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14. ABSTRACT This proposal represents our continuing our efforts to investigate the molecular mechanisms of Parkinson's disease (PD), a progressive neurodegenerative disorder that affects about 1 million people in the United States alone. Recent evidence suggests that inflammation plays a major role in the progressive nature of this disease. Thus, we are investigating the contributions of several pro-inflammatory enzymes that we found up-regulated in microglia following the use of 1-methyl-4-phenyl-1,2 ,3,6-tetrahydropyridine (MPTP), the neurotoxin that is the cornerstone of PD research. We found that the number of SNpc DA neurons in MPTP-treated IL-1 beta and IL-1R1 knockout mice did not differ from their non-knockout littermates. This suggests that IL-1 beta and IL-1R1 may not be involved in the inflammatory response related to the necrotic form of neuronal death characteristic of the acute MPTP dosing schedule. This suggests that IL-1 beta and IL-1R1 may not be involved in the inflammatory response related to the necrotic form of neuronal death characteristic of the acute MPTP dosing schedule. However, we did note that cyclooxygenase-2 (COX-2) and cPLA2 are both up-regulated in SNpc DA neurons following acute MPTP. Because the acute MPTP dosing schedule is quite harsh, Core B characterized a subacute MPTP model of PD and found that this model exhibits at least 3 forms of neuronal death in the SNpc that are time dependent, necrosis, apoptosis and autophagy. The subacute model seems to be closer to human PD in that it does not present an immediate end-stage situation characteristic of many neurotoxin-based PD models. We have also found in this subacute model that the number of SNpc DA neurons in MPTP-treated IL-1 beta and IL-1R1 knockout mice did not differ from their non-knockout littermates. during the inflammatory process which presents somewhat of a confusing situation. Thus, we are now using our neuronal/glial cultures and our neuronal and glial conditioned media to sort out the mechanisms involved in the initiation of the inflammatory response related to SNpc DA neuronal death.					
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Abstract

Parkinson's Disease (PD) is the second most common neurodegenerative disease that affects our aging population. One of the most consistent pathological features of this disorder is neuroinflammation which is characterized by a significant presence of activated microglia, a prominent astrocytosis and the up-regulation of certain enzymes known to produce toxic compounds in the substantia nigra pars compacta (SNpc) of the brain. We, as researchers of PD, have asked the question what does the presence of neuroinflammation have to do with PD? The answers may be that this neuroinflammation is the root cause of the progressive nature of PD. Based on what we know from pathological studies and using the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of PD, which replicates almost all of the hallmarks of PD and produces an oxidative stress in the substantia nigra (SN) of the brain, we examined the role of inflammation in MPTP-induced dopaminergic neuron death in the MPTP-treated mouse. We found that indeed both microglia and astrocytes are activated in the SNpc, that certain enzymes, such as NADPH oxidase and inducible nitric oxide synthase that produce toxic compounds such as the superoxide and nitric oxide are up-regulated in this area following MPTP administration and that the cyclooxygenase type II (COX-2) enzyme is involved here. These findings were documented to be present in the human PD brain. Furthermore, we showed that we could mitigate the MPTP-induced up-regulation of these toxic producing enzymes by pharmacological means through the use of specific blockers of these enzymes. In covering all bases, noting that it is the neuromelanin-containing dopaminergic neuron in the SNpc that is dying in PD, thus spilling their contents into the microenvironment surrounding the dopaminergic neuron, we investigated the role of neuromelanin in the production of inflammation in the SNpc of rats. We found that neuromelanin produces an inflammatory response that is consistent with the pathological findings of TH-positive neuron death, microglial activation, alpha-synuclein concentration within the neurons and a significant accumulation of ubiquitin in the DA-containing cells. From our studies, we believe that neuroinflammation is a major part of the progressiveness of PD in that the disease starts with neurons dying and ends with the dying neuron initiating inflammation, making the progressiveness of PD a cyclic process.

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Role of Inflammation in MPTP-Induced Dopaminergic Neuronal Death

Final Report for Award DAMD-17-03-1-0002

Principle Investigator: Serge Przedborski, MD, PhD

Prepared by Vernice Jackson-Lewis, PhD

Date: 31 December 2008

Introduction

Most neurodegenerative diseases involve specific subsets of neurons. In the case of Parkinson's disease (PD), a common neurodegenerative disorder characterized behaviorally by resting tremor, rigidity, akinesia/bradykinesia and postural instability (Fahn and Przedborski, 2000), these are mainly, though not exclusively, the dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski, 2000). However, we do not know the etiology of PD. What we do know, aside from the death of the dopaminergic neurons in the SNpc, is 1) that PD affects mainly those individuals over 50 years of age and the risk of developing PD increases as we age (Fahn and Przedborski, 2000); 2) that there is a greater loss of dopaminergic terminals in the striatum than the loss of dopaminergic neurons in the SNpc (Fahn and Przedborski, 2000); 3) that there is an inflammatory component to PD that we and others suspect to be the cause of PD's progressive nature (McGeer et al, 1988; Banati et al, 1998); 4) that there is an up-regulation of certain cytokines in the SNpc (Mogi et al, 2000; Mogi et al, 1996; Mogi et al, 1994) 5) that the superoxide radical and nitric oxide have been implicated in PD (DiMauro, 1993; Hunot et al, 1996) and 6) that dysregulation of mitochondria is somehow in play here (ref). The central hypothesis that encompasses what we know is that following the initiation of the disease, a cascade of deleterious events leading to oxidative injury, macromolecule damage and evoked inflammation all conspire to produce mitochondrial dysfunction and energy failure which ends in the death of the dopaminergic neuron. In order to investigate the mechanisms that are involved in the death of the dopamine neuron in the SNpc, we have used MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin that replicates, thus far, in humans (Langston et al, 1999), in non-human primates (Snow et al, 2000) and in other mammals such as mice (Jackson-Lewis et al, 1995), most of the hallmarks of PD. Like in PD, MPTP causes a greater loss of dopaminergic terminals in the striatum than of dopaminergic neurons in the SNpc (Moratalla et al, 1992). Also noted, is that MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area of the brain (Muthane et al, 1994). Furthermore, as in PD, MPTP causes a selective defect in mitochondrial electron transport chain trafficking such that there is a blockage at the complex 1 site of the mitochondrial electron transport chain (Gluck et al, 1994) which suggests an overproduction of free radicals.

Aside from the severe loss of dopaminergic neurons in the SNpc, PD exhibits a marked gliosis in this area of the brain. Astrocytes and microglia, the two main components of the glial response are both up-regulated (Langston et al, 1999), however, the magnitude of their responses is quite different. The astrocytic response, both in number and in immunoreactivity, is, in general, mild and in only a few instances, has this response been dramatic (Forno et al, 1992; Mirza et al, 2000). In contrast to this, the activation of microglia has been consistently strongest in the SNpc the area of the brain most affected by the neurodegenerative process (Vila et al, 2001). Moreover, activated microglia are found in close proximity to the remaining dopaminergic neurons (McGeer et al, 1988), around which they sometimes cluster to produce what resembles neurophagia. This same situation has been noted in the three autopsied brains that were recovered from those individuals who self-administered MPTP (Langston et al, 1999). Furthermore, for reasons that we do not know, the SNpc seems to contain a significant number of microglia in its microenvironment.

Microglial activation and neurophagia are indicative of an active ongoing process of cell death which is consistent with the progressive nature of PD. Yet, data from PD brains and from the brains of the few individuals who injected MPTP do not provide us with information about the temporal relationship between SNpc dopaminergic neuron death and microglial activation. To this end, we have used the MPTP mouse model of PD to glean information about and to sort out the process of gliosis as it relates to the time course of microglial and astrocytic activation in SNpc dopaminergic neuron death. We have found that while microglia are up-regulated early on in the MPTP neurotoxic process and reaches maximum before the peak of dopamine neuron death in the SNpc, astrocytes, while mildly activated early in the neuronal death process, are further up-regulated and remain up-regulated for a lengthy period of time following MPTP administration (Liberatore et al, 1999). Thus, it is reasonable to think that glia participate in the death of the dopaminergic neurons in the SNpc both in PD and in the MPTP mouse model of PD.

Microglial cells are the resident macrophage cells in the brain that have the ability to react promptly in response to brain injury and to subtle changes in the microenvironment surrounding their charges, the neurons (Kreutzberg, 1996). In the normal brain, microglial cells are in a resting state in which they are barely visible and very few, if any, ramified processes are detected. In contrast, in a pathological situation, microglia quickly proliferate, become hypertrophic, increase in size to resemble spiders and produce a number of marker molecules that are either pro- or anti-inflammatory in nature. Thus, microglial cells are a double-edged sword type of cell producing such proinflammatory compounds as the superoxide radical, nitric oxide (NO), prostaglandin E₂ (PGE₂), excitatory amino acids, and proinflammatory cytokines such as interleukin-1-beta (IL-1-β), or such anti-inflammatory compounds as interleukin-4 (IL-4) and transforming growth factor-beta-1 (TGF-β1) (Banati et al, 1993; Gehmann et al, 1995; Hopkins and Rothwell, 1995). Since activated microglia have been found in PD and in the MPTP mouse model of PD, and microglia can produce such damaging molecules, attempts to block their activation seem reasonable. However, while blocking microglial activation is indeed important to the treatment and alleviation of a number of conditions, it is urgent that we elucidate the mechanisms by which microglia are activated in PD as this will help us to better identify those therapies that can dampen or prevent the progressive nature of PD and possibly other neurodegenerative disorders. Thus, our goal is to examine the role of inflammation in the death of dopaminergic neurons in the SNpc using the MPTP mouse model of PD. Since this is a Program Project with several components, we will concern ourselves with the Project Core (Core A and Core B) and Project 1 (Przedborski).

Neuromelanin

In searching for answers to the role of inflammation in the death of dopaminergic neurons in the substantia nigra (SN), one answer may lie with the pigment neuromelanin (NM) as a possible instigator or mediator of the neuroinflammatory process. Most PD researchers know that PD is characterized by the loss of the neuromelanin-containing dopaminergic neurons in the SNpc (Fahn and Przedborski, 2000). It has been noted that a chronic inflammatory situation exists in PD as postmortem findings in PD brains consistently show an abundance of activated microglia (Forno et al, 1992; Damier et al, 1993; Banati et al, 1998; Mizra et al, 2000). We now know that activated microglia produce a host of toxic substances, among which are the superoxide radical and nitric oxide. And these two compounds are at the heart of the oxidative stress, mitochondrial

dysfunction and energy failure that are proposed to be part of the PD neurodegenerative process. Another consistent finding in PD brains is the presence of significant amounts of neuromelanin (Double et al, 2002). NM is a pigment found in specific areas in the CNS of mammals. The greatest amounts of neuromelanin, which increases with age, are found in the neurons of the substantia nigra (SN) and the locus coeruleus (LC), two areas of the brain that are affected in PD (Fedorow et al, 2005). Neuromelanin is formed essentially by the metabolism of dopamine and norepinephrine and its synthesis appears to be driven by an excess of cytosolic catecholamines that have not been taken up into the synaptic vesicle by VMAT2 (Fedorow et al, 2005). Since NM is released into the cytosol during the death of the SNpc dopaminergic neurons, it is possible that this release may be a cause of the inflammatory response. Thus, it is logical to examine the part played by NM in the inflammatory process in PD as a link to the neurotoxicity of catecholamines, particularly dopamine, and as a cause of the inflammatory response in PD pathogenesis. It is during this extension of our work on inflammation that we will examine the role of NM in the PD inflammatory response.

Body of Research

Our overall long-term goal is the study of the pathogenesis of PD based on the oxidative stress hypothesis of PD. A number of observations have led us to believe that microglia are in play in this disorder and that they play an important role in the MPTP neurotoxic process and in the progressive nature of PD. These observations are as follows: (1) MPTP, a neurotoxin that damages DA neurons in the SNpc as seen in PD, stimulates a robust activation of microglia which have been shown to produce cytotoxic factors; (2) NADPH oxidase, activated in microglia after MPTP administration, stimulates the production of the superoxide radical; (3) immunologic nitric oxide synthase (iNOS) is induced in microglia following MPTP administration resulting in increased nitric oxide (NO) production; (4) following MPTP intoxication, other noxious mediators are produced by microglia such as the pro-inflammatory cytokine interleukin-1 β (IL-1 β); (5) we have demonstrated that mediators such as the pro-inflammatory prostaglandin PGE₂ is also increased following MPTP administration. In our continuing efforts to investigate the pathogenesis of PD, we want to understand how microglia contribute to the degeneration and death of the DA neurons in the SNpc in PD and in the MPTP mouse model of PD. Thus, in **Specific Aim I of our proposal**, we planned to gauge the neuroprotective effects of minocycline, a second generation tetracycline antibiotic that blocks microglial activation independent of its antimicrobial effects, on MPTP-induced SNpc DA neuron death in mice using HPLC, immunostaining and Western blot techniques. Also planned were cell culture experiments using neuronal/microglial cultures to assess the individual mechanisms of microglial activation here. For **Specific Aim II**, the role of NADPH oxidase in the MPTP neurotoxic process at different time points in the MPTP mouse model of PD using both normal and NADPH oxidase-deficient mice was the plan. This included assessing neuroprotection with exogenously applied superoxide dismutase (SOD) on the process of microglial activation. In **Specific Aim III**, we assessed the contribution of the superoxide radical. In our last goal for this project, **Specific Aim IV** was designed to examine the role of prostaglandin PGE₂ in the death of the SNpc DA neurons by assessing the contributions of the PGE₂-synthesizing enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) to the MPTP neurotoxic process in different brain regions and at different time points. COX-1 and COX-2-deficient mice are used in

these studies. Furthermore, as an extension to this project, because the neuromelanin-containing neurons are the ones that are degenerating in the SNpc of PD patients, we investigated how neuromelanin might contribute to the inflammatory process in this debilitating disease. Together, these planned specific aims have provided valuable information about the mechanisms involved in the inflammatory response related to the MPTP neurotoxic process and to PD and have identified targets for therapeutic intervention.

