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Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity

(nitric oxide synthase/superoxide radical/superoxide dismutase/Parkinson disease)

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ABSTRACT **1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes nigrostriatal dopaminergic pathway damage similar to that observed in Parkinson disease (PD). To study the role of NO radical in MPTP-induced neurotoxicity, we injected MPTP into mice in which nitric oxide synthase (NOS) was inhibited by 7-nitroindazole (7-NI) in a time- and dose-dependent fashion. 7-NI dramatically protected MPTP-injected mice against indices of severe injury to the nigrostriatal dopaminergic pathway, including reduction in striatal dopamine contents, decreases in numbers of nigral tyrosine hydroxylase-positive neurons, and numerous silver-stained degenerating nigral neurons. The resistance of 7-NI-injected mice to MPTP is not due to alterations in striatal pharmacokinetics or content of 1-methyl-4-phenylpyridinium ion (MPP^+), the active metabolite of MPTP. To study specifically the role of neuronal NOS (nNOS), MPTP was administered to mutant mice lacking the nNOS gene. Mutant mice are significantly more resistant to MPTP-induced neurotoxicity compared with wild-type littermates. These results indicate that neuronally derived NO mediates, in part, MPTP-induced neurotoxicity. The similarity between the MPTP model and PD raises the possibility that NO may play a significant role in the etiology of PD.**

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is used extensively in various mammalian species to produce an experimental model of Parkinson disease (PD) (1–3), a common and disabling neurodegenerative disorder (4) of unknown cause (5). In humans and nonhuman primates, MPTP induces irreversible and severe motor abnormalities almost identical to those observed in PD (1, 3). In both monkeys and mice, MPTP replicates many of the biochemical and neuropathological changes in the nigrostriatal dopaminergic pathway found in PD (1–3). This includes a marked reduction in the levels of striatal dopamine and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (1–3). In addition, as in patients with PD, animals that receive MPTP show significant reductions in the number of dopamine cell bodies in the substantia nigra pars compacta (SNpc) (1–3, 6). In monkeys, MPTP induces the formation of intraneuronal eosinophilic inclusions resembling Lewy bodies, a neuropathological hallmark of PD (7). Thus, these similarities provide appealing hints that the MPTP model may lead to important new insights into the pathogenesis of PD.

Previously, we reported that transgenic mice that overexpress the copper/zinc-containing superoxide dismutase (SOD) (8) were more resistant to MPTP-induced dopamine neurotoxicity (9). Thus far, the only known function for SOD is the protection of cells against reactive oxygen species by means of the detoxification of superoxide radicals (10). Consequently, we concluded that superoxide radicals are involved in the deleterious cascade that leads to the death of SNpc dopamine neurons following MPTP administration (9). Consistent with this notion is the demonstration that 1-methyl-4-phenylpyridinium ion (MPP^+), the active metabolite of MPTP, stimulates the production of the superoxide radical *in vitro* (11). However, superoxide is a poorly reactive radical and it is the general consensus that this radical cannot cause serious direct injury (10). Instead, the superoxide radical is believed to exert many or most of its toxic effects through the generation of other reactive oxygen species (10). For instance, superoxide radical can react at an extremely fast rate with the NO radical to produce peroxynitrite ion, which, in turn, can decompose rapidly at physiological pH to the tissue-damaging hydroxyl radical (12). In light of this, we wondered whether NO plays an important role in MPTP-induced toxicity. Previous studies suggest that inhibition of nitric oxide synthase (NOS) activity can attenuate MPTP-induced decreases in striatal dopamine levels (13). However, it has yet to be shown that inhibition of NO production can actually prevent MPTP-induced dopaminergic neuronal death. We show here that 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice (14), is profoundly neuroprotective against MPTP-induced dopaminergic neuronal death. Moreover, we show that neuronally derived NO is responsible for MPTP neurotoxicity because mice that lack the gene for neuronal NOS (nNOS) are protected against MPTP neurotoxicity.

MATERIALS AND METHODS

Animals and Treatment. Eight-week-old male C57/b1 mice (22–25 g; Charles River Breeding Laboratories) were housed three per cage in a temperature-controlled room under a 12-hr light/12-hr dark cycle with free access to food and water. On the day of the experiment, mice received four i.p. injections of MPTP·HCl (20 or 15 mg of free base per kg; Research Biochemicals, Natick, MA) in saline at 2-hr intervals; control mice received saline only. Mice also received s.c. injections of 7-NI (25 or 50 mg/kg; Research Biochemicals) in peanut oil

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Abbreviations: DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium ion; 7-NI, 7-nitroindazole; NOS, nitric oxide synthase; nNOS, neuronal NOS: PD, Parkinson disease; SNpc, substantia nigra pars compacta; SOD, superoxide dismutase; TH, tyrosine hydroxylase.

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(Sigma) 30 min before each MPTP injection and every 8 hr for the next 4 days; control mice received peanut oil only.

Assay of NOS Catalytic Activity. At different time points (0, 5, 15, 30, 60, and 90 min and 2, 4, 8, and 12 hr) after a single s.c. injection of 50 mg of 7-NI per kg and 30 min after a single s.c. injection of different doses of 7-NI (3–75 mg/kg), mice (4–6 per group) were decapitated. Their brains were quickly removed, and the striatum and cerebellum were dissected out freehand on an ice-cold glass Petri dish (15). Samples were immediately frozen on dry ice and stored at -80°C until analysis. NOS catalytic activity was assayed by measuring the Ca^{2+}-dependent conversion of [^{3}H]arginine to [^{3}H]citrulline (16). On the day of the assay, tissue samples were sonicated in 20 vol (wt/vol) of 50 mM Tris·HCl (pH 7.4) buffer containing 1 mM EDTA and 1 mM EGTA. After centrifugation (18,000 × *g* for 15 min at 4°C), 25 μl of supernatant was added to 100 μl of 50 mM Tris·HCl (pH 7.4) buffer containing 1 mM NADPH, 1 mM EDTA, 1 mM EGTA, 2.25 mM $CaCl_2$, and 0.1 μCi of L-[2,3-^{3}H]arginine (specific activity: 36.8 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN) and incubated for 15 min at 25°C. The reaction was terminated by the addition of 3 ml of 20 mM Hepes,pH 5.5/1 mM EDTA/1 mM EGTA and applied onto a 0.5-ml column of Dowex AG50WX-8 (Pharmacia). [^{3}H]Citrulline was quantified by liquid scintillation counting of the eluate. No significant [^{3}H]citrulline production occurred in the absence of calcium. Protein concentrations were determined by the method of Lowry *et al.* (17) using bovine serum albumin as standard.

