Departments of^aNeurology and^bPathology and the^cCenter for Neurobiology and
Behavior, Columbia University, New York, New York 10032, USA*

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 E₂ (PGE₂) 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 PGH₂, the prostaglandin precursor of PGE₂ 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 disease³ 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-

Address for correspondence: Dr. Serge Przedborski, BB-307, Columbia University, 650 West 168th Street, New York, NY 10032. Voice: 212-305-1540; fax: 212-305-5450.
SP30@columbia.edu

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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-tetrahydropyridine (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)-1 β , 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 PGE₂ 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 develops⁸ 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 PGE₂ 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 cysteine residues in proteins to form protein-bound S-S-cysteinyl dopamine, a type of posttranslational modification that can seriously affect protein functions.

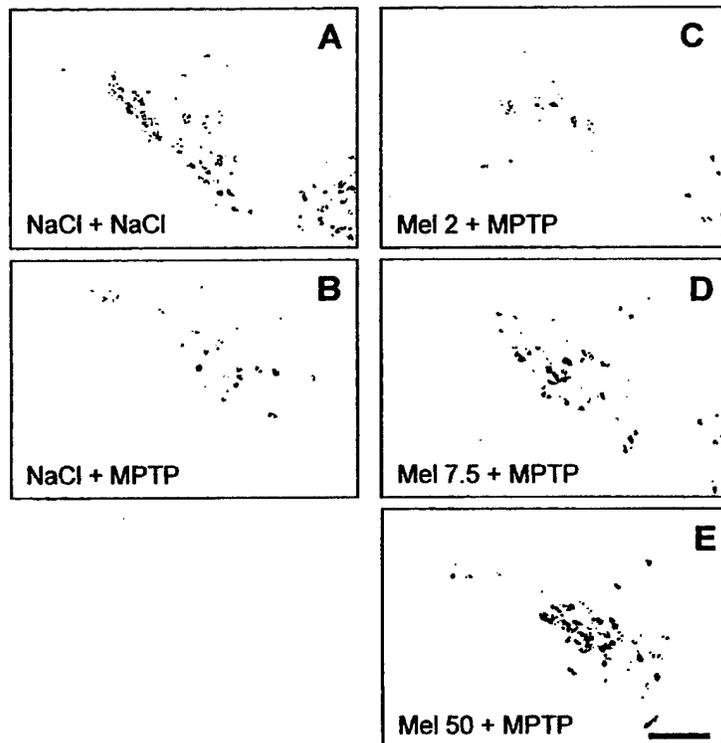


FIGURE 1. Effect of meloxicam on MPTP-induced SNpc dopaminergic neuronal death. (A) In saline-injected control mice, there are numerous SNpc tyrosine hydroxylase (TH)-positive neurons. (B) In mice treated with MPTP (30 mg/kg subcutaneous injection), the number of SNpc TH-positive neurons is reduced. (C-E) In mice treated with both MPTP and meloxicam, there is a noticeable attenuation of SNpc TH-positive neuronal loss. *Scale bar:* 200 μ m. (Reproduced from Teismann & Ferger¹³ 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.

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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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8

Free Radical and Nitric Oxide Toxicity in Parkinson's Disease

*†Serge Przedborski, *Vernice Jackson-Lewis, *Miquel Vila, *Du Chu Wu,
*Peter Teismann, *Kim Tieu, *Dong-Kug Choi, and *Oren Cohen

*Departments of *Neurology and †Pathology, Columbia University, New York, New York*

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?