Key Accomplishments

Core A.

Core A, the administrative arm of this Research Program, has provided the centralized scientific leadership necessary for this program to succeed. Its members were in constant contact with each other, be it by physical presence, telephone or e-mail. They routinely discussed the various experiments of the project with the involved researchers, dissected results, and guided the researchers involved in this project to the completion of the various experiments and to the publication of their results. Because of their guidance, this project was fruitful and has provided significant insights into the role of MPTP in the inflammatory response as it relates to PD. These are played out in a number of interesting and significant publications.

Core B.

Core B, the centralized MPTP facility of this project, is located on the 19th floor of the College of Physicians and Surgeons building 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.

At present, the MPTP facility enjoys a significant presence on the medical center campus. It has become a stand-alone unit that is not only consulted by Columbia faculty, but also by outside investigators who want to do MPTP experiments but are not equipped to do so or who are really not knowledgeable of the workings of MPTP. Dr. Vernice Jackson-Lewis is the PI of this facility. She is not only consulted worldwide for MPTP use in animals but also teaches researchers the proper and safe use of MPTP and the procedures that the Przedborski lab uses to assess the results of MPTP use in mice. This facility also analyzes brain levels of MPTP and MPP+. For in-house experiments, whenever there is a delivery of animals to Core B, it is informed of the delivery by the Institute of Comparative Medicine (ICM) and a member of the Core B staff puts the housed mice into the MPTP facility. 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 only. 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. 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). For consultation outside of the university, Dr. Jackson-Lewis will either invite the outside researcher to Columbia to learn the various techniques or travel to the requesting site to show them how to set up a MPTP facility. We normally perform about 4-6 consultations a year.

Specific Aims

Cell Culture Facility.

Our cell culture facility is now in full operation and has conducted the cell culture studies necessary for this project. We have developed our primary post-natal ventral midbrain culture system and these are used to sort out individual mechanisms in the inflammatory response in PD using MPP⁺ (1-methyl-4-phenyl pyridium ion), the active metabolite of MPTP as well as other neurotoxins..

In vivo Experiments

All of the in vivo experiments relating to this project (Specific Aims I-IV) have been performed and completed. These involved the neuroprotective effect of minocycline, a second generation tetracycline that inhibits microglial activation independent of its antibiotic activity, against the toxic effects of MPTP (Specific Aim I), the essential role of NADPH oxidase, an enzyme up-regulated in microglia, that produces significant amounts of reactive oxygen species in the MPTP neurotoxic process (Specific Aim II), an assessment of M40401, a SOD mimetic, against MPTP-induced microglial activation in the SNpc of MPTP-treated mice (Specific Aim III) and the contribution of PGE₂ and the COX-2 enzyme to an MPTP-associated microglial activation. All of these experiments gave insights into the inflammatory response following MPTP treatment which were documented to also occur in human PD tissues.

Reportable Outcomes

Specific Aim I: Minocycline

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 (figure 1g) 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 an indicator that nitration of specific proteins has occurred (ref). 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 and Western blot 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 4) 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 (Liberatore et al, 1999), 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.

This series of experiments and its results were published in the Journal of Neuroscience, 2002.

Specific Aim II

Specific AIM II addressed the role of NADPH oxidase in MPTP toxicity.

Epidemiological studies suggest that inflammation increases the risk for developing neurodegenerative condition such as Parkinson 's Disease (Chen et al, 2003). Consistent with this is the fact that experimental models of PD demonstrate significant microglial activation (Liberatore et al, 1999, Babior, 1999) 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 (Chen et al, 2003). 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} (Babior, 1999). 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 (Babior, 1999). The superoxide radical, in turn, gives rise to secondary reactive oxygen species (Babior, 1999). Although NADPH oxidase is known to be instrumental in the killing of invading microorganisms in infections through the sustained production of the superoxide (Babior, 1999), 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 (Wu et al, 2003). 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 GP⁹¹ in mice treated with MPTP. On Western blot analyses, ventral midbrain GP⁹¹ levels patterned those of NADPH oxidase (2a, b). In the immunostaining studies, saline-injected mice showed a mild immunoreactivity to the GP⁹¹ 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 GP⁹¹ immunoreactivity was noted in the SNpc in larger cells (figure 2g,h) with thick short ramifications reminiscent of activated microglia (figure 2j). Similar GP⁹¹ immunoreactivity alterations were noticed in the striatum of these same mice. On confocal examination of the MPTP-treated tissues, GP⁹¹ 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.

GP⁹¹ expression is increased in PD midbrains.

Using both Western blot analyses (figure 3a, b) and GP⁹¹ immunostaining (figure 3c, d), we have observed increases in GP⁹¹ activity in post-mortem tissues from the SNpc of PD brains when compared to control brains. Results here were consistent with our MPTP data.

Lack of ROS production in GP⁹¹-deficient mice after MPTP administration.

To demonstrate ROS production, particularly the production of superoxide radicals 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

The Contribution of superoxide radical in MPTP-induced Neurotoxicity

M40401 Studies

Reactive oxygen species (ROS) have been implicated in a number of neurodegenerative disorders including Parkinson's disease (PD) (Przedborski, 2007). Theory has it that within the DA neuron, ROS, like the superoxide radical and the hydroxyl radical as well as reactive nitrogen species (RNS) like nitric oxide (NO), are instrumental in causing damage to essential cellular components such as mitochondria, lipids, DNA, RNA and proteins (Przedborski and Jackson-Lewis, 2000) which disrupts normal cellular function and eventually leads to the death of the neuron. In the course of metabolism within the DA neuron, the superoxide radical can be produced in several different ways. First of all, DA itself is metabolized by monoamine oxidase (MAO), an outer mitochondrial membrane enzyme, which results in a two-electron reduction of oxygen and the production of both the superoxide and the hydroxyl radical (Przedborski and Jackson-Lewis, 2000). In PD, as a result of the loss of the DA neurons, the remaining DA neurons become hyperactive and tend to exhibit increased DA turnover which results in increased ROS production (Jackson-Lewis and Smeyne, 2005). Furthermore, presumably, since L-DOPA therapy increases brain levels of DA (Przedborski and Jackson-Lewis, 2000), DA can auto-oxidize to increase the ROS load in the brain which includes the superoxide radical (Przedborski and Jackson-Lewis, 2000). Also, NADPH oxidase and cyclo-oxygenase-2 (COX-2) both inducible enzymes in pathological situations such as PD (Wu et al, 2003; Teismann et al, 2003), generate the superoxide radical. Moreover, reduction of complex I of the mitochondrial electron transport chain results in leakage of the superoxide radical (Jackson-Lewis and Smeyne, 2005) into the cytosol of the neuron. These scenarios represent at least three oxidative stress situations (mitochondrial, cytosolic and inflammatory) during which the superoxide radical is produced. To mimic the situation theorized to occur in PD, we have used the MPTP mouse

model of this disorder. Our previous studies have demonstrated that both the superoxide radical (Przedborski et al, 1992) and NO (Przedborski et al, 1996; Liberatore et al, 1999) are involved in oxidative stress produced by the MPTP neurotoxic process and that the source of both the superoxide radical and the NO appear to be microglia (Liberatore et al, 1999; Wu et al, 2003). Thus, it is not only necessary to find out what causes the death of the dopaminergic neurons in the substantia nigra pars compacta in PD, it is also necessary to find therapies which can attenuate or even prevent the death of these important neurons.

Attempts have been made to decrease the assault leveled at the brain by the superoxide radical using conventional peptidyl enzymes such as the systemic administration of native SOD (Salvemini et al, 1999) and have met with little success due to limitations associated with enzyme therapies (Salvemini et al, 1999). Recently, using molecular modeling systems studies, a stable and active class of SOD mimetics which catalyze the dismutation of the superoxide radical with rates similar to that of native MnSOD has been synthesized (Salvemini et al, 1999). These low molecular weight non-peptidyl compounds, which lack catalase activity (Salvemini et al, 1999), distribute widely in the body, keep their chemical identity and can be recovered intact in the urine and feces (Salvemini et al, 1999). The parent compound, M40403, does not react with NO, H₂O₂ or OONO and has been shown to counteract edema, inhibit neutrophil infiltration and the release of such proinflammatory cytokines as TNF- α and IL1- β (Salvemini et al, 1999). A related compound, M40401, which possesses a catalytic rate constant at least equal to or greater than that of the native SOD enzymes, showed a significant protective effect against the neuropathological and ultrastructural changes effected by bilateral occlusion of the common carotid artery ischaemia-reperfusion injury in the brain. Both injuries generate the superoxide radical (Cuzzocrea et al, 2001), thus they throw the brain into an oxidative stress situation. Key to the M40401 molecule is its manganese II (MnII) center which is difficult to oxidize to the MnIII state (Salvemini et al, 1999) (figure 1). What is interesting and so attractive about M40401 is that it has no reactivity until it is oxidized by protonated superoxide. Once reactive, the complex is rapidly reduced back to the MnII state by the superoxide anion at diffusion-controlled rates. The oxidative stress situation in the cell generated following MPTP might be the ideal situation in which to test the in vivo effectiveness of M40401 in dampening the effects of the superoxide radical as MPTP mimics the assault of superoxide radicals on the DA neuron that we see in PD. Thus, we examined the effectiveness of M40401 in attenuating cell death in the MPTP mouse model of PD.

Animals and treatment

Ten to twelve weeks old male C57Bl/6 mice from Charles River Laboratories (Wilmington, MA) were used in all experiments. Mice were housed 3-4 per cage in a temperature-controlled room with a 12 hour light/dark cycle and with free access to food and water. For MPTP administration, mice (n= 6-8 per group and per condition) received four intraperitoneal (i.p.) injections of MPTP-HCL, 16-20 mg/kg free base, in saline at 2 hour intervals. For M40401, M40404 and M40435 treatments, mice received a single subcutaneous (s.c.) injection of either compound dissolved in water pHed to 7.5 with 0.2M NaHCO₃ at a dose of 15mg/kg 30 mins prior to the first MPTP injection, then once daily for four additional days. For the MPP⁺ levels, mice received M40401 either 30 mins prior to or 30 mins after a single dose of MPTP administration. Control mice received i.p.saline/s.c. in water pHed to 7.5 only. Mice (n =6-8 per group; saline-water, M40401-saline, water-MPTP and M40401-MPTP) were sacrificed at selected time points

and their brains were used for morphological and biochemical studies. All procedures were in accordance with the National Institute 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 procedures (ref).

Results (See figures below)

M40401 attenuates dopamine neuron death in the SNpc of MPTP-treated mice.

SNpc neuron counts indicate that MPTP, 20 mg/kg free base in four doses over eight hours caused more than a 60% decrease in the number of DA neurons in the nigrostriatal DA pathway as evidenced by TH immunostaining (Figure 2). Whereas M40401 at the maximum dose of 15 mg/kg per day times 5 days had no effect on DA neurons, it significantly reversed the effect of MPTP on SNpc DA neurons. The two M40401 analogs, M40404 and M40435, at doses equal to those of M40401, were not effective in protecting SNpc DA neurons from the toxic insult by MPTP.

In PD, striatal terminal loss is around 70% which translates into impaired function of dopaminergic transmission maintenance. To determine whether M40401 might also protect against striatal DA nerve terminal loss, using TH immunostaining, we assessed the density of TH immunostained fibres in the striatum of these same groups of mice. MPTP injections (20 mg/kg x 4 doses over 8 hours) caused a dramatic decrease in striatal TH immunostaining (Figure 2) which was not reversed by the M40401 treatment regimen (15 mg/kg s.c. daily x 5 days). To examine whether M40401 might offer some protection

in the striatum against a lower dose of MPTP, we used a 16 mg/kg x 4 dose treatment schedule of MPTP. This dose of MPTP caused a reduction in striatal TH immunostaining that was not significantly different from the higher dosing schedule of MPTP, yet there was a significant, though not tremendous, sparing of TH fibres with this lower dose of MPTP in the presence of M40401 treatment. Neither saline nor M40401 by themselves affected TH immunoreactivity in the striatum.

M40401 reduces the microglial response to MPTP in the SNpc of mice.

Previously, we have shown that MPTP elicits a strong microglial response in the SNpc of mice which is maximal at 2 days after the last dose of MPTP for microglia and at 7 days after for astrocytes (ref). Thus, we examined the effect of M40401 on the microglial response to MPTP in the SNpc of mice using MAC-1, a specific marker for microglia and GFAP, a specific marker for astrocytes. As shown in Figure 4b, maximal MAC-1 immunostaining in the SNpc in response to MPTP (20 mg/kg x 4 doses over 8 hours) is indeed a strong response at the 48 hours time point indicating upregulation of both the numbers of activated microglia and the appearance of individual microglial cells. M40401 reduced significantly the number of activated microglia present in the SNpc of MPTP-treated mice (Figure 4d) as well as their appearance. At 24 hours after MPTP administration, MAC-1 upregulation was evident (data not shown). There was no evidence of MAC-1 upregulation in the SNpc in either saline-treated (Figure 4a) or M40401-treated (Figure 4c) mice. In fact, MAC-1 immunostaining in the M40401-treated mice was even less than that of the saline-treated mice (Figure 4c vs 4a). GFAP immunostaining showed some upregulation at 24 hours after the last dose of MPTP, however, maximal GFAP upregulation following MPTP administration was not observed until 7 days after the last dose of MPTP.

M40401 had no effect on GFAP immunostaining in the SNpc either at 2 days after or at 7 days after combined drug administration (data not shown).