Measurement of Striatal Dopamine, DOPAC, and HVA Levels. HPLC with electrochemical detection was used to measure striatal levels of dopamine, DOPAC, and HVA (18). Seven days after the last MPTP injection, mice (4–6 per group) were killed and striata were dissected out as above, immediately frozen, and stored at −80°C until analysis. On the day of the assay, tissue samples were sonicated in 50 vol (wt/vol) of 0.1 M perchloric acid containing 25 ng/ml dihydrobenzylamine (Sigma) as internal standard. After centrifugation (15,000 × *g* for 10 min at 4°C), 20 μl of supernatant was injected onto a C18-reverse-phase HR-80 catecholamine column (ESA, Bedford, MA). The mobile phase consisted of 90% 50 mM sodium phosphate/0.2 mM EDTA/1.2 mM heptanesulfonic acid (pH = 3.5) solution and 10% methanol. Flow rate was 1.0 ml/min. Peaks were detected by an ESA model Coulochem 5100A detector (E1 = −0.04 V, E2 = +0.35 V). Data were collected and processed on a computerized Dynamax data manager (Rainin Instruments).

Measurement of Striatal MPP^+ Levels. HPLC with UV detection (wavelength = 295 nm) was used to measure striatal MPP^+ levels (9). Groups of 7-NI- and peanut oil-injected mice (4–5 per time point and per treatment condition) were killed 90 min after each of the four MPTP injections and also 3 and 6 hr after the fourth injection. Striata and cerebella were dissected out as above, immediately frozen, and stored at −80°C until analysis. On the day of the assay, cerebella were used for NOS catalytic activity determination while striata were prepared for MPP^+ measurements by sonicating the tissue samples in 5 vol (wt/vol) of 5% trichloroacetic acid containing 5 μg/ml of 4-phenylpyridine (Sigma) as internal standard. After centrifugation (as for catecholamines), 50–100 μl of supernatant was injected onto a cation-exchange Ultracyl-CX column (Beckman). The mobile phase consisted of 90% 0.1 M acetic acid/75 mM triethylamine·HCl (pH 2.35 adjusted with formic acid) solution and 10% acetonitrile. The flow rate was 1.5 ml/min. Data were collected and processed as above.

Immunohistochemistry for Tyrosine Hydroxylase (TH) and Silver Staining. At 2 days (silver staining) and at 7 days (TH immunohistochemistry) after the last dose of MPTP, mice (4–5 per group) were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.1) as described (6). After postfixation in the same fixative solution and cryoprotection in 20% sucrose/phosphate buffer, brains were frozen and serially sectioned (20 μm) through the entire mesencephalon in a cryostat (6). Neurons containing TH, the rate-limiting enzyme in dopamine synthesis, were shown by incubating the tissue sections successively with a rabbit polyclonal anti-TH antibody (1:1000; Eugene Tech, Ridgefield Park, NJ), a biotin-conjugated polyclonal goat anti-rabbit antibody (1:200; Vector Laboratories), and a horseradish-conjugated avidin/biotin complex (Vector Laboratories) as described (6). For silver staining (6, 19), sections were incubated in pretreating solution (equal volumes of 9% NaOH/1.2% NH_4NO_3) followed by impregnating solution (60 ml of 9% NaOH/40 ml of 16% NH_4NO_3/0.5 ml of 50% $AgNO_3$) before color development in the developing solution.

Quantitative Morphology. SNpc neuronal counts were performed manually by workers blinded to the treatment schedule as described (i.e., saline, peanut oil, MPTP, and/or 7-NI) (6, 20). For each mouse, at least two TH-immunostained and silver-stained sections for each of the five representative mesencephalic planes (plane numbers: 321, 335, 343, 351, and 361) (21) were analyzed by scanning the entire SNpc on both sides (light microscopy; ×400). The average number of neurons in each plane was added to provide a measure of the total

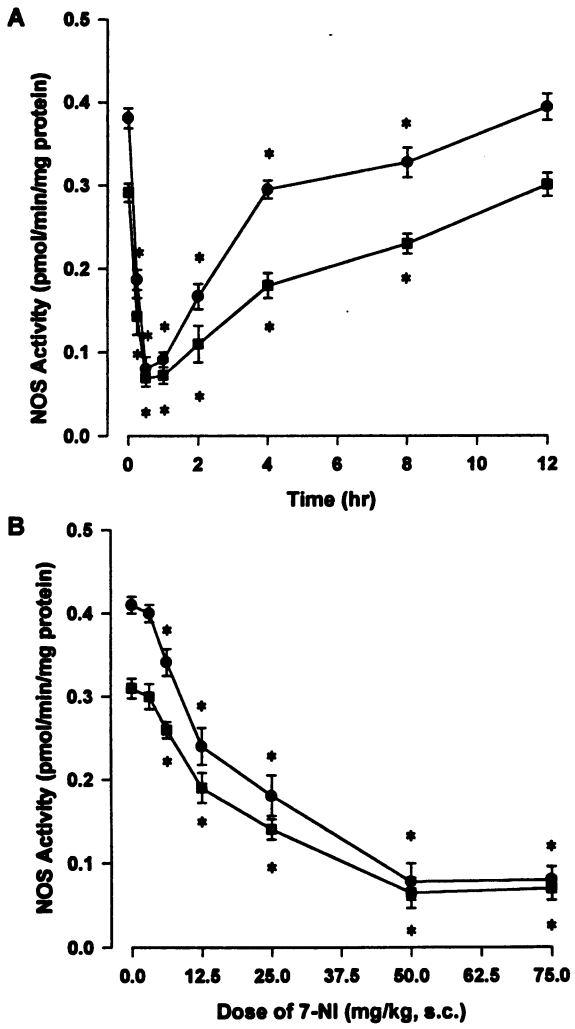

FIG. 1. (*A*) Time course of 7-NI inhibition of striatal (■) and cerebellar (●) NOS activity in C57B6 mice. (*B*) Dose response of 7-NI inhibition of NOS catalytic activity in both cerebellum (●) and striatum (■). Values are mean ± SEM for 3–5 mice per group. *, Significantly different (Newman–Keuls post-hoc test, $P < 0.05$) from 0 min postinjection (*A*) or 7-NI at 0 mg/kg (*B*).