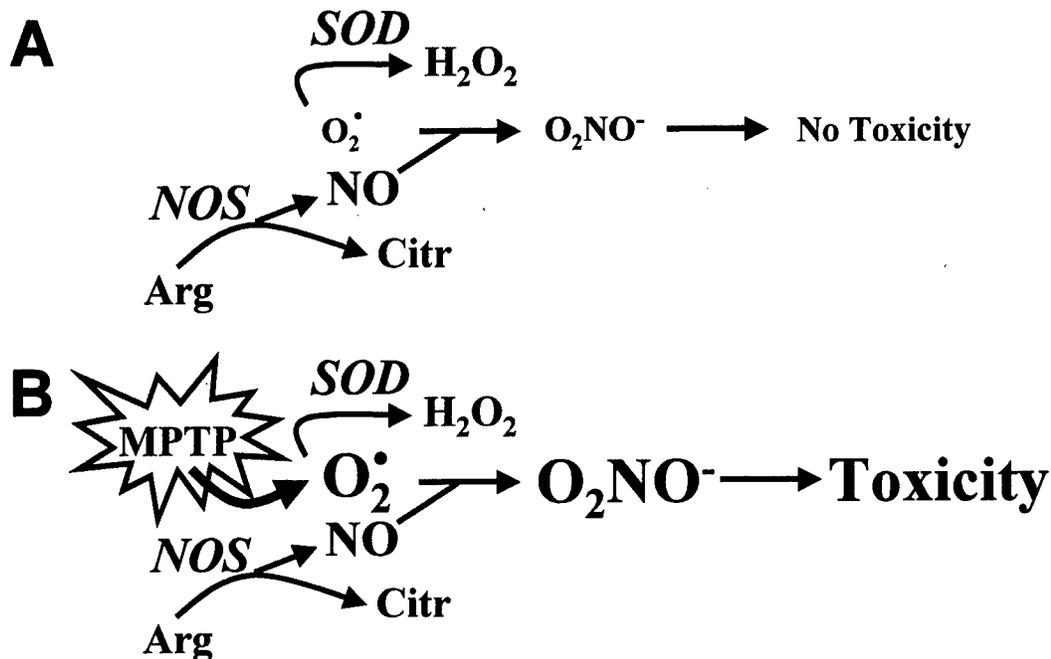


FIGURE 8.1. Superoxide radical (O_2^{\bullet}) reacts with nitric oxide (NO) to produce peroxynitrite (O_2NO^-). In normal situations (**A**), superoxide dismutase detoxifies most of the produced superoxide radicals. On the other hand, NO , which is produced by the oxidation of L-arginine into L-citrulline, 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, peroxy-nitrite is known to be very unstable and therefore to have a very short half-life. Conse-

quently, it is virtually impossible to measure the actual content of peroxy-nitrite in biological samples collected and processed for laboratory measurements. To circumvent this problem, several investigators have exploited the fact that peroxy-nitrite 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 peroxy-nitrite 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