M40401 suppresses superoxide radical formation in the SNpc of MPTP-treated mice.

Previously, we have demonstrated that MPTP stimulates the production of the superoxide radical in the SNpc of mice which seems to peak at 2 days after the last dose of drug and has been tied to the activated microglia in the SNpc (Wu et al, 2002; Liberatore et al, 1999). Thus, we examined the effect of M40401 on superoxide radical production in the SNpc at 1 and 2 days after MPTP administration using our previously published protocol for superoxide radical detection. As shown in Figures 5a and c, a significant presence of the superoxide radical was noted at 1 and 2 days after MPTP administration which was suppressed on both days by M40401 administration (Figures 5b and d). Superoxide radical production was at normal levels in the SNpc of the saline-treated mice and was even lower than normal in the M40401 only mice (data not shown).

M40401 reduces MPTP-induced carbonyl content elevations in the striatum of MPTP-treated mice.

Protein carbonyl content is evidence that modification of cellular proteins has occurred. Normal levels of carbonyl in neurons is quite low as seen in saline-treated mice (Figure 3). However, at 2 days after the last dose of MPTP, significant elevations of carbonyl content in the striatum of MPTP-treated mice was noted (Figure 3). These elevations at this time point were reduced significantly by M40401 (Figure 3). This compound by itself showed a slight though insignificant effect on normal levels of striatal carbonyl content. At 7 days after MPTP treatment, both MPTP only and MPTP plus M40401 were no different from saline-treated mice (Figure 3).

M40401 Does Not Affect Tyrosine Hydroxylase Biochemistry.

Tyrosine hydroxylase (TH) is the key enzyme for the proper function of DA in the nigrostriatal pathway. Thus, we examined whether M40401 might have any effect on TH biochemistry in our MPTP-treated mice. On Western blot analysis, TH in the presence of M40401 was the same as TH in the presence of saline only (Figure 6a). Western blots also show significant decreases in TH levels at 2 days and at 7 days after MPTP administration and these level decreases were not altered by M40401 (Figure 6a). Densitometry of the TH Western blots (Figure 6b) are in total agreement with TH Western blot analyses.

Immunoprecipitation studies of TH shows a decrease in TH protein at 2 days after MPTP administration and these changes were not affected by M40401 (Figure 6c). TH protein continued to decrease as evidenced by the further disappearance of TH protein at the 7 day time point after MPTP treatment (Figure 6c). M40401 did not affect TH protein disappearance at this time point either. Densitometric analysis here (Figure 6d) represents a clear visualization of the behavior of TH protein with and without M40401.

M40401 does affect MPTP-induced Striatal Dopamine Metabolism.

HPLC with electrochemical detection showed that MPTP at 20 mg/kg in 4 doses over 8 hours caused about a 90% depletion in striatal dopamine levels (Figure 7a) which is in agreement with our previously published data on MPTP-induced dopamine depletion (ref) and which was slightly but significantly attenuated by the presence of M40401 (Figure 6a). Like dopamine, both DOPAC and HVA were also decreased by MPTP administration (Figures 7b and c, respectively)

which was indeed significant though not to the same degree as dopamine. M40401 reversed somewhat the MPTP-induced effect on DOPAC and HVA (Figures 7b and c). M40401 and saline had no effect on dopamine metabolism in the striatum (Figures 7a, b and c).

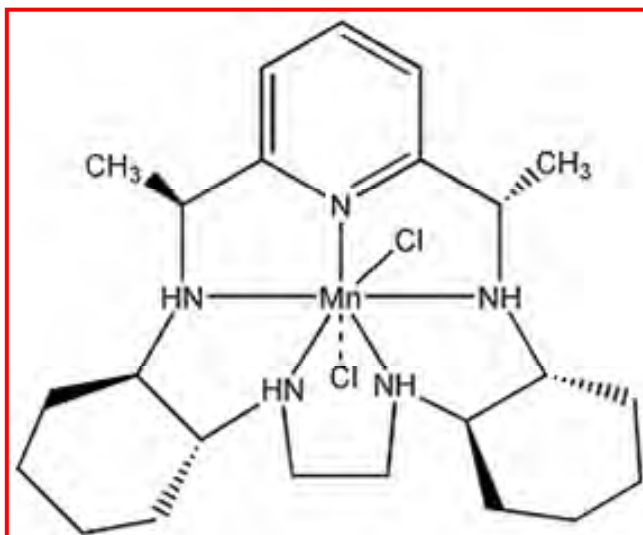
M40401 does not affect MPTP metabolism in the striatum.

The metabolism of MPTP to MPP⁺ and MPP⁺ entry into dopaminergic neurons are key to the neurotoxic effects of MPTP and interference in any of these processes compromises any neuroprotective effect of the agent being used for such. Thus, we examined whether M40401 might interfere with the key steps of MPTP metabolism in the brain. Figure 8a shows no differences in striatal levels of MPP⁺ between the MPTP only and the MPTP-M40401 groups of mice 90 mins after an acute dose of MPTP (Figure 8a). This same lack of differences in MPP⁺ levels is also seen in ventral midbrain (Figure 8b). Furthermore, when we examined blood levels of MPP⁺, we found the levels of MPP⁺ equal between the two groups of mice as were the blood levels of MPTP (Figure 8c). When we looked at the synaptosomal uptake of MPP⁺ in the presence of M40401 or its two isoforms, M40404 and M40435, no interference in synaptosomal uptake of MPP⁺ was noted (Figure 8d). Moreover, MPTP-induced lactate levels in striatal tissues were unchanged even with the highest concentration of M40401 used (Figure 8e).

The first leg of the studies using M40401 in the MPTP mouse model of Parkinson's disease were completed, however the additional studies involving the chronic use of M40401 were not done as we were unable to obtain a continuing supply of the compound.

This work on M40401 in the MPTP mouse model of PD was presented at the Society for Neuroscience Meetings in 2004. Since we could not obtain any more of this compound, we began investigations on TEMPOL, another SOD mimetic compound. The manuscript for the work involving M40401 is now in preparation.

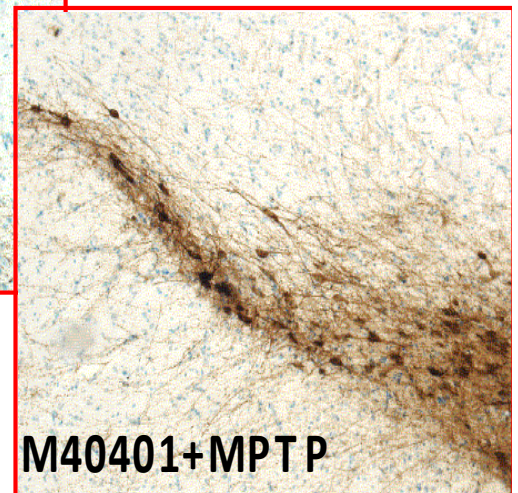
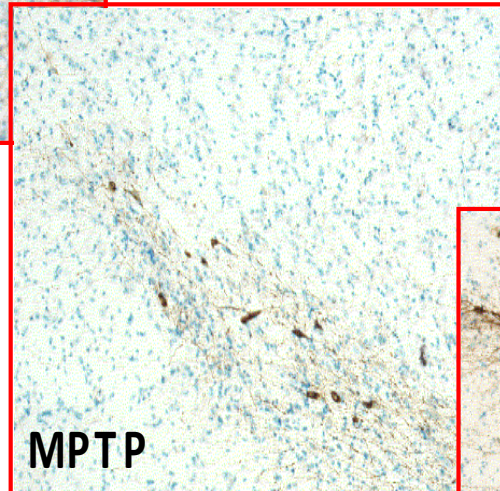
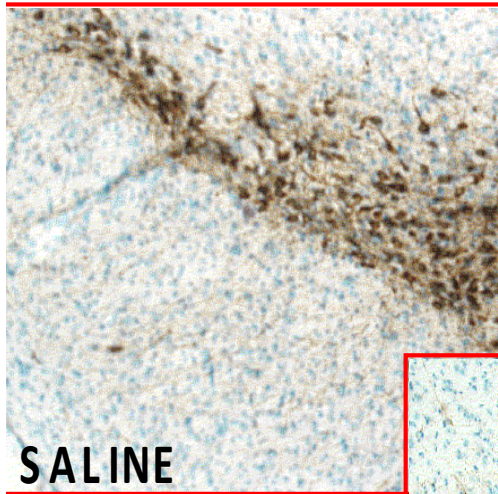
FIGURE 1. M40401 STRUCTURE



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FIGURE 2. TH IMMUNOSTAINING IN M40401 + MPTP-TREATED MICE



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FIGURE 3. M40401 ATTENUATES TYROSINE HYDROXYASE (TH)-POSITIVE NEURON LOSS IN THE SNPC OF MPTP-TREATED MICE.

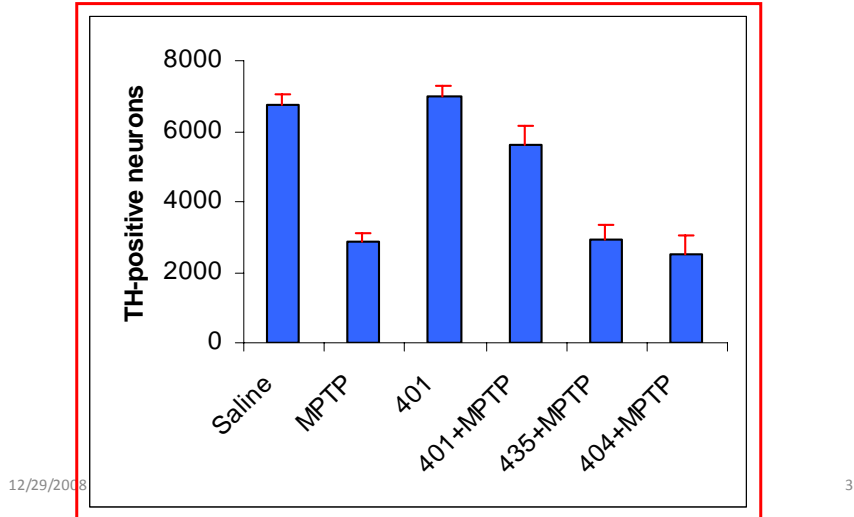


FIGURE 4. EFFECT OF M40401 ON MPTP-INDUCED STRIATAL NERVE TERMINAL DAMAGE.