number of spared (TH immunostaining) or degenerating (silver staining) SNpc neurons for each animal. In preliminary studies, we measured the area of 15–30 representative SNpc TH-immunostained and silver-stained neurons for each mouse (6). The mean cross-sectional areas for SNpc TH-positive neurons among the five groups were: control, 274 ± 6 μm^2; MPTP, 266 ± 8 μm^2; 7-NI, 278 ± 9 μm^2; 7-NI/MPTP, 271 ± 6 μm^2 (F[3,116] = 0.55, $P = 0.65$). The mean cross-sectional areas for SNpc silver-stained neurons among the five groups were: MPTP, 120 ± 7 μm^2; 7-NI/MPTP, 131 ± 8 μm^2 (t[21] = 0.98, $P = 0.34$); no silver-stained neurons were detected in control or 7-NI-injected mice. Because no significant differences in cross-sectional areas were found, no Abercrombie correction (22) was required to compare neuronal counts among the different treatment groups.

Mice Lacking nNOS (nNOS$^-$). nNOS$^-$ mice were obtained from a breeding colony established at Johns Hopkins University using animals previously produced by homologous recombination (16). Because the nNOS$^-$ mutation was initially made on a 129 SvEv agouti mouse and C57B6 mouse background and our preliminary studies indicated that 129 SvEv agouti mice are markedly resistant to MPTP-induced neurotoxicity (unpublished observations), we backcrossed the nNOS$^-$ mice to the sensitive C57B6 strain (The Jackson Laboratory). Progeny from an F_1 heterozygote × F_1 heterozygote mating and an F_2 heterozygote × F_2 heterozygote were used in this study and were genotyped by Southern blot analysis or PCR as described (16). Thus, all the mice used in this study are littermate controls from the heterozygote matings.

On the day of the experiment, 4- to 6-month-old nNOS$^-$ ($n = 6$), heterozygous ($n = 6$), and wild-type ($n = 6$) mice received four i.p. injections of MPTP (15 mg of free base per kg) in saline at 2-hr intervals; control mice ($n = 27$; 10 wild-type, 10 heterozygotes, and 7 nNOS$^-$) received saline only. Seven days after the last injection, all mice were killed and striatal extracts were prepared for HPLC monoamine measurement (18).

Statistics. All values are expressed as the mean ± SEM. Differences between means were analyzed using a two-tailed Student's *t* test. Differences among means were analyzed using one-way ANOVA with the different treatments as the independent factor. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Newman–Keuls post-hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using SIGMASTAT for Windows (version 1.0, Jandel, San Rafael, CA).

RESULTS

7-NI Inhibits Striatal and Cerebellar NOS Activity *in Vivo*. Before assessing the effect of NOS inhibition by 7-NI on MPTP-induced toxicity, we performed a series of experiments aimed at determining some of the pharmacokinetic characteristics of 7-NI in mouse brain. Basal NOS activity in the cerebellum is significantly higher than in the striatum of peanut oil-injected control mice. Administration of a single s.c. injection of 50 mg of 7-NI per kg produces a significant and comparable time-related inhibition of NOS activity in both brain regions (Fig. 1*A*). Maximum inhibition is observed 30 min after injection of 7-NI. By 4 and 8 hr after injection, NOS inhibition is less pronounced but still significantly reduced in both regions. By 12 hr after injection, striatal and cerebellar NOS activities are comparable with control values (Fig. 1*A*). NOS activity is significantly inhibited in the cerebellum and striatum in a dose-dependent fashion (Fig. 1*B*). At 30 min after injection, 7-NI at 25 mg/kg produces about a 50% inhibition of NOS activity, whereas maximal inhibition is produced by 50 mg/kg. 7-NI did not elicit any behavioral abnormalities. Since we showed that the neurodegenerative phase of SNpc dopamine neurons occurs during the first 4 days after the last injection of MPTP in the mouse model (6), we decided to inject 7-NI 30 min before each MPTP injection and every 8 hr after the last injection of MPTP for 4 days.

NOS Inhibition Protects Against MPTP-Induced Reduction of Striatal Dopamine, DOPAC, and HVA Levels. Four injections of MPTP at 20 mg/kg cause a reduction of more than 90% in striatal dopamine, and of more than 70% in striatal DOPAC and HVA (Table 1) (20, 23, 24). 7-NI significantly attenuates the reduction in striatal catecholamine levels induced by four injections of MPTP at 20 mg/kg (Table 1). The protective effect of 7-NI is dose-dependent as striatal catecholamine levels are better preserved in mice pretreated with multiple injections of 7-NI at 50 mg/kg compared with 25 mg/kg. To test whether 7-NI administration could provide complete protection, we examined another group of mice with less severe striatal damage by injecting a lower dose of MPTP (4 × 15 mg/kg). In mice that received MPTP only, this regimen reduces striatal dopamine levels by 72% and its metabolites by 66%. Multiple injections of 7-NI at 50 mg/kg provide complete neuroprotection against four injections of MPTP at 15 mg/kg (Table 1).

NOS Inhibition Protects Against MPTP-Induced Loss of Nigral Neurons. Key features of the neuropathology of PD are the destruction of dopamine nerve terminals in the striatum as well as the loss of dopamine cell bodies in the SNpc (7). Studies on neurotoxins such as methamphetamine (25), and even MPTP (23, 26), indicate that destruction of dopamine nerve terminals does not necessarily equate with loss of the corresponding dopamine cell bodies. Therefore, it is important to examine whether NOS inhibition protects against the actual loss of SNpc dopamine neurons in the MPTP mouse model. To

Table 1. 7-NI protects against MPTP neurotoxicity

Treatment	Striatal level, ng/mg of tissue		
	Dopamine	DOPAC	HVA
Control	12.73 ± 0.93	2.50 ± 0.41	1.33 ± 0.18
7-NI (25 mg/kg)	12.97 ± 0.84	2.73 ± 0.19	1.25 ± 0.06
7-NI (50 mg/kg)	13.76 ± 0.98	2.49 ± 0.16	1.31 ± 0.07
MPTP (20 mg/kg)	0.78 ± 0.11*†	0.23 ± 0.30*†	0.39 ± 0.02*†
+ 7-NI (25 mg/kg)	3.73 ± 0.95*‡	0.63 ± 0.04*‡	0.47 ± 0.07*‡
+ 7-NI (50 mg/kg)	9.63 ± 0.65*	2.05 ± 0.13	0.96 ± 0.03
MPTP (15 mg/kg)	3.55 ± 0.86*§	0.86 ± 0.30*§	0.72 ± 0.04*§
+ 7-NI (50 mg/kg)	12.29 ± 0.75	2.65 ± 0.28	1.23 ± 0.09