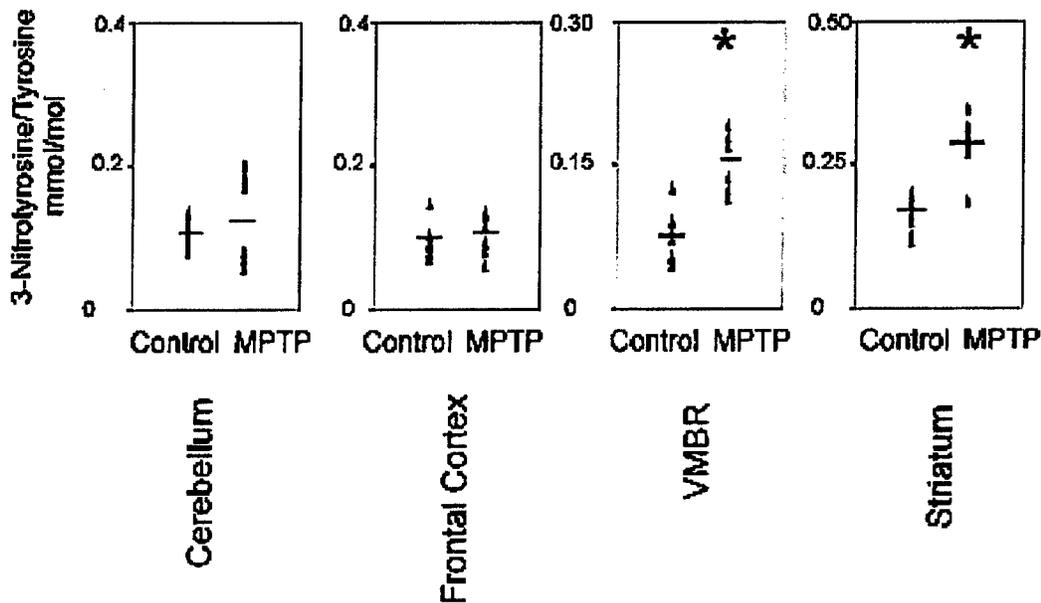


FIGURE 8.2. Gas chromatographic/mass spectroscopic quantification of nitrotyrosine 24 hours after methylphenyltetrahydropyridine (MPTP) administration in cerebellum, frontal cerebral cortex, ventral midbrain (VMBR), and striatum. (*Significantly higher than controls [$p < .05$, Student's t test].) (From 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

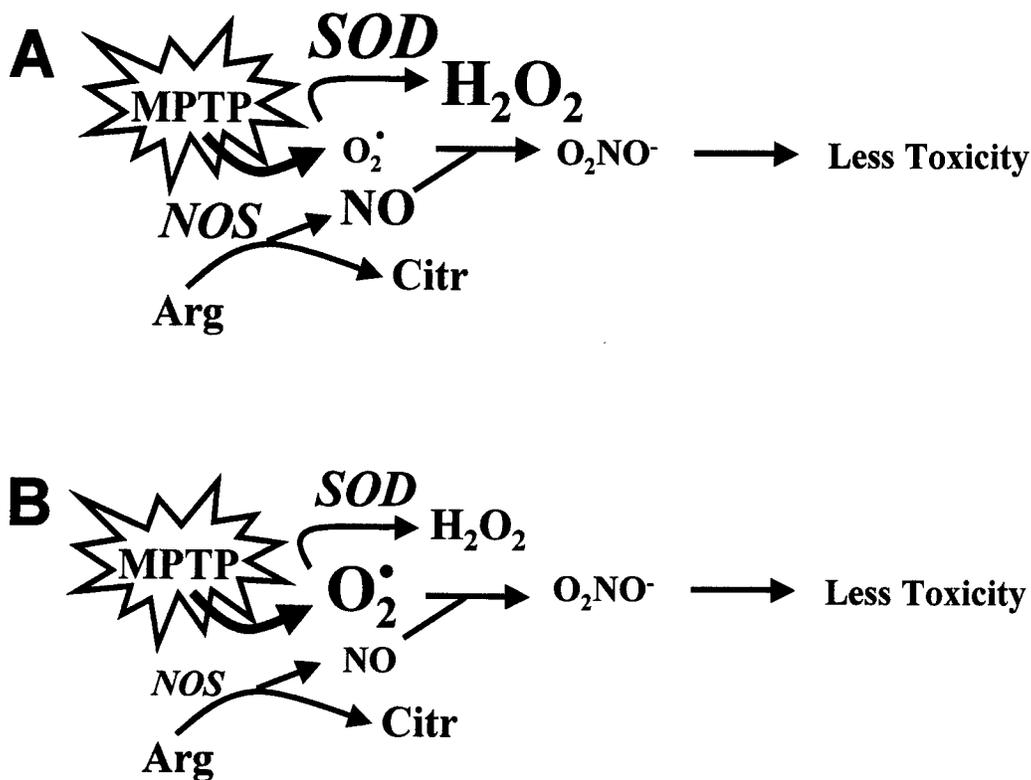


FIGURE 8.3. Effects of genetic interventions on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-related production of peroxynitrite (O_2NO^-) and cytotoxicity. By increasing superoxide dismutase (SOD) activity (A), MPTP-related superoxide radical ($O_2^{\cdot -}$) production is reduced. Thus, less 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 re-

gions, 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 as-

sess 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 loses 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-