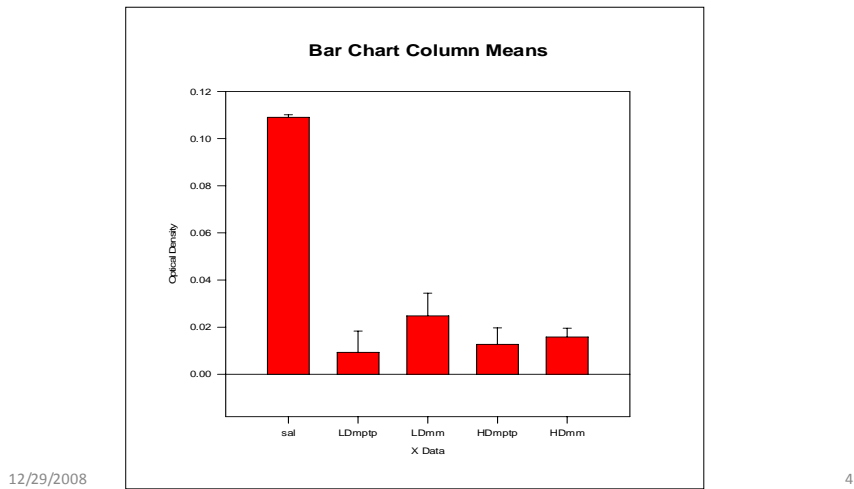


FIGURE 5. M40401 ATTENUATES MICROGLIAL ACTIVATION

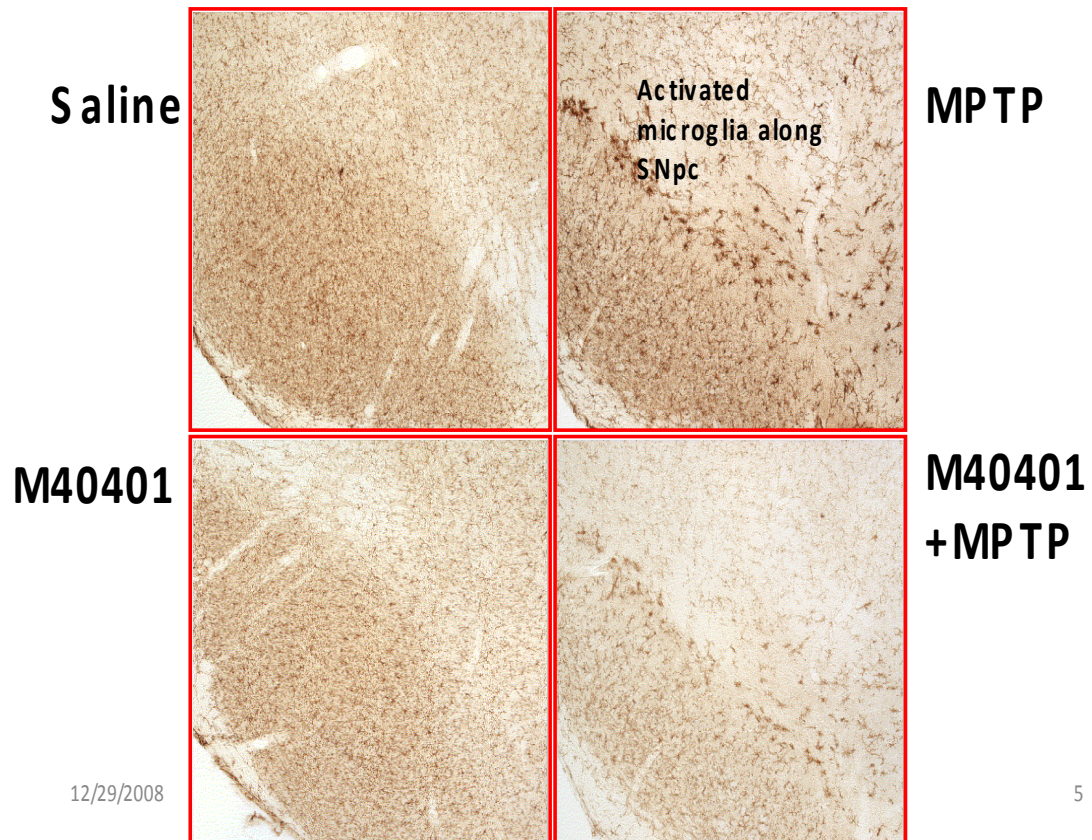
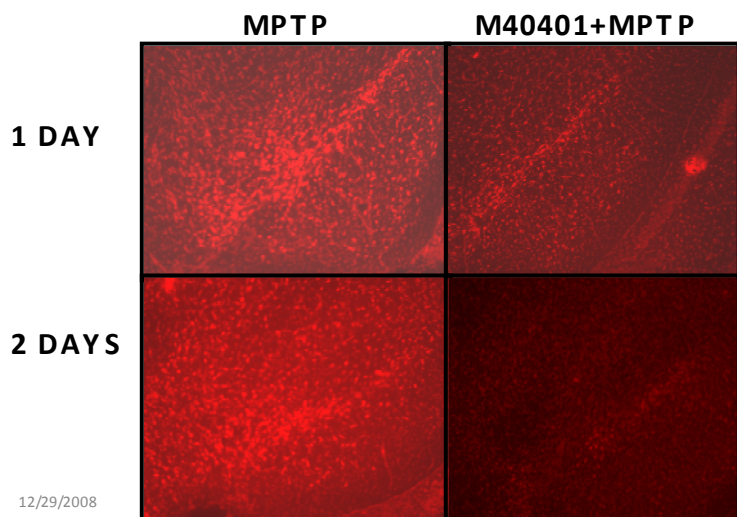


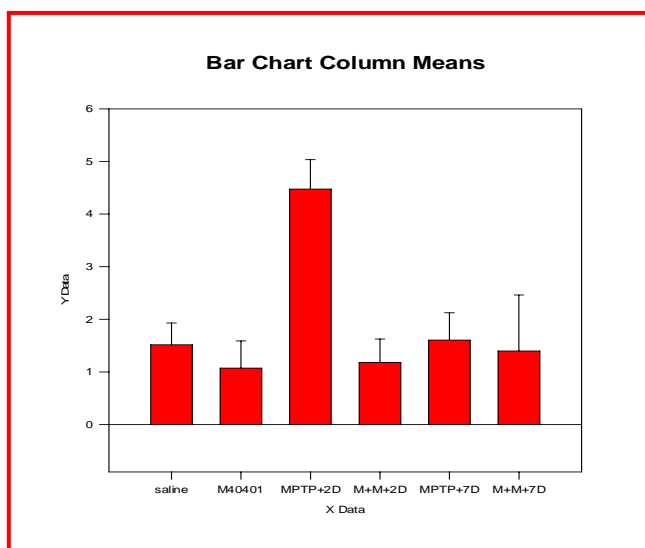
FIGURE 6. M40401 SUPPRESSES SUPEROXIDE FORMATION



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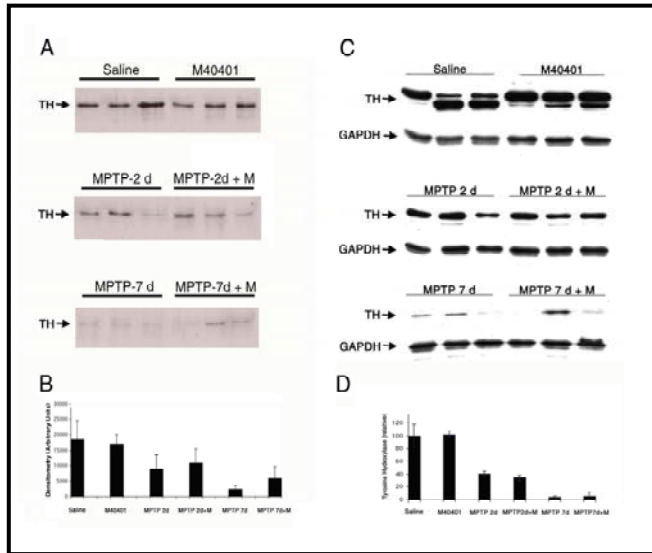
FIGURE 7. M40401 SUPPRESSES MPTP-INDUCED STRIATAL CARBONYL CONTENT.



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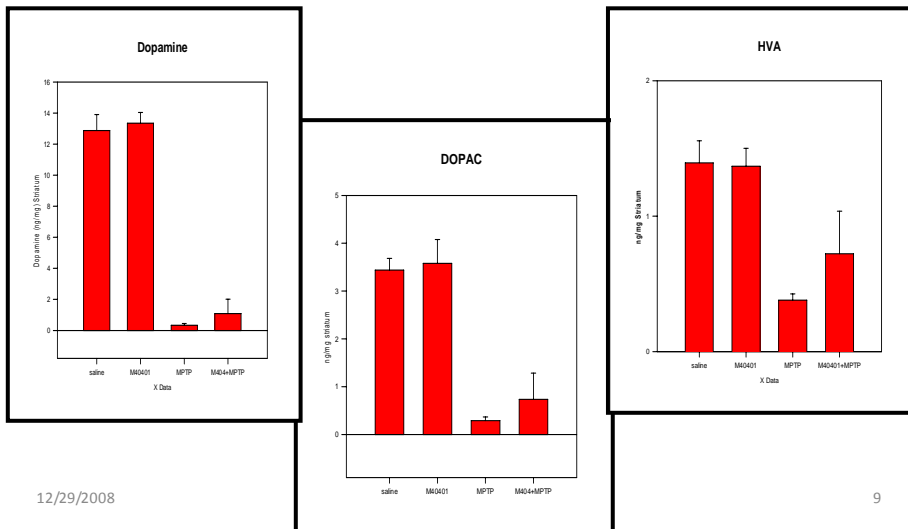
FIGURE 8. M40401 DOES NOT AFFECT MPTP-INDUCED NITROTYROSINE FORMATION



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FIGURE 9. EFFECT OF M40401 ON STRIATAL DOPAMINE METABOLISM IN MPTP-TREATED MICE.



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FIGURE 10. M40401 DOES NOT AFFECT MPTP METABOLISM IN THE STRIATUM, VENTRAL MIDBRAIN OR BLOOD.

MPTP AND MPP+ (UG/G OR UG/ML)

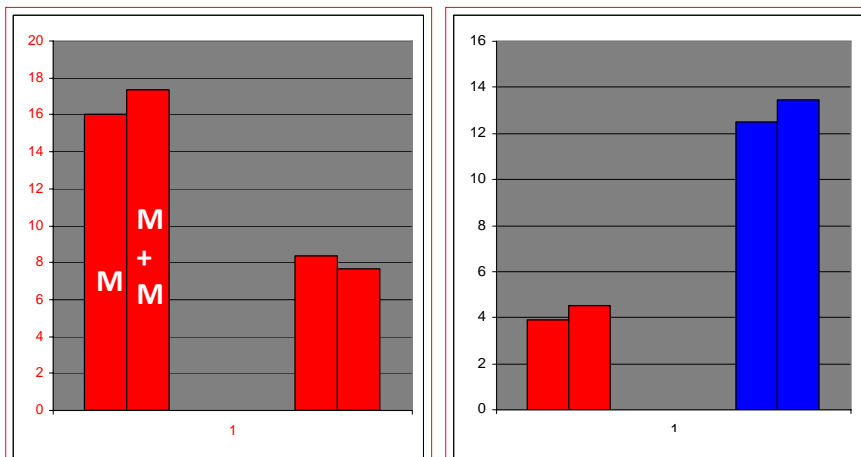
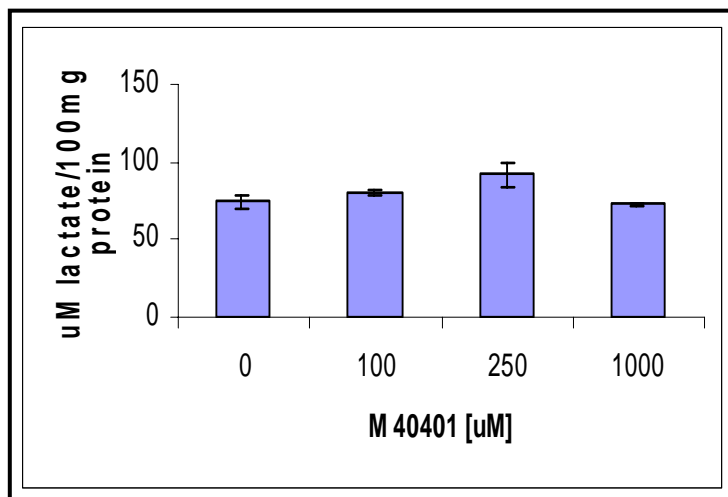


FIGURE 11. SYMPTOSOMAL UPTAKE OF M40401 VS. INACTIVE SOD-MIMETICS IN THE PRESENCE OF MPP+.

Concentration (μ M)	IC ₅₀ of MPP ⁺ uptake
M-40401	23.30 \pm 1.73
M-40404	0.82 \pm .22
M-40435	36.25 \pm 7.91

FIGURE 12. EFFECT OF M40401 IN THE PRESENCE OF MPP+ ON LACTATE LEVELS.



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Tempol

In our continuing efforts to find and examine SOD mimetics that might protect the TH-positive neurons in the SNpc of MPTP-treated mice, we investigated the neuroprotective effects of TEMPOL. TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl, figure 1) a stable nitroxyl antioxidant that exhibits SOD mimetic characteristics (Samuni et al, 1990), is thought to be neuroprotective in disorders involving reactive oxygen species. TEMPOL reacts directly with carbon-centered and peroxy radicals (Rak et al, 2000) and prevents the reduction of hydrogen peroxide to the hydroxyl radical (Samuni et al, 1991). It can oxidize reduced transition metals (Samuni et al, 1991) and decrease the mortality related to intraperitoneal 6-hydroxydopamine (6-OHDA) (Purpura et al, 1996; Weinberg et al, 2004). Furthermore, TEMPOL has been shown to protect MN9D DA neurons against 6-OHDA in cell culture and DA and its metabolites against the intrastriatal application of 6-OHDA in vivo. Since recent studies demonstrated the neuroprotective effects of TEMPOL against both central and peripheral nervous system damage (Liang et al, 2005), we thought it prudent to examine whether TEMPOL could counteract the damaging effects of MPTP in mice. In our basic studies, we found (1) that TEMPOL does not interfere with MPTP metabolism; (2) that it protects significantly SNpc TH-positive neurons against MPTP-induced damage; (3) that TEMPOL partially attenuates the MPTP-induced up-regulation of microglia and astrocytes; and (4) that TEMPOL suppresses superoxide radical formation to a significant degree. Although more work needs to be done to clarify the usefulness of TEMPOL as a possible therapeutic agent against PD, this drug does show some promise

Animals and Treatment

C57bl/6 mice from Charles River Laboratories, aged 12 weeks, were used in all experiments. Mice were treated with Tempol, 50-250 mg/kg i.p. 0.5-1.0 hour prior to MPTP, 18 mg/kg in 4 doses over an 8 hour period, each dose 2 hours after the previous dose. Tempol dosing was continued daily for an additional 4 days after MPTP treatment. Mice were sacrificed by perfusion-fixation at 2 and 7 days after the last dose of MPTP for immunohistochemistry studies. For the HPLC analysis of catecholamines, mice were sacrificed by decapitation without anesthesia for fresh striatum and ventral midbrain. As to whether Tempol interferes or not with MPTP metabolism, mice were injected with Tempol either 30 mins before or 30 min after a single dose of MPTP, 18 mg/kg. Mice were then sacrificed 90 mins after MPTP administration and striatum was taken for MPTP and MPP+ levels. Furthermore, to demonstrate the presence of the superoxide radical using fluorescence, treated mice at 2 days after MPTP only and Tempol plus MPTP, were injected with dihydroethidium, 1 mg/kg, and sacrificed 15 mins later. Mice were decapitated without anesthesia and whole brains were quickly removed and frozen in dry-ice cooled isopentane until the day of the assay.

Results (See figures below)

Tempol protects TH-positive neurons in the mouse SNpc against MPTP-induced damage.

Seven days after the last dose of MPTP, the SNpc of the mice showed a significant loss of TH-positive neurons (figure 2c), which is consistent with our previous findings in this model. And which was reversed (figure 2d) by treatment with Tempol. Tempol alone (figure 2b) appears to have no effect on TH-positive neurons when compared to saline-injected mice.(figure 2a). Thus, Tempol is believed to have a protective effect in the SNpc against MPTP-induced DA neuronal death.

TEMPOL partially suppresses microglial activation in the MPTP-treated mouse.

In saline-injected mice (figure 3a), on MAC-1 immunostaining, microglia are quiescent, appearing small with few or no processes. In the presence of Tempol alone.(figure 3b), microglia seem to have even less of a presence than in the saline treated mice. At 2 days after the last dose of MPTP, microglia are ramified and exhibit thick processes (figure 3c), both of which are partially suppressed with Tempol (figure 3d). With no Tempol present in the central nervous system at 7 days after the last injection of MPTP (figure 3e), microglia are intensely activated not only appearing ramified but also giving the appearance of clumping in the area of the SNpc. From these data, it is apparent that Tempol is effective in partially attenuating the microglial activation caused by MPTP.