Values are mean ± SEM of 4–6 mice per group. Mice were injected s.c. with 7-NI at 50 or 25 mg/kg dissolved in peanut oil and/or i.p. with MPTP at 15 or 20 mg/kg dissolved in saline as described; control mice received vehicle only. Seven days after the last injection, mice were killed and striata were used for HPLC dopamine, DOPAC, and HVA level determinations. Significantly different (Newman–Keuls post-hoc test, $P < 0.05$) from: *, vehicle and 7-NI at 25 and 50 mg/kg; †, MPTP at 20 mg/kg 7-NI at 25 mg/kg and MPTP at 20 mg/kg/7-NI at 50 mg/kg; ‡, MPTP at 20 mg/kg/7-NI at 50 mg/kg; and §, MPTP at 15 mg/kg/7-NI at 50 mg/kg.

ascertain whether inhibition of NOS activity can prevent not only MPTP-induced damage of dopaminergic nerve terminals in the striatum but also the loss of dopaminergic cell bodies in the SNpc, we counted the number of nigral TH-positive neurons in the different groups of mice (6) (Table 2 and Fig. 2*A–C*). In control mice, there is a large number of TH-positive cell bodies intermingled with a dense network of TH-positive nerve fibers (Fig. 2*A*). Four injections of MPTP at 20 (Fig. 2*B*) and 15 mg/kg reduce the number of SNpc TH-positive neurons as compared with saline-injected controls by 74% and 39%, respectively (Table 2). Mice that received either mutiple injections of 7-NI at 25 or 50 mg/kg and four injections of MPTP at 20 mg/kg show a significantly greater number of TH-positive neurons in the SNpc (Table 2 and Fig. 2*C*). Mice that received multiple injections of 50 mg/kg 7-NI and four injections of 15 mg/kg MPTP showed complete protection (Table 2).

To identify degenerating cells in the SNpc, mesencephalic tissue sections were silver-stained (6). A large number of degenerating SNpc neurons is observed 2 days after four injections of MPTP at 20 mg/kg (Table 2 and Fig. 2*E*) and, to a lesser extent, after four injections of 15 mg/kg (Table 2). Mice that received multiple injections of 7-NI at either 25 or 50 mg/kg and four injections of MPTP at 20 mg/kg show significantly fewer degenerating neurons scattered along the long axis of the SNpc (Fig. 2*F*). Even more striking is that no silver-stained neurons are observed in mice that received multiple injections of 7-NI at 50 mg/kg and four injections of MPTP at 15 mg/kg (Table 2).

7-NI Does Not Affect MPTP Metabolism. After systemic administration, MPTP rapidly enters the brain (27), where it is converted to MPP^+ by the enzyme monoamine oxidase B (28). MPP^+ is then taken up by dopamine neurons (29) and is concentrated in mitochondria by an active mechanism (30). In the mitochondria, MPP^+ inhibits complex I of the electron transport chain (31, 32), which is believed to play a central role in the ensuing cell death. Although alterations at any of these steps can potentially reduce the effects of MPTP, only contents of striatal MPP^+ correlate significantly with the degree of dopamine neurotoxicity (33). Because 7-NI can reduce cerebral blood flow (34), we thought it important to verify that the apparent resistance to MPTP of 7-NI-injected mice is not related to lower striatal MPP^+ levels secondary to a lower MPTP inflow into the brain. Initially, we examined several early time points, including 15, 30, 60, 90, and 120 min after a single injection of MPTP (20 mg of free base per kg, i.p.). Striatal MPP^+ contents reached a peak level ≈90 min after MPTP injection in both groups of mice (7-NI-injected mice, 31.06 ± 5.21 nmol/g of tissue; peanut oil-injected controls, 32.90 ± 2.93 nmol/g of tissue; $t[6] = 0.31$, $P > 0.05$). We then determined striatal MPP^+ content at 90 min after each of the four injections of MPTP as well as 3 and 6 hr after the fourth MPTP injection (Fig. 3*A*). At no time point was the striatal MPP^+ level significantly different between 7-NI-injected and peanut oil-injected mice (Fig. 3*A*), despite the significant difference in cerebellar NOS activity between the two groups (Fig. 3*B*). A dose of 7-NI comparable to that used in the

Table 2. 7-NI protects against dopaminergic cell death and degeneration

Treatment	No. of neurons	
	TH-positive	Silver-stained
Control	686.4 ± 9.4	0
7-NI (25 mg/kg)	662.8 ± 10.4	0
7-NI (50 mg/kg)	679.8 ± 14.3	0
MPTP (20 mg/kg)	178.8 ± 12.8*†	568.5 ± 32.1*†
+ 7-NI (25 mg/kg)	342.1 ± 14.3*‡	315.2 ± 20.1*‡
+ 7-NI (50 mg/kg)	556.6 ± 14.2*	138.3 ± 17.2*
MPTP (15 mg/kg)	420.1 ± 12.4*§	238.5 ± 14.0*§
+ 7-NI (50 mg/kg)	671.7 ± 14.4	0

The numbers of SNpc TH-positive and silver-stained neurons in mice injected with MPTP and/or 7-NI are tabulated. Values are mean ± SEM of 4–6 mice per group. Significantly different (Newman–Keuls post-hoc test, $P < 0.05$) from: *, vehicle and 7-NI at 25 and 50 mg/kg; †, MPTP at 20 mg/kg/7-NI at 25 mg/kg and MPTP at 20 mg/kg/7-NI at 50 mg/kg; ‡, MPTP at 20 mg/kg/7-NI at 50 mg/kg; and §, MPTP 15 mg/kg/7-NI at 50 mg/kg.