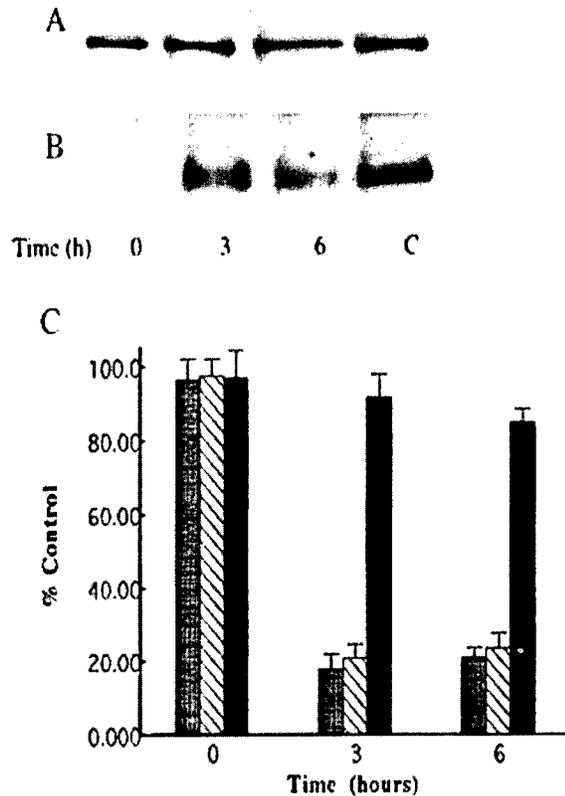


FIGURE 8.4. Inactivation of tyrosine hydroxylase (TH) by tyrosine nitration after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration. At selected times (0-, 3-, and 6-hour postexposure), TH was immunoprecipitated from the striatum of MPTP-injected mice and then visualized using an anti-TH 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.)