TEMPOL attenuates MPTP-induced up-regulation of astrocytes in the SNpc of mice.

Astrocytes, thought to supply nutrients to neurons in the SNpc., are up-regulated at 2 days after MPTP, as evidenced by a small increase in GFAP immunostaining in the SNpc of the MPTP-treated mouse (figure 4b). This response in the SNpc is even stronger at the 7 days timepoint (figure 4d) after MPTP and is indicative of the up-regulation of astrocytes. At both the 2 days

and the 7 days timepoints after MPTP application, figures 4c and 4e show that this astrocyte up-regulation is partially attenuated by Tempol.

TEMPOL suppresses MPTP-induced superoxide production.

We have previously shown that MPTP induces superoxide production, which peaks at 2 days after MPTP administration. Consistent with our previous data, we demonstrate this same effect as before using dihydroethidium (figure 5a). Furthermore, we also show that superoxide production at the 2 day timepoint after MPTP is suppressed by Tempol (figure 5b) administration.

TEMPOL does not interfere with MPTP metabolism.

The metabolism of MPTP to its active metabolite MPP⁺ is key to the damaging effects of MPTP. Interference in any of MPTP's metabolic steps by a compound can negate that compound's neuroprotective effect. Thus, we injected Tempol both before and after MPTP. We found that although there was a slight though non-significant increase in striatal levels of MPP⁺ when Tempol was administered 30 mins after MPTP, both dosing schedules of Tempol did not affect MPTP metabolism (figure 5).

This work was presented at the Society for Neuroscience Meetings in 2006

FIGURE 1. TEMPOL STRUCTURE

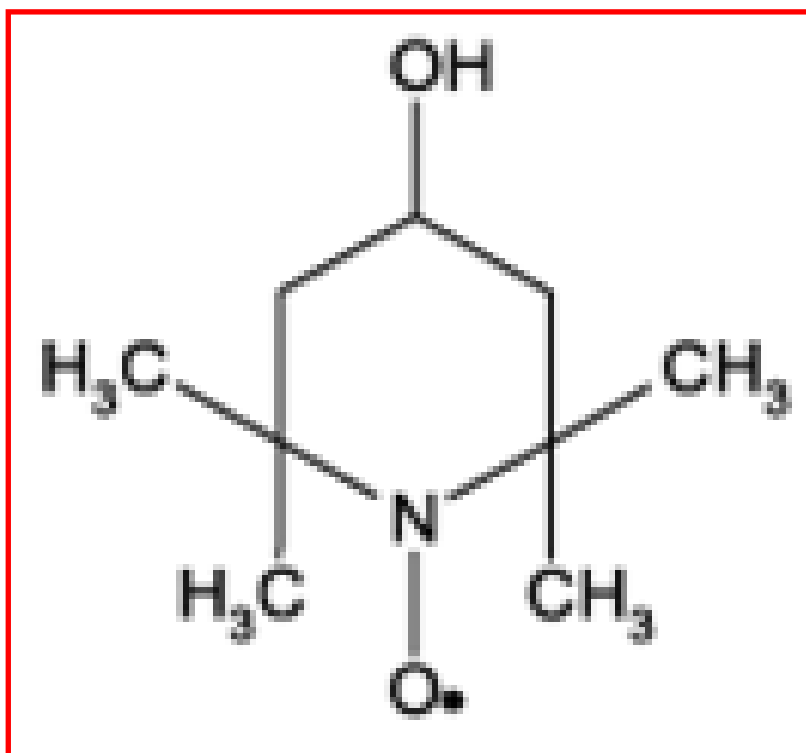


FIGURE 2. TEMPOL PROTECTS DOPAMINERGIC NEURONS IN THE MOUSE SNPC AGAINST MPTP-INDUCED DAMAGE.

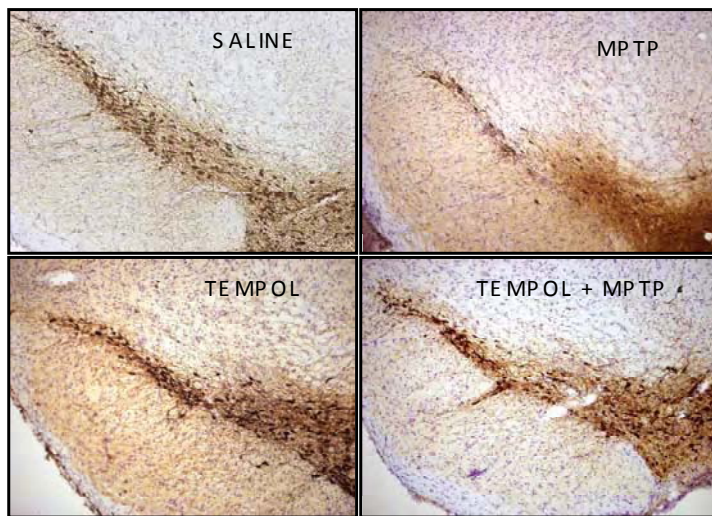


FIGURE 3. TEMPOL PARTIALLY SUPPRESSES MAC-1 IMMUNOSTAINING IN THE SNPC AT 2 DAYS BUT NOT AT 7 DAYS AFTER MPTP.

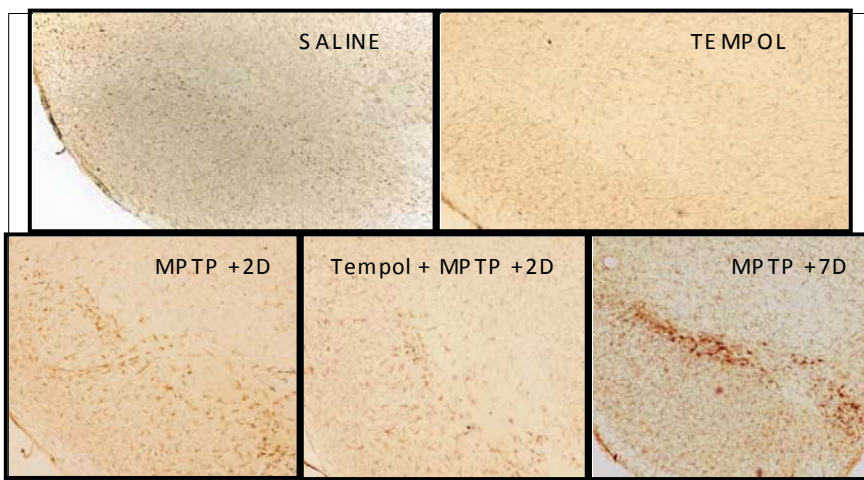


FIGURE 4. TEMPOL ATTENUATES MPTP-INDUCED UP-REGULATION OF ASTROCYTES IN THE MOUSE SNPC AT 7 DAYS AFTER MPTP.

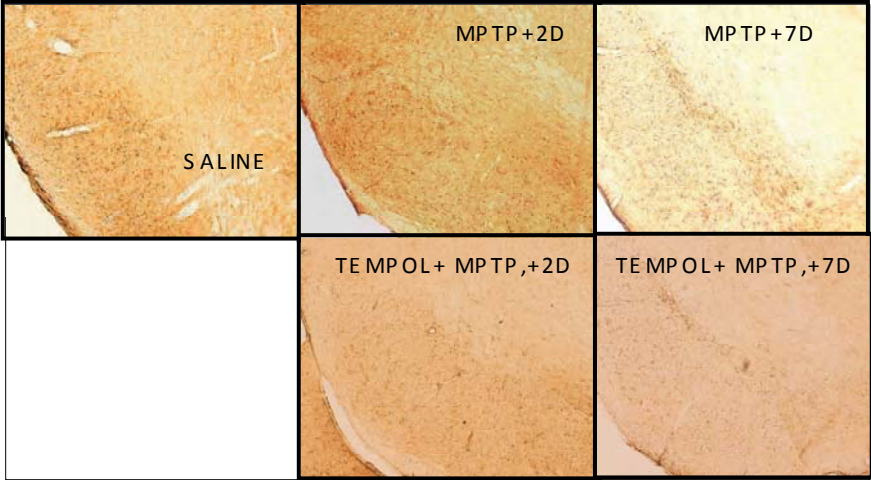


FIGURE 5. TEMPOL SUPPRESSES MPTP-INDUCED SUPEROXIDE PRODUCTION.

Hydroethidium was injected i. p. at +2D after MPTP and during Tempol administration. Mice were sacrificed 15 mins after Heth, brains fresh frozen and cut (14 um) on a cryostat. Tissues were assessed under a fluorescent microscope.

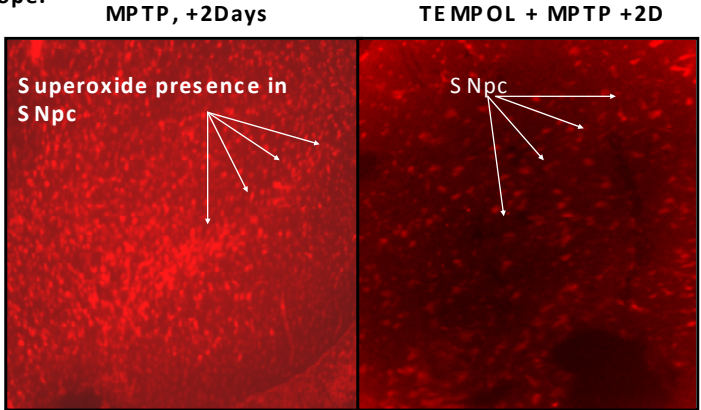
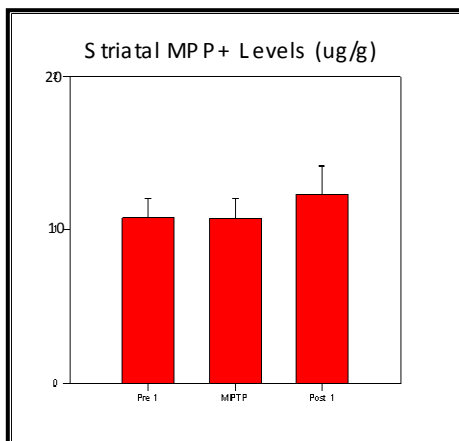


FIGURE 6. TEMPOL DOES NOT INTERFERE WITH MPTP METABOLISM.



Specific Aim IV

The contribution of the cyclooxygenase enzymes to the MPTP neurotoxic process.

The inflammatory response in the brain is thought to contribute to the neurodegenerative process that is now a hallmark of PD in that it may be at the root of its progressive nature (Przedborski, 2007). Parts of this response include microglial activation and proinflammatory and cytokine up-regulation (Banati et al, 1993; Gehrman et al, 1995; Rothwell, 1995). Since we are investigating the contribution of inflammation to the neurodegenerative process, it is necessary to examine the separate components which contribute to this inflammation. 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 (Teismann et al, 2003). Whereas COX-1 is constitutively expressed in many eukaryotic cells and is relevant to the production of prostanoids in physiological processes (O'Banion, 1999), COX-2 expression is induced and, along with the elevation of PGE₂, has been implicated in degeneration in a variety of situations (O'Banion, 1999). 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. Male mice, 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 levels 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 COX-1 protein content at 2 and 4 days after MPTP, these alterations were not significant. In contrast, however, 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 co-localize 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 our 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 TH-positive neurons in the SNpc and surviving TH 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 TH neurons in the SNpc and TH terminals in the striatum.

MPTP-induced toxicity requires COX-2 catalytic activity.

Since COX-2 can exert deleterious effects in transfected cells in absence of its catalytic activity (O'Banion, 1999), 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, at oral doses of 25 and 50 mg/kg and 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 TH-positive 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 events to occur in SNpc TH-positive 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 (Subbaramaiah et al, 1998). 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 TH-positive neuronal death, it did not afford the same protection to TH-positive striatal fibres (figure 4C, E and Table 1). Thus, our data show 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.

Since we 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, the

next logical step was to examine whether any of these manipulations might affect MPTP uptake and metabolism. Thus, we measured 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 in these various situations. 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.

Our next question to answer was 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.

COX-2 can damage intracellular protein-bound sulfhydryl groups by the oxidation of catechols such as DA (Hastings, 1995). Thus, we asked whether this is 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 2 times higher in the MPTP only mice than the levels of this compound in mice that received Rofecoxib along with MPTP (figure 6I).

This work was presented at the Society for Neuroscience Annual Meeting in 2002 in Orlando, FL and was published in the Proceedings of the National Academy of Science in 2003.

Extension: Neuromelanin

In Parkinson's Disease (PD), there is a significant loss of the neuromelanin-containing neurons in the substantia nigra pars compacta (SNpc) of the brain (Fahn and Przedborski, 2000). NM consists mainly of dopamine (DA)-derived compounds including cysteinyl-DA in significant quantities. Since both DA and cysteinyl-DA are thought to be toxic, and these are sequestered in NM, it is possible that dying DA neurons release these toxic compounds into the extracellular space (Zhang et al, in press). Such a release may induce a neuroinflammatory response which can possibly push the DA neuron death process. Furthermore, NM as well as several of the toxic compounds that are contained within NM, such as iron and synuclein, individually have already been shown to elicit a cell death response in vitro (Smythies, 1996; Halliday et al, 2005). Thus, we thought it prudent to examine this phenomenon in an in vivo situation. We injected human

NM (figure 1) from PD patients into the SNpc of rats and found that it indeed induces an inflammatory response that is time-dependent as well as the loss of TH-positive immunostaining. Also noted was a decrease in TH-positive neuron numbers and an increase in the number of Nissl-stained cells. GABA neurons were not affected by the NM injection. We conclude that NM may contribute significantly to the progressive nature of PD through the production of a neuroinflammatory response as a result of the release of toxic compounds.