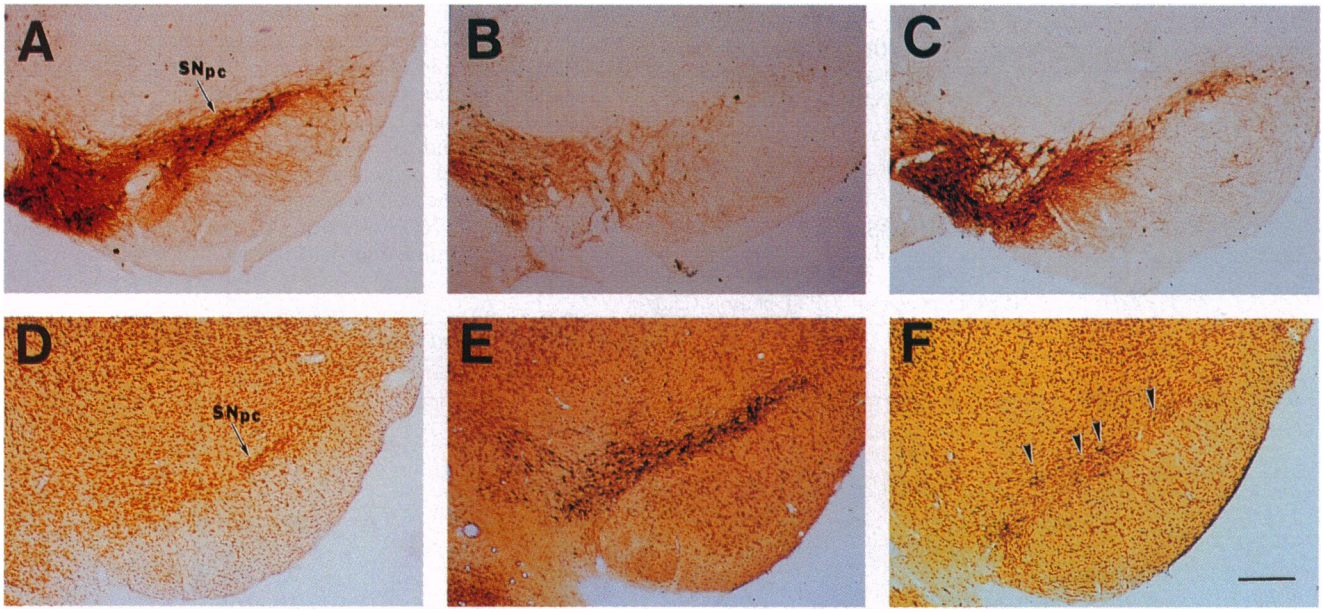

FIG. 2. Color photomicrographs of the SNpc TH-positive and silver-stained neurons in a mouse injected with saline and peanut oil (*A* and *D*), with MPTP at 20 mg/kg (*B* and *E*), and with both MPTP at 20 mg/kg and 7-NI at 50 mg/kg (*C* and *F*). Normal control mice show numerous TH-positive cell bodies and a dense TH-positive nerve fiber network (arrow in *A*) and no degenerating neurons, as evidenced by the lack of silver-stained neurons (arrow in *D*). MPTP administration causes a marked reduction in both the number of cell bodies and density of nerve fiber network in the SNpc (*B*) and a large number of silver-stained neurons (*E*). Note the clear partial preservation of both the number of cell bodies and density of nerve fiber network in the SNpc (*C*) and the presence of only a few scattered degenerating neurons in mice injected with 7-NI and MPTP (arrowheads in *F*). (Bar = 220 μm.)

present study was also found not to alter significantly either brain monoamine oxidase B activity or striatal [^{3}H]dopamine uptake (13), two other important steps in MPTP metabolism (1). Moreover, the protective effects were not due to temperature alteration, as 7-NI seems to produce only small changes in body temperature (13). Therefore, we feel confident to conclude that inhibition of NOS is responsible for the observed resistance to MPTP in the 7-NI-injected mice.

nNOS$^-$ Mice Are Resistant to MPTP. Although 7-NI is touted as a relatively selective nNOS inhibitor, it may inhibit all three isoforms of NOS in the brain, which include nNOS, endothelial NOS, and inducible NOS (34–37). To clarify the role of nNOS in MPTP-induced dopaminergic neurotoxicity, we examined the effects of MPTP in mice lacking the neuronal isoform (16). This approach is more advantageous than the pharmacological inhibition of NOS because it allows for the study of the neuronal enzyme independent of other NOS enzymes.

In preliminary experiments, administration of four injections of MPTP at 20 mg/kg killed all the wild-type controls, but the nNOS$^-$ mice were completely spared from the lethal effects of MPTP (data not shown). The death rate was much less after the administration of four injections of MPTP at 15 mg/kg. It is worth noting that 3 of 6 (50%) wild-type mice and 2 of 6 (33%) heterozygous mutant mice died less than 24 hr after the last injection of MPTP, while all 6 nNOS$^-$ mice survived through the 7 days of the experiment. Four injections of MPTP at 15 mg/kg caused a significant reduction in striatal dopamine, DOPAC, and HVA levels in wild-type mice compared with saline-injected controls (Fig. 4). Heterozygous mutant mice exhibited profound reductions in striatal catecholamine levels as well. In contrast, nNOS$^-$ mice showed significantly lower reductions in dopamine, DOPAC, and HVA levels than either wild-type or heterozygous mutant mice (Fig. 4).

DISCUSSION

This study provides several lines of evidence indicating that neuronally derived NO mediates, in part, MPTP-induced SNpc cell death and decrements in striatal dopamine and its metabolites DOPAC and HVA. 7-NI, a putative selective nNOS inhibitor, prevents MPTP neurotoxicity in proportion to its potency as a NOS inhibitor. In mice that received 7-NI, MPTP caused significantly less neuronal death in the SNpc, as evidenced by the greater number of TH-positive neurons and the smaller number of silver-stained degenerating neurons in these animals compared with those that received MPTP only. A similar observation is made for striatal dopamine, DOPAC, and HVA levels. The degree of resistance to MPTP in mice appears to result from a balance between the dose of 7-NI and the dose of MPTP, with the highest neuroprotection in mice that received the regimen of 7-NI at 50 mg/kg and MPTP at 15 mg/kg and the lowest neuroprotection in mice that received the regimen of 7-NI at 25 mg/kg and MPTP at 20 mg/kg. Thus, these findings indicate that the greater the inhibition of NOS activity, the stronger the neuroprotection against MPTP-induced nigrostriatal dopamine neurotoxicity. Moreover, nNOS$^-$ mice are 2-fold less affected by MPTP than wild-type and heterozygous mice, thus establishing the role of nNOS in MPTP-induced neurotoxicity. Our findings are consistent with other evidence that neuronally produced NO causes MPTP neurotoxicity. Significant attenuation of MPTP-induced damage to striatal dopamine nerve terminals was also reported by Schulz *et al.* (13) using a different protocol of 7-NI injection and mice with less severe striatal dopamine decrements, and by Smith *et al.* (38) using L-nitroarginine, another but less selective NOS inhibitor (39). Together, these observations establish neuronal NO as a prinicipal mediator of MPTP neurotoxicity.