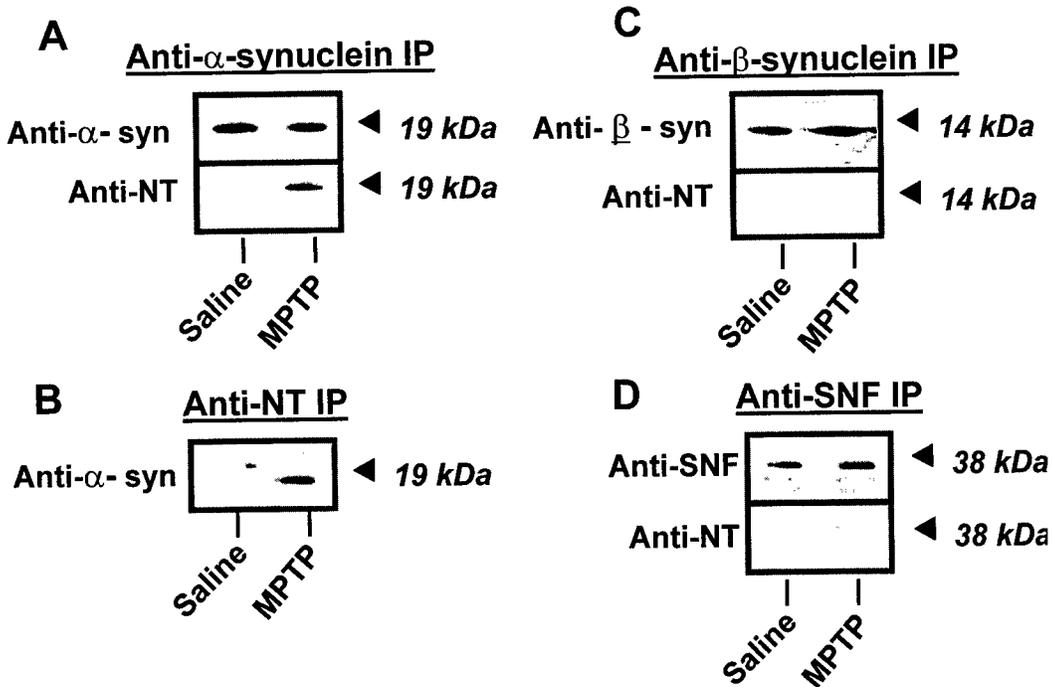


FIGURE 8.5. Tyrosine nitration of striatal α -synuclein, but not of β -synuclein, or synaptophysin, after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection to mice. After MPTP administration (4 hours after the last injection) or vehicle (saline), striatal proteins were immunoprecipitated 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-phenylpyridinium (MPP^+). As such, this putative molecule has to enter dopaminergic neurons via the plasma membrane dopamine transporter (DAT). Once inside dopaminergic neu-

rons, 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

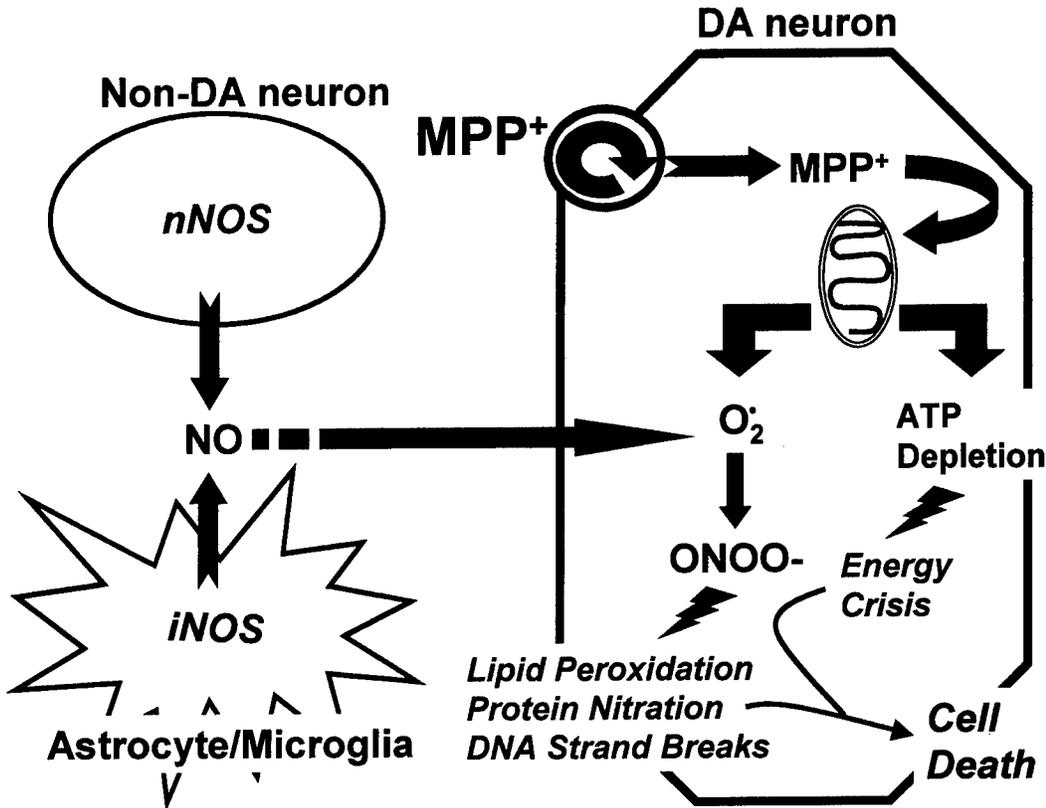


FIGURE 8.6. Proposed scenario of methylphenyltetrahydropyridine neurotoxic process and pathogenesis of Parkinson's disease.

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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