Animals and Treatment

Rats, Sprague-Dawley males, 200-225 grams at the beginning of the experiment, were rotated with amphetamine, 1mg/kg, i.p. to ascertain whether there were any imbalances in the nigrostriatal system. After 1 week of rest, rats received a stereotaxic injection of 3.4 micrograms of human NM (0.85mg/ml PBS) into the left SN at the rate of 1 microliter per min. The needle was left in place for an additional 2 mins to allow for diffusion of the NM. At 3 weeks after NM injection, rats were rotated to amphetamine (1 mg/kg, i. p.), followed by apomorphine rotation at 4 weeks after NM injection. Following rotation studies, rats were sacrificed either by perfusion for immunostaining or decapitated for monoamine measurements and Western blot studies in the striatum and the ventral midbrain.

Results (See attached figures)

Rotational Behavior following NM injection into the SNpc.

As noted in figure 2, rotational behavior to the same side of the lesion was elicited with 1mg/kg of amphetamine 3 weeks after a 4 ug injection of NM into SNpc of rats indicating a left-right asymmetry in the nigrostriatal DA system. In contrast, no apomorphine-induced rotation was seen in these animals.

DA and metabolites are altered following NM injection into the SNpc.

On HPLC analyses, 4 weeks after NM injection into the SNpc of rats, a small but significant decrease in DA and DOPAC was found in the left striatum (figure 3a,b). This same situation was found in the left SN (figures 3c,d) of the rats. NM injection did not affect the right striatum or SN.

TH immunostaining and cell counts alterations after NM injection into the SNpc of rats.

Figure 4a depicts TH immunostaining in the injected SN 4 weeks after NM injection. On appearance, NM injection here seemed to have killed a significant number of TH-positive neurons. Figure 4b shows the actual TH-positive neuron number lost. Counts indicate about a 65% neuron number loss, however, a significant portion of the decrease in TH-positive neuron number loss correlates with a dramatic increase in Nissl-stained neuron numbers. Thus, a large number of the TH-positive neurons were not lost; what was lost was their phenotype.

Alpha-synuclein immunostaining is altered in the SNpc of rats after NM injection.

In the SN of the PD brain, alpha-synuclein accumulation has been shown to be quite prominent. Most likely, this is the result of some kind of injury. In keeping with the injury theory, it was noted that alpha-synuclein accumulation occurs as early as 2days after NM injection into the SN

(figure 5a) and still exhibits immunostaining (figure 5b) as late as 21 days after NM injection compared to PBS injected rats.

Ubiquitin accumulates in the SNpc of NM-injected rats.

Ubiquitin accumulation is usually associated with impaired protein degradation. At 21 days after NM injection into the SNpc of the rats, figure 6 depicts prominent ubiquitin immunoreactivity in contrast to the PBS injected control SNpc.

NM injection into the rat SNpc induces microglial activation.

Our original premise was that NM contributes to neuroinflammation in PD possibly through its release into the SN microenvironment following the death of the melanin-containing TH-positive neurons. At 2 days after the injection of NM into the SNpc of rats, there was a significant activation of microglia on Iba-1 immunostaining (figure 7a) which was still ever present at 21 days after NM injection (figure 7b). Also noted is the clumping of the microglia around the injection site.

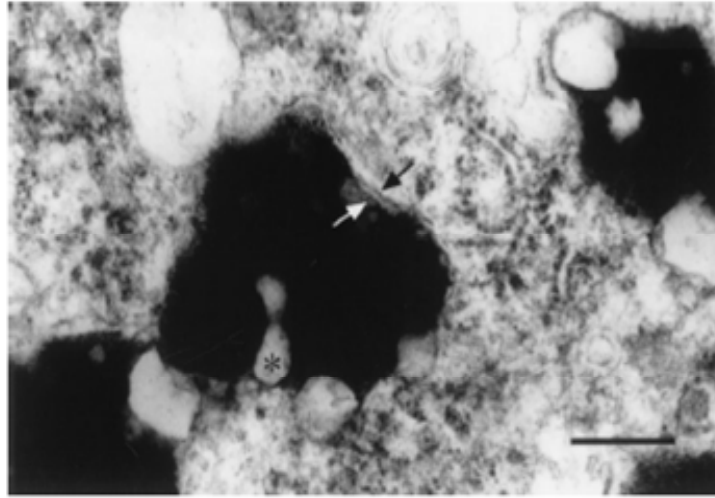
NM induces astrocytosis in the rat SNpc.

Part of the inflammatory response is astrocyte activation. In keeping with this, we noted that the astrocyte response on GFAP immunostaining was present although mild in the SNpc at 2 days after NM injection (figure 8). This astrocytic response still exhibited a strong presence at 21 days after NM injection. (figure 8)

This work was presented at the Society for Neuroscience Annual Meeting in Washington, DC in 2008.

NM figures

FIGURE 1. NM IN THE HUMAN SN



Sulzer et al, PNAS 97: 11869-11874 (2000)

FIGURE 2. ROTATION IN NM-INJECTED RATS

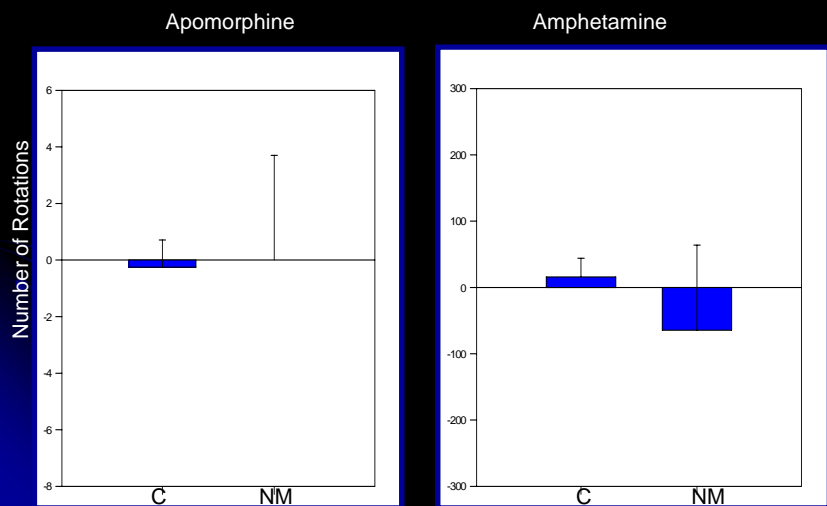


FIGURE 3A. DA LEVELS (NG/MG): LEFT AND RIGHT STRIATA

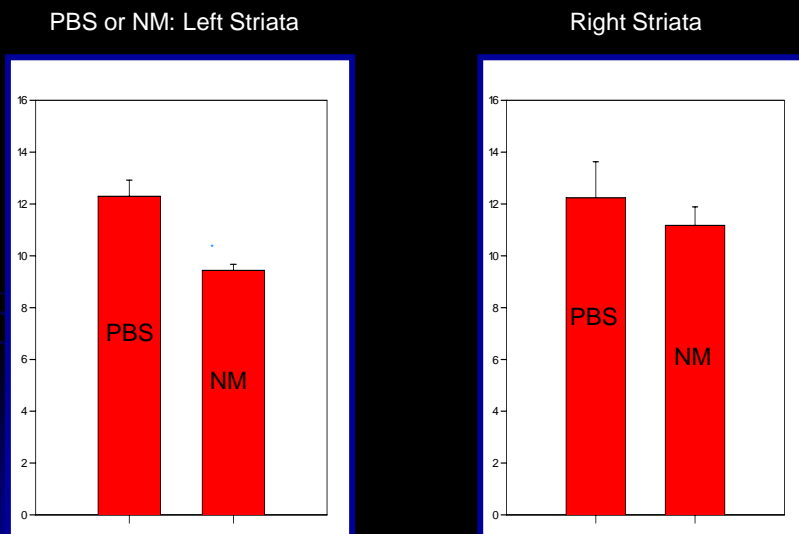


FIGURE 3B. DOPAC LEVELS: LEFT AND RIGHT STRIATA

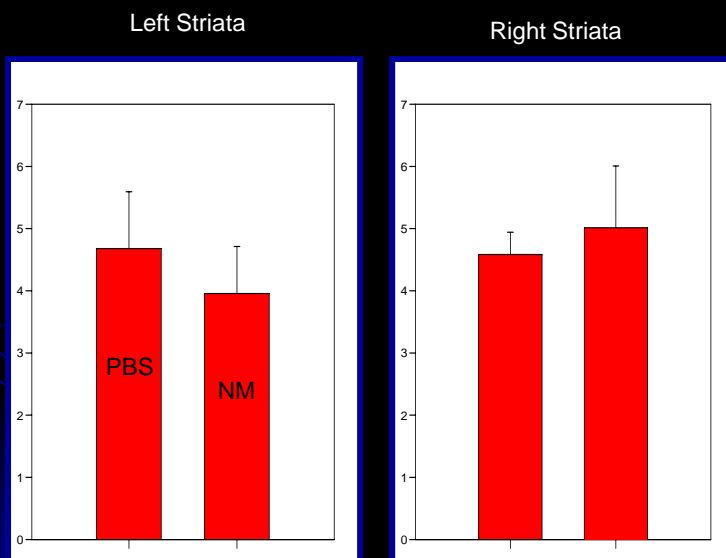


FIGURE 3C: DA LEVELS IN THE SUBSTANTIA NIGRA (SN)