The resistance of nNOS$^-$ mice to MPTP illustrates the importance of nNOS in the MPTP neurotoxic mechanism. However, it is important to note that nNOS$^-$ mice were significantly, but not completely, protected against MPTP. nNOS$^-$ mice possess a low amount of residual NOS activity that is thought to be due to the other isoforms that exist in the brain, including endothelial and inducible NOS (16, 40). It is conceivable that these enzymes could generate sufficient NO for MPTP to exert some degree of dopamine neurotoxicity. Consistent with this notion is that 7-NI provides complete neuroprotection at the lower doses of MPTP, whereas the nNOS$^-$ mice are only partially protected. Microglia and astrocytes are activated following MPTP administration (41, 42); thus 7-NI may provide complete neuroprotection through inhibition of inducible NOS.

A striking observation is that the rate of acute death is significantly greater among wild-type and heterozygous mice compared with the nNOS$^-$ mice. The cause of acute death that occurs within 24 hr after the last injection of MPTP is not known, but it is probably not due to the acute depletion of dopamine from the brain. Death is most likely due to a fatal cardiac arrhythmia or blood pressure changes, in that MPTP

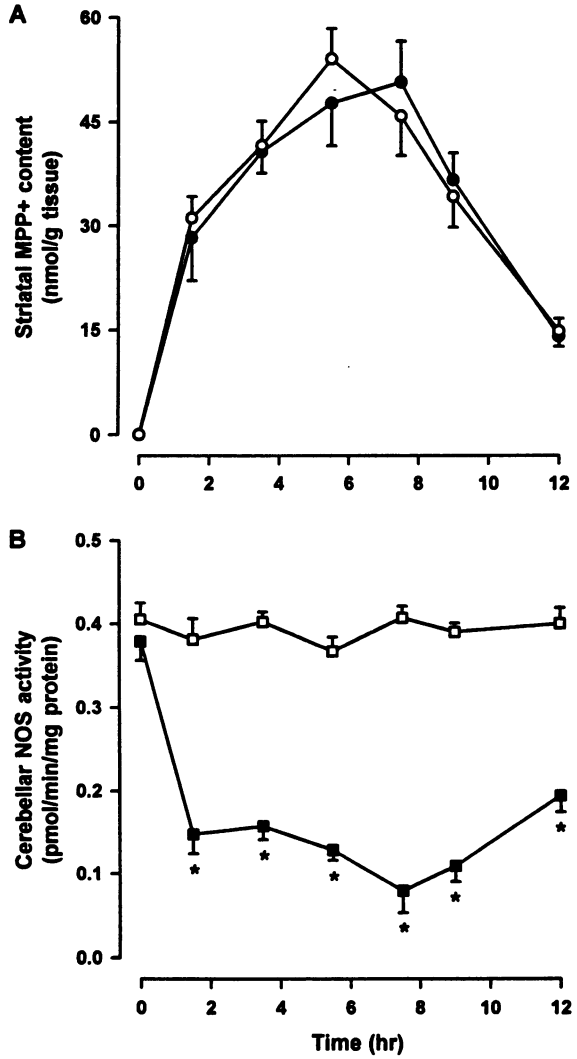

FIG. 3. Effect of 7-NI administration on striatal MPP$^+$ content and cerebellar NOS catalytic activity. (*A*) MPP$^+$ content in mice that received 7-NI (●) and peanut oil (○). (*B*) NOS catalytic activity in mice that received 7-NI (■) and mice that received peanut oil (□). Values are mean ± SEM for 4–5 mice per group. *, Significantly different (Newman–Keuls post-hoc test, $P < 0.05$) from peanut oil-injected mice.

administration causes acute depletion of heart norepinephrine in mice (43). Immunohistochemical studies have shown positive nNOS immunoreactivity in the cells of the intermediolateral column of the spinal cord (44) and of various nuclei of the medulla (45). Because all of these structures are involved in the regulation of the cardiovascular function, nNOS$^-$ mice may have biochemical or anatomical alterations in their autonomic nervous systems that hamper the MPTP-induced cardiovascular response. Telemetric recordings of cardiac function in the nNOS$^-$ mice indicate that there is loss of central vagal tone with substantial tachycardia that is unaffected by atropine treatment (J. P. Dinerman, T.M.D., and S. H. Snyder, unpublished observations). Perhaps the loss of central vagal tone in the nNOS$^-$ mice confers resistance to the putative cardiotoxic effects of MPTP.

What is the source of neuronally derived NO that contributes to MPTP neurotoxicity? SNpc TH-positive neurons show no immunoreactivity for nNOS (46), and there is no evidence for nNOS immunoreactivity in neurons or nerve fibers in the vicinity of the SNpc (46), thus the source of neurotoxic NO may be in the striatum, the major projection of the SNpc. Indeed, the striatum contains a rich density of nNOS-positive neurons and fibers (46) that colocalize with somatostatin, neuropeptide Y, and NADPH diaphorase (47). Consequently, dopamine nerve terminals may be the primary target of MPTP neurotoxicity and may die first followed by a slower and secondary death of the SNpc dopamine cell bodies. Consistent with this notion are the observations that MPP$^+$ accumulates primarily in striatal dopamine terminals and not in the SNpc dopamine cell bodies after MPTP administration (48) and intrastriatal injection of 6-hydroxydopamine, another dopamine neurotoxin, destroys dopamine nerve terminals and leads to a retrograde degeneration of the SNpc dopamine cell bodies (49).

Recent studies indicated that the majority of NO-mediated neurotoxicity is dependent on local "factors" produced within the susceptible target neurons (47, 50). Because transgenic mice with increased SOD activity are also more resistant to MPTP (9), it is likely that the contribution of NO to MPTP neurotoxicity evolves from its interaction with superoxide radical to form peroxynitrite (12). NO will readily react with the superoxide radical to produce the potent oxidant, peroxynitrite (51, 52), at a rate three times faster than the reaction rate of SOD in catalyzing the dismutation of the superoxide radical to hydrogen peroxide (52). Accordingly, a simple increase in the concentration of either NO or superoxide radical can lead to the formation of peroxynitrite and subsequent cell injury. Thus, following MPTP administration, MPP$^+$ accumulates in dopamine terminals and, through blockade of the mitochondrial electron transport chain (31), stimulates the production of superoxide radicals (11, 53). NO, which is freely diffusible, out competes with SOD for the increased superoxide radical contained within the dopamine terminals to form peroxynitrite. Peroxynitrite, a potent oxidant, causes direct injury to the cell (47) (Fig. 5); it also decomposes to the reactive hydroxyl radical (10, 54), which leads to further injury (Fig. 5). In addition, peroxynitrite can nitrate tyrosine residues (55), which may serve as a biomarker for peroxynitrite actions. In support of this latter neurotoxic pathway is the demonstration that MPTP treatment causes significant increases in 3-nitrotyrosine in the mouse striatum (13). Because of the similarity between the MPTP model and PD, a comparable cascade of events may be relevant to the pathogenesis of PD.

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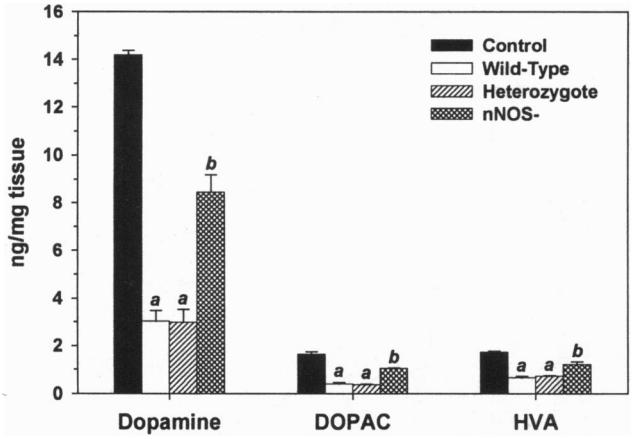

FIG. 4. nNOS$^-$ mice are resistant to MPTP neurotoxicity. Values are mean ± SEM [wild-type (n = 3), heterozygotes (n = 4), and nNOS$^-$ (n = 6)]. All mice used in these experiments are littermates of heterozygote matings of heterozygotes obtained from a nNOS$^-$ backcross to C57B6. Mice were injected i.p. with MPTP at 15 mg/kg dissolved in salined; control mice received vehicle only. Seven days after the last injection, mice were killed and striata were used for HPLC determination of dopamine, DOPAC, and HVA levels. Dopamine, DOPAC, and HVA levels did not differ among wild-type (n = 10), heterozygous (n = 10), and nNOS$^-$ (n = 7) saline-injected mice; thus these values were pooled. Significantly different (Newman–Keuls post-hoc test, $P < 0.05$) from (a) saline-injected controls and MPTP-injected nNOS$^-$, and (b) MPTP-injected wild-type, heterozygous mice, and saline-injected controls.

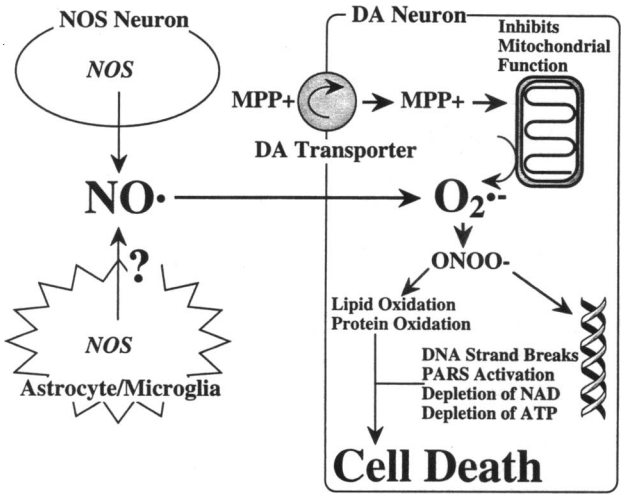

FIG. 5. Proposed scheme for selective MPTP-induced dopaminergic neurotoxicity. MPTP is converted to MPP$^+$, which is transported into dopamine (DA) neurons via the dopamine transporter. MPP$^+$ inhibits enzymes in the mitochondrial electron transport chain, resulting in increased "leakage" of superoxide anion ($O_2^{\cdot-}$) from the respiratory chain. $O_2^{\cdot-}$ cannot readily cross cellular membranes and so remains in the cell and organelle in which it is produced. In contrast, nitric oxide (NO$^\cdot$) is membrane permeant and diffuses to neighboring neurons. If the neighboring cell has elevated levels of $O_2^{\cdot-}$, then there is an increased probability of reacting with NO$^\cdot$ to form the potent oxidant, peroxynitrite (ONOO$^-$). ONOO$^-$ can initiate cell death by a host of toxic insults to the cell. It can cause DNA strand breaks, leading to activation of the nuclear enzyme poly(ADP-ribose) synthetase (PARS) (56). Activation of PARS can deplete the cell of NAD and then ATP (57). If mitochondrial activity is impaired the cell cannot replace these energy substrates and dies. ONOO$^-$ can also initiate lipid peroxidation, and protein oxidation and inactivation. All of these events can contribute to cell death. In this scheme it is the site of generation of $O_2^{\cdot-}$ which determines whether a cell will succumb to NO$^\cdot$- and ONOO$^-$-mediated cell death. Because, after MPTP administration, dopamine neurons selectively accumulate MPP$^+$, which in turn, stimulates $O_2^{\cdot-}$ production, these neurons are selectively at risk.