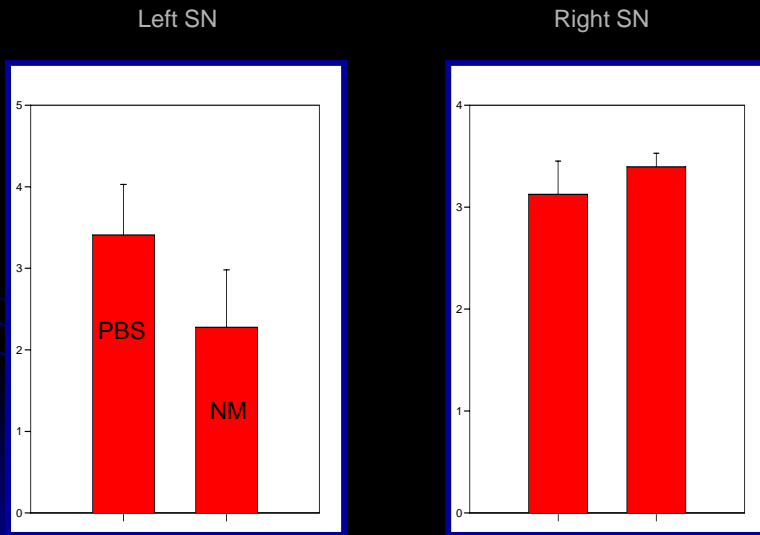


FIGURE 3D: DOPAC LEVELS IN THE SN

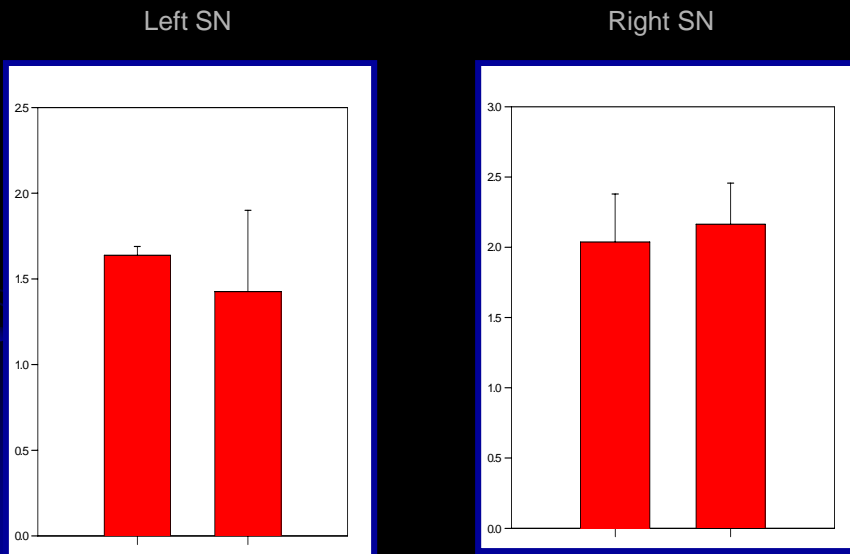


FIGURE 4A. TH IMMUNOSTAINING IN NM-INJECTED RATS AT 4 WEEKS AFTER LESION

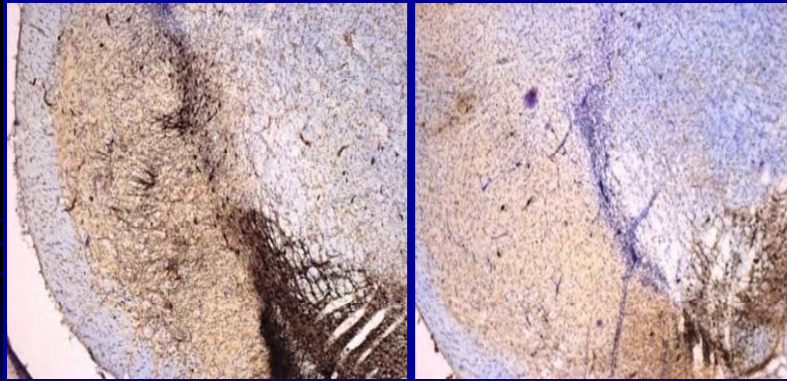


FIGURE 4B. TH AND NISSL CELL COUNTS IN THE INJECTED SN

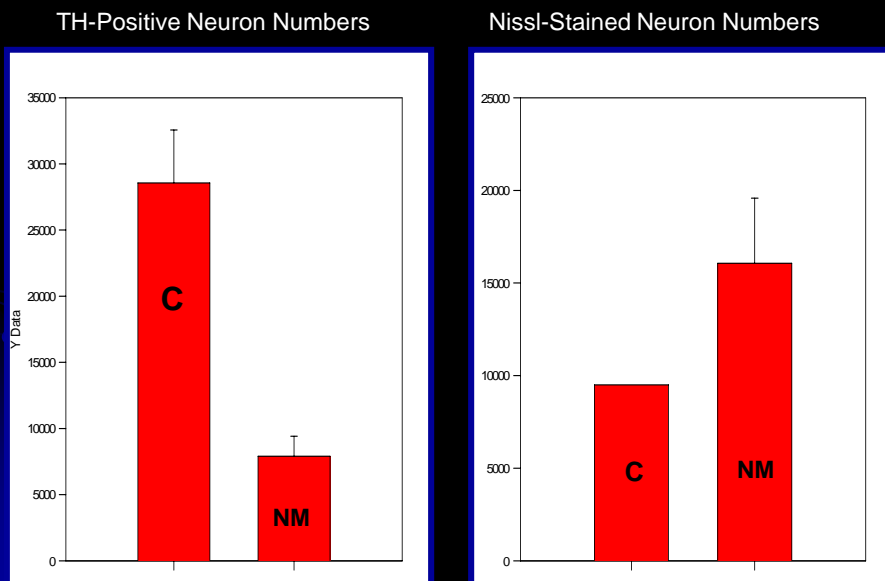


FIGURE 5: ALPHA-SYNUCLEIN IMMUNOSTAINING IN NM-INJECTED RATS

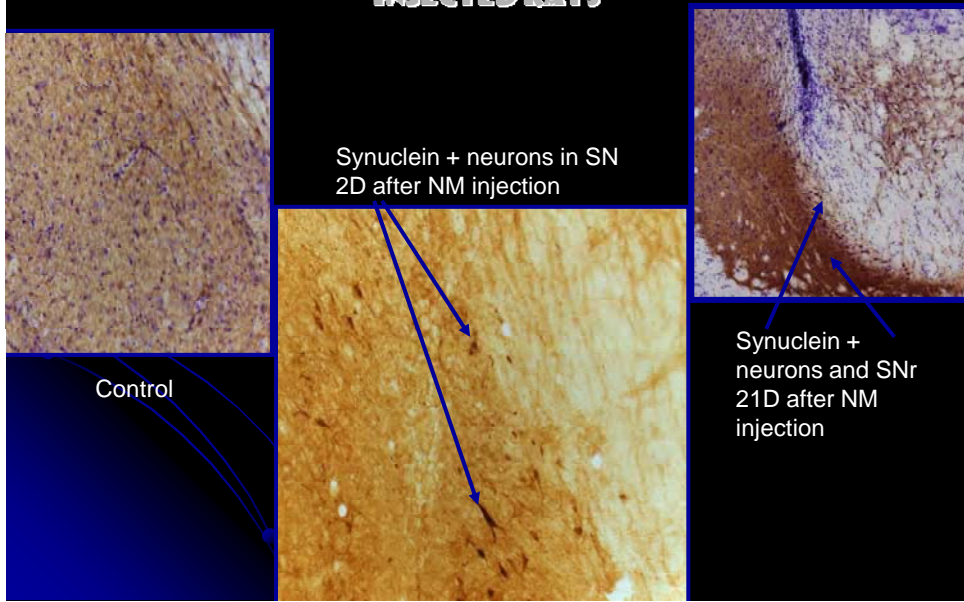


FIGURE 6: UBIQUITIN IMMUNOSTAINING IN NM-INJECTED RATS

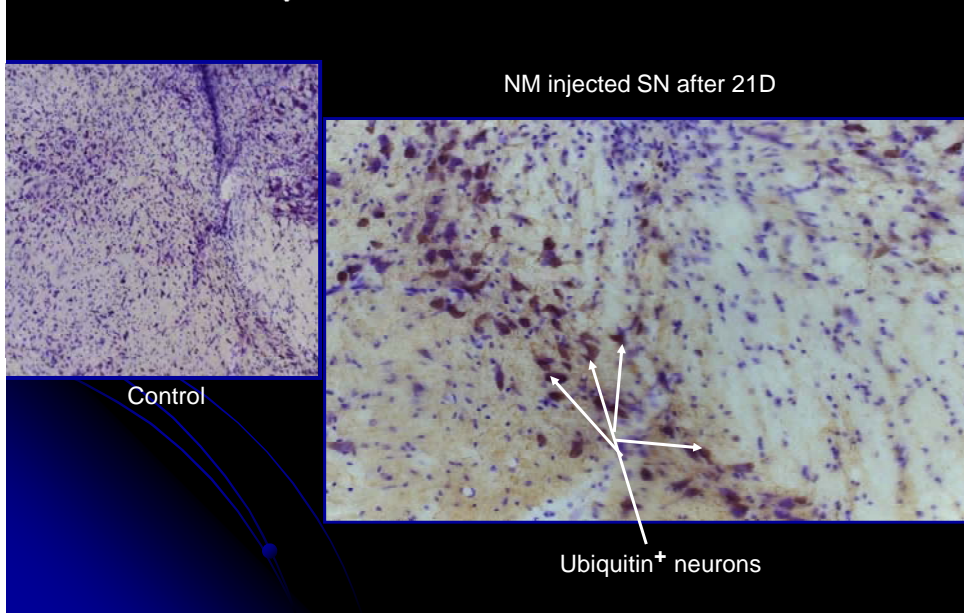


FIGURE 7. MICROGLIAL ACTIVATION AT NM INJECTION SITE

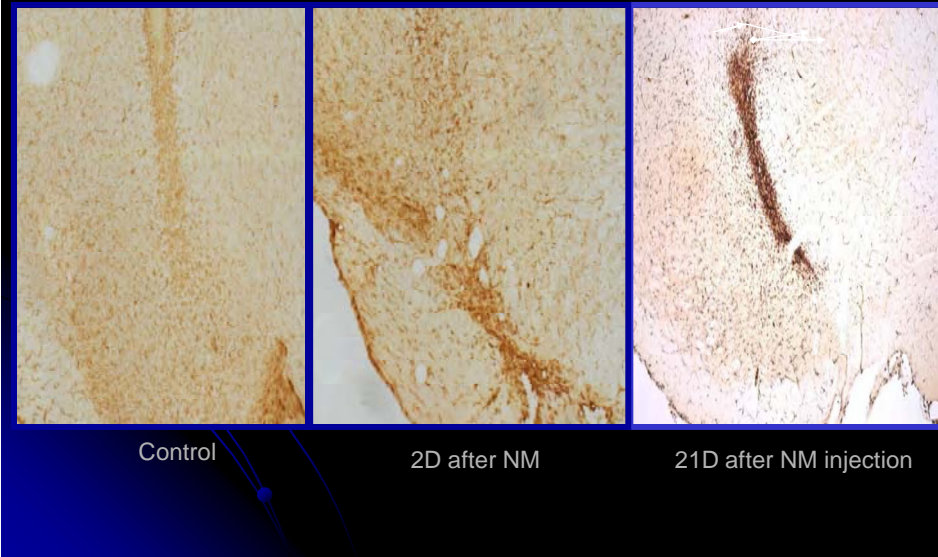
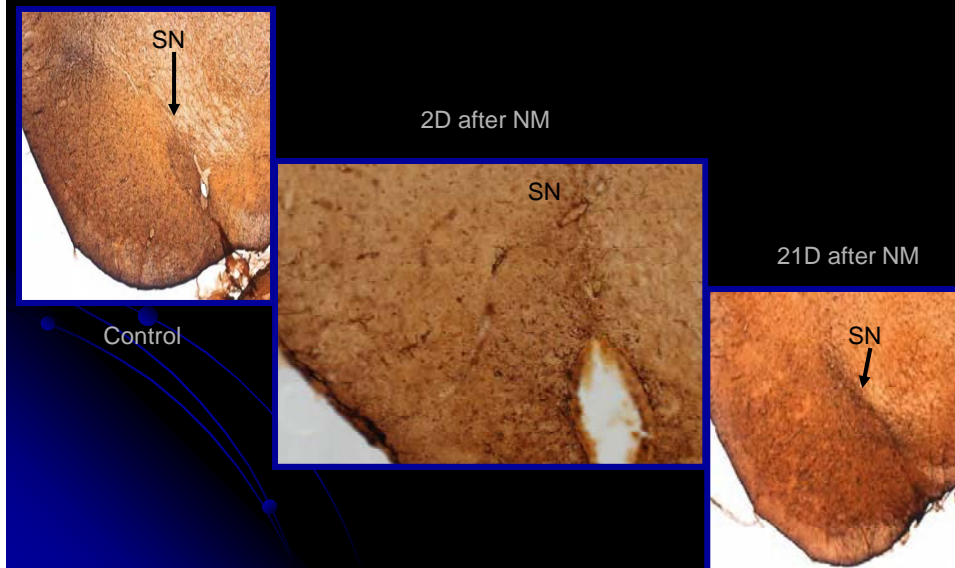


FIGURE 8. ASTROCYTE RESPONSE TO NM INJECTION



Discussion

For decades, the gliosis noted as part of the neuropathological picture in PD brains was relegated to a secondary and insignificant feature of PD (ref). In recent times however, epidemiological studies have suggested that inflammation may increase the risk of developing PD (Chen et al, 2003). Brain imaging and neuropathological studies by Langston et al (1999) indicate that, after an acute phase of DA neuron death in the SN of individuals who had injected MPTP 3-16 years prior to death, DA neurons continued to die, albeit at a much lower rate than the original neuronal death event. In experimental models of PD, it has been demonstrated that inflammation can influence nigrostriatal dopaminergic neuronal death (Gao et al, 2002, Wu et al, 2002). Although the observed gliosis in the SN of PD brains is comprised of both microglia and astrocytes, the magnitude of the responses to injury from these cells is quite different. On autopsy, many PD brains exhibit a mild astrogliosis both in the number of astrocytes and in GFAP immunoreactivity (Forno et al, 1992). In contrast to this mild astrocytic response, the microglial activation is quite dramatic (Banati et al, 1998). In PD brains, microglial cells are found in close proximity to free neuromelanin in the neuropil and to the remaining DA neurons around which clumping of microglia has been noticed (Mizra et al, 2000). Our own time studies have shown that a strong microglial activation occurs prior to the peak of TH-positive neuron death and prior to astrocytosis in the MPTP-treated mouse (Liberatore et al, 1999). Given our MPTP data and the findings from imaging, neuropathological and epidemiological studies, it seems that microglia are indeed important to the pathogenesis of PD.

In our studies on the role of microglia in MPTP-induced dopaminergic neuron death, we examined the neuroprotective effect of minocycline in the MPTP mouse model of PD. We found that minocycline indeed attenuated TH-positive neuron death in the SNpc of this model and mitigated microglial activation, but did not affect the increase in astrocyte number and GFAP immunostaining. From a neuropathological point of view, microglial activation and neuronophagia in the SN are indicative of an active ongoing process of cell death (Langston et al, 1999). Microglia behave like a 2-edged sword in that on the one hand, they can produce elements such as glial-derived neurotrophic factor (Batchelor et al, 2000) and brain-derived neurotrophic factor (Batchelor et al, 1999) which are essential to the wellbeing of the neuron. On the other hand, these same cells can produce toxins such as nitric oxide (Liberatore et al, 1999) and the superoxide radical (Wu et al, 2003) which can be damaging to the neuron. In our studies, we found that microglial activation is time-dependent in that activation takes place prior to any major neuronal death and prior to any astrocytosis (Liberatore et al, 1999). Minocycline dampened microglial activation, thus decreasing the death of the TH-positive neurons as demonstrated by the increase in the survival numbers of these same neurons. The protection afforded here is related to the ability of minocycline, a second-generation tetracycline antibiotic, to block the up-regulation of inducible nitric oxide synthase (iNOS) in the microglia as well as block the production of other noxious mediators of neuronal death such as interleukin-1 β (IL-1 β) (Banati et al, 1993), thus decreasing the oxidative stress inflicted upon the neurons. Furthermore, minocycline decreased 3-nitrotyrosine (3-NT) in the SNpc. 3-NT is a stable marker of protein modification that is indicative of the presence of peroxynitrite (Pennarthur et al, 1999; Jackson-Lewis and Smeyne, 2005). Thus, we believe that minocycline not only blocks microglial activation and nitric oxide (NO) production independent of its antimicrobial activity, but also acts specifically on the microglia to prevent the production of cytotoxic substances other than NO.

The afforded protection to SNpc DA neurons by minocycline, although not a complete protection, was dose –dependent as doses lower than 11.25 mg/kg twice a day were less effective in countering the damaging effects of MPTP than the doses greater than 11.25 mg/kg given twice daily. However, because we focused our studies on the 2 day and the 7 day timepoints, we cannot exclude that a greater protection could have been achieved had we used higher doses of minocycline or had we used it for greater lengths of time. In our dosing schedule of minocycline, because we noted that minocycline given before MPTP can affect MPTP metabolism by decreasing striatal levels of MPP+ by about 20%, we injected minocycline after we administered MPTP which did not affect striatal MPP+ levels or any other key aspects of MPTP metabolism, (Przedborski et al, 2000) such as entry into the dopaminergic neurons and inhibition of mitochondrial respiration.

The incomplete protection of DA neurons in the SNpc by minocycline against MPTP-induced microglial activation (Wu et al, 2003) suggests that other factors produced in the microglia are involved in the MPTP neurotoxic process (Przedborski et al, 2003). Among these other factors, based on the oxidative stress hypothesis, reactive oxygen species (ROS) are of particular interest as in a previous study (Przedborski et al (1992), we showed that the superoxide radical is part of the oxidative stress produced by MPTP administration, although where this radical emanated from was not known. As stated above, NADPH oxidase is a significant source of ROS. It is a multimeric enzyme composed of a number of subunits (Babior, 1999) which when separated render the enzyme inactive as in resting microglia. When these subunits assemble, as in a stressful situation, NADPH oxidase becomes elevated and microglia are activated in a time-dependent manner giving rise to secondary oxidants such as the superoxide radical. In these MPTP studies, we found that NADPH oxidase subunit levels and subunit mRNA in the SNpc increase dramatically at 2 days after MPTP treatment indicating that NADPH oxidase is an inducible enzyme in the SNpc and that this induction coincides with the activation of microglia here. Our studies using GP⁹¹-deficient mice confirmed the role of NADPH oxidase in activated microglia following MPTP treatment in that TH neurons seemed to be protected in these mice and there was a dampening of the microglial response as well as a significant decrease in the carbonyl content (a marker of protein modification) in the ventral midbrain of the GP⁹¹-deficient mice compared to their wild-type littermates. Furthermore, when we tested for the presence of the superoxide radical in the GP⁹¹-deficient mice, we noted that while MPTP elicited a strong response on ethidium bromide fluorescence in the SNpc at both 1 and 2 days following MPTP administration in the littermates, GP⁹¹-deficient mice showed very little ethidium bromide immunofluorescence in this area of the brain which is indicative of a reduced level of NADPH oxidase activity and a decrease in the level of microglial activation. Moreover, when we infused CuZnSOD (extracellular SOD) directly into the striatum, both TH-positive fibres in the striatum and TH-positive neurons in the SNpc were protected against the damaging effects of MPTP. These studies confirmed several of the findings in human PD related to the up-regulation of NADPH oxidase, which is only one of the many contributors to oxidative stress in the brain.

Preventing iNOS up-regulation to decrease the presence of NO is only half of the battle in attenuating microglial activation. As we have demonstrated in Wu et al, 2003, the other half of microglial activation in the SNpc rests with NADPH oxidase which produces the superoxide radical and probably releases it into the microenvironment of the DA neuron. While individually, these two cause little damage to the neurons in the SNpc, their interaction produces peroxynitrite

(Przedborski and Jackson-Lewis, 2000; Jackson-Lewis and Smeyne, 2005), one of the most damaging compounds known to man. The primary defense against the superoxide radical is the superoxide dismutase (SOD) enzyme of which there are three isoforms. Two of the three are of concern to PD as these seem to play a role in maintenance of the microenvironment in and around the SNpc DA neuron. The copper-zinc (CuZnSOD; SOD1) form is found in extremely high amounts in the extracellular space of SNpc (Fridovich, 1978) and has been shown to be unchanged in the SNpc of PD brains (Martilla et al, 1988). The manganese (MnSOD; SOD2) form is found in mitochondria within the neuron, is known to be inducible in response to ROS production and has been shown to be increased in both striatum and SNpc in PD brains (Martilla, 1988). The sole purpose of SOD is to metabolize the superoxide radical to hydrogen peroxide. In this respect, because it is inducible, MnSOD is thought to be neuroprotective as mice lacking this enzyme have a very short lifespan and have mitochondria that do not function properly (Melov et al, 2001). Furthermore, the SNpc of mice overexpressing MnSOD is protected against MPTP-induced neurodegeneration (Klivenyi et al, 1998). Thus, while both isoforms of SOD are necessary for the cell to survive, MnSOD seems to be the more important of the two in oxidative stress situations.

Attempts have been made to decrease the assault leveled at the brain by the superoxide radical using conventional peptidyl enzymes such as the systemic administration of native SOD and have met with little success due to limitations associated with enzyme therapies (Salvemini et al, 1999). Recently, using molecular modeling systems studies, a stable and active class of SOD mimetics which catalyze the dismutation of the superoxide radical with rates similar to that of native MnSOD has been synthesized (Salvemini et al, 1999). These low molecular weight non-peptidyl compounds, which lack catalase activity (Salvemini et al, 1999), distribute widely in the body, keep their chemical identity and can be recovered intact in the urine and feces (Salvemini et al, 1999). The parent compound, M40403, does not react with NO, H₂O₂ or OONO and has been shown to counteract edema, inhibit neutrophil infiltration and the release of such proinflammatory cytokines as TNF- α and IL1- β (Salvemini et al, 1999). A related compound, M40401, which possesses a catalytic rate constant at least equal to or greater than that of the native SOD enzymes, showed a significant protective effect against the neuropathological and ultrastructural changes effected by bilateral occlusion of the common carotid artery (Cuzzocrea et al, 2001) and ischaemia-reperfusion injury in the brain (Murata et al, 2004). Both injuries generate the superoxide radical, thus they throw the brain into an oxidative stress situation.

Key to the M40401 molecule is its manganese II (MnII) center which is difficult to oxidize to the MnIII state (Salvemini et al, 1999). What is interesting and so attractive about M40401 is that it has no reactivity until it is oxidized by protonated superoxide. Once reactive, the complex is rapidly reduced back to the MnII state by the superoxide anion at diffusion-controlled rates (Salvemini et al, 1999). The oxidative stress situation in the cell generated following MPTP was an ideal situation in which to test the in vivo effectiveness of M40401 in dampening the effects of the superoxide radical as MPTP mimics the assault of superoxide radicals on the DA neuron that we see in PD. Dopaminergic neurons in the SNpc were protected to a significant degree against the damaging effects of MPTP by M40401 whereas its isoforms M40435 and M40404 offered no such protection. In mice that received our acute regimen of MPTP along with M40401, significantly more TH-positive neurons were noted at 7 days after drug administration compared to the MPTP only mice. Neuroprotection of TH-positive neurons in the SNpc is due,

primarily, to a significant suppression of the superoxide radical by M40401 administration. M40401 did not interfere with MPTP uptake nor did it interfere with MPTP metabolism as evidenced by our demonstration that, in the presence of M40401, MPP⁺ levels in the striatum, ventral midbrain and blood were not significantly different between the MPTP only and the MPTP-M40401 groups of mice.

This protective effect, however, did not extend to the TH-positive fibres in the striatum as at the 7 day time point, striatal optical density in the M40401-MPTP mice was not different from the MPTP only mice. We did notice that, in mice given less MPTP (16 mg/kg x 4 doses), there was a tendency toward some protective effect in the striatum. Given this, it is possible that M40401, administered more frequently or at higher doses, might indeed offer more neuroprotection to striatal fibres. There are several possible reasons why no neuroprotection was afforded to DA fibres in the striatum by M40401: 1) MPTP may exact a huge release of DA from DA terminals in the striatum; 2) it may be that DA itself is neurotoxic; 3) metabolites in the pathway of DA metabolism such as 6-hydroxydopamine are known to be neurotoxic (Graham, 1978); 4) one of the products of the dismutation of the superoxide radical is the hydroxyl (OH \cdot) radical which can (Slivka and Cohen, 1985) attack DA directly, thus forming 6-OHDA

M40401 also suppressed MPTP-induced superoxide production in the SNpc as indicated on hydroethidium fluorescence and mitigated the activation of microglia. The superoxide radical in the MPTP-treated mouse is the result of the blockade of complex I of the mitochondrial chain (Przedborski et al, 1992) within the neuron. Thus, its interaction with NO is probably intracellular as NO can not only travel through membranes, it can travel great distances as well (Przedborski and Jackson-Lewis, 2000) to interact with the superoxide radical to form peroxynitrite. We do not know how M40401 dampened the MPTP-induced activation of microglia as we did not test its effect on this enzyme at all. We can only surmise that it may not have had any effect at all here.

Finally, it was noted that M40401 did not affect the MPTP-induced 3-NT formation, thus it had no effect on the iNOS enzyme. However, it did reduce the MPTP-induced carbonyl content indicating that M40401 affected MPTP-induced protein oxidation. We did not, however, examine its effect on NADPH oxidase, thus we cannot say what effect M40401 might have had on this enzyme. Nor did we test its effect on MnSOD activity. Furthermore, we did not examine the combined effect of minocycline and M40401 on MPTP-induced microglial activation. We did, however, conduct preliminary studies using another SOD mimetic compound, Tempol, which is a stable piperidine nitroxyl SOD mimetic (Rak et al, 2000). Preliminary data shows that this compound attenuates MPTP-induced microglial activation and protects TH-positive neurons to the same degree as M40401. Although M40401 shows significant promise as a possible therapeutic agent for PD, this compound is no longer available to us.

In trying to cover all of the bases that relate to inflammation and microglial activation in PD, we noted, in the literature, that the increased expression of the cyclooxygenase enzyme (COX) and the elevation of prostaglandin E₂ (PGE₂) levels in the brain have been implicated in the cascade of deleterious events leading to neurodegeneration in a variety of pathological settings (ref). The COX enzyme converts arachidonic acid to prostaglandin H₂, the precursor of PGE₂ (Teismann et al, 2003). Although two isoforms of the COX enzyme exist, it is the COX-2 enzyme that has

been linked to pathological events. In fact, we found that the COX-2 enzyme is indeed up-regulated in the SN of post-mortem PD brains. Thus, we wished to clarify this finding and elucidate the meaning of this finding using the MPTP mouse model of PD. In this model, we found that MPTP does induce an up-regulation of both the COX-2 enzyme and mRNA in the ventral midbrain of the treated mice which was specific to the SNpc DA neurons. Here, MPTP toxicity depends on COX-2 activity as rofecoxib, a COX-2 inhibitor that did not affect MPTP metabolism, completely blocked the formation of PGE₂ and increased the number of surviving TH-positive neurons in the SNpc. Interestingly, elevated PGE₂ levels in the ventral midbrain of the treated mice associated more with the COX-1 isoform of the enzyme than with COX-2. In examining COX-2 involvement in the activation of microglia, we found that COX-2 modulation did not attenuate MPTP-induced microglial activation based upon the elevation of Mac-1, iNOS, gp91 and ICE mRNA in the face of both COX-2 inhibition and abrogation. Thus, we concluded that the COX-2 enzyme had no role in microglial activation. However, since TH-positive neurons do die when under the oxidative stress produced by MPTP in the ventral midbrain and COX-2 is up-regulated here, we asked what initiates the COX-2 up-regulation. What we found was that JNK activation, known to regulate the COX-2 enzyme, influenced the MPTP-induced changes in COX-2 within TH-positive neurons as blocking JNK activation by CEP-11004 eliminated COX-2 induction which attenuated TH-positive neuron loss. COX-2 does stimulate the production of the superoxide radical (Przedborski and Jackson-Lewis, 2000), thus, we surmised that it may influence oxidative stress within the neuron through its stimulation of superoxide radical production. In the oxidative stress produced by treatment with MPTP, we quantified 5-cysteinyl-DA, a stable catechol modification engendered by the COX-2-related oxidation of DA. We found significantly higher levels of 5-cysteinyl-DA in the striatum of the treated mice than in control mice.

Conclusion

Microglia can influence both the life of and the death of DA neurons in the SN as their numbers in the microenvironment surrounding these neurons are immense. In reviewing the data from this entire study, we can now propose a scenario which points to microglia as being at the root of the progressive nature of PD. Although what initiates PD is unknown, when the first aging neuromelanin-containing neuron dies, it releases its contents into the surrounding microenvironment in the SNpc. Contents of these neurons include NM which sequesters toxic substances such as metals, the superoxide radical, and oxidized catecholamines. These substances accumulate over time and as the neuron ages, it may eventually reach critical mass in terms of sequestering such substances. Once critical mass is reached, the neuron bursts, releasing its contents, which are essentially toxins, into the neuronal microenvironment. This release alters the neuronal environment such that it is no longer friendly, but hostile. Environmental changes most likely to occur are pH alterations and salt content changes. This, of course, will have to be examined.

Release of the neuronal contents acts like an injury and initiates an inflammatory response, meaning that microglia within the immediate area of the neuron are activated. Once activated, our studies show that, morphologically, microglia become amoeboid-like, increasing in size. On immunostaining and Western blot analyses, microglia present with increased levels of NADPH oxidase and iNOS, which produce copious amounts of the superoxide radical and NO. By

themselves, these compounds are not toxic, however, when they interact with each other, either within the microglia or in the microenvironment, they produce peroxynitrite, one of the most damaging compounds that we know of. Peroxynitrite, released into the neuronal microenvironment, can damage proteins, lipids and DNA. This means that the membranes surrounding the neurons are subjected to a hostile environment, are damaged by peroxynitrite and will eventually die, releasing their contents into the neuronal microenvironment. The process now becomes cyclic, thus the progressive nature of PD. A speeded-up version of this scenario is achieved with MPTP and we have been able to recapitulate in this model many of the hallmarks of PD. Although we found no involvement of the COX-2 enzyme in microglial activation, we did find that this enzyme seemed to be controlled by the JNK pathway which has been shown to be involved in the apoptotic machinery. Apoptosis has been suggested as the mode of DA neuronal death in PD.

Based on our study and on the studies of others, we believe that any neuroprotective strategy in PD has to include the prevention of microglial activation, if we are to slow the progression of PD. As noted in our studies, increases in surviving DA neuron number went along with suppression of microglial activation. With minocycline, protection was related to the ability of minocycline to partially block iNOS up-regulation, which in turn, limited the superoxide radical-NO interaction. The same can be said for M40401 and Tempol as both of these SOD mimetics lessened the production of the superoxide radical as evidenced by dihydroethidium fluorescence experiments. Thus, most likely microglial activation attacks the neuron from the outside, the superoxide radical may be in play inside of the neuron.

Addendum

Full Papers Related to the Project

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