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1. Langston, J. W. & Irwin, I. (1986) *Clin. Neuropharmacol.* **9,** 485–507.
2. Heikkila, R. E., Sieber, B. A., Manzino, L. & Sonsalla, P. K. (1989) *Mol. Chem. Neuropathol.* **10,** 171–183.
3. Kopin, I. J. & Markey, S. P. (1988) *Annu. Rev. Neurosci.* **11,** 81–96.
4. Fahn, S. (1988) in *Parkinsonism*, eds. Wyngaarden, J. B. & Smith, L. H., Jr. (Saunders, Philadelphia), Vol. 18, pp. 2143–2147.
5. Jenner, P., Schapira, A. H. V. & Marsden, C. D. (1992) *Neurology* **42,** 2241–2250.
6. Jackson-Lewis, V., Jakowec, M., Burke, R. E. & Przedborski, S. (1995) *Neurodegeneration* **4,** 257–269.
7. Forno, L. S., DeLanney, L. E., Irwin, I. & Langston, J. W. (1993) *Adv. Neurol.* **60,** 600–608.
8. Przedborski, S., Jackson-Lewis, V., Kostic, V., Carlson, E., Epstein, C. J. & Cadet, J. L. (1992) *J. Neurochem.* **58,** 1760–1767.
9. Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A. B., Simonetti, S., Fahn, S., Carlson, E., Epstein, C. J. & Cadet, J. L. (1992) *J. Neurosci.* **12,** 1658–1667.
10. Halliwell, B. & Gutteridge, J. M. (1991) *Free Radicals in Biology and Medicine* (Clarendon, Oxford).
11. Hasegawa, E., Takeshige, K., Oishi, T., Murai, Y. & Minakami, S. (1990) *Biochem. Biophys. Res. Commun.* **170,** 1049–1055.
12. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.
13. Schulz, J. B., Matthews, R. T., Muqit, M. M. K., Browne, S. E. & Beal, M. F. (1995) *J. Neurochem.* **64,** 936–939.
14. Moore, P. K., Wallace, P., Gaffen, Z., Hart, S. L. & Babbedge, R. C. (1993) *Br. J. Pharmacol.* **110,** 219–224.
15. Reches, A., Wagner, H. R., Jiang, D., Jackson, V. & Fahn, S. (1982) *Life Sci.* **31,** 37–44.
16. Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. & Fishman, M. C. (1993) *Cell* **75,** 1273–1285.
17. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
18. Przedborski, S., Levivier, M., Raftopoulos, C., Naini, A. B. & Hildebrand, J. (1995) *Mov. Disord.* **10,** 28–36.
19. Gallyas, F., Wolff, J. R., Bottcher, H. & Zaborszky, L. (1980) *Stain Technol.* **55,** 299–306.
20. Muthane, U., Ramsay, K. A., Jiang, H., Jackson-Lewis, V., Donaldson, D., Fernando, S., Ferreira, M. & Przedborski, S. (1994) *Exp. Neurol.* **126,** 195–204.
21. Sidman, R. L., Angevine, J. B., Jr., & Pierce, E. T. (1971) *Atlas of the Mouse Brain and Spinal Cord* (Harvard Univ. Press, Cambridge, MA).
22. Abercrombie, M. (1946) *Anat. Rec.* **94,** 239–247.
23. Hallman, H., Lange, J., Olson, L., Stromberg, I. & Jonsson, G. (1985) *J. Neurochem.* **44,** 117–127.
24. Heikkila, R. E., Hess, A. & Duvoisin, R. C. (1984) *Science* **224,** 1451–1453.
25. Ricaurte, G. A. & McCann, U. D. (1992) *Ann. N.Y. Acad. Sci.* **648,** 371–382.
26. Hallman, H., Olson, L. & Jonsson, G. (1984) *Eur. J. Pharmacol.* **97,** 133–136.
27. Markey, S. P., Johannessen, J. N., Chiueh, C. C., Burns, R. S. & Herkenham, M. A. (1984) *Nature (London)* **311,** 464–467.
28. Heikkila, R. E., Manzino, L., Cabbat, F. S. & Duvoisin, R. C. (1984) *Nature (London)* **311,** 467–469.
29. Javitch, J. A., D'Amato, R. J., Strittmatter, S. M. & Snyder, S. H. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2173–2177.
30. Ramsey, R. R. & Singer, T. (1986) *J. Biol. Chem.* **261,** 7585–7587.
31. Nicklas, W. J., Yougster, S. K., Kindt, M. V. & Heikkila, R. E. (1987) *Life Sci.* **40,** 721–729.
32. Vays, I., Heikkila, R. E. & Nicklas, W. J. (1986) *J. Neurochem.* **46,** 1501–1507.
33. Giovanni, A., Sieber, B. A., Heikkila, R. E. & Sonsalla, P. K. (1991) *J. Pharmacol. Exp. Ther.* **257,** 691–697.
34. Kelly, P. A. T., Ritchie, I. M. & Arbuthnott, G. W. (1995) *J. Cerebr. Blood Flow Metab.* **15,** 766–773.
35. Bland-Ward, P. A. & Moore, P. K. (1995) *Life Sci.* **57,** PL131–PL135.
36. Medhurst, A. D., Greenlees, C., Parsons, A. A. & Smith, S. J. (1994) *Arch. Biochem. Biophys.* **314,** 360–366.
37. Wolff, D. J. & Gribin, B. J. (1994) *Arch. Biochem. Biophys.* **311,** 300–306.
38. Smith, T. S., Swerdlow, R. H., Parker, W. D., Jr., & Bennett, J. P., Jr. (1994) *NeuroReport* **5,** 2598–2600.
39. Rees, D. D., Palmer, R. M. J., Schulz, R., Hodson, H. F. & Moncada, S. (1990) *Br. J. Pharmacol.* **101,** 746–752.
40. O'Dell, T. J., Huang, P. L., Dawson, T. M., Dinerman, J. L., Snyder, S. H., Kandel, E. R. & Fishman, M. C. (1994) *Science* **265,** 542–546.
41. Francis, J. W., Von Visger, J., Markelonis, G. J. & Oh, T. H. (1995) *Neurotoxicol. Teratol.* **17,** 7–12.
42. Viswanathan, M., de Oliveira, A. M., Wu, R. M., Chiueh, C. C. & Saavedra, J. M. (1994) *Cell. Mol. Neurobiol.* **14,** 99–104.
43. Fuller, R. W., Hahn, R. A., Snoddy, H. D. & Wikel, J. H. (1984) *Biochem. Pharmacol.* **19,** 2957–2960.
44. Dun, N. J., Dun, S. L., Wu, S. Y., Förstermann, U., Schmidt, H. H. H. W. & Tseng, L. F. (1993) *Neuroscience* **54,** 845–857.
45. Dun, N. J., Dun, S. L. & Forstermann, U. (1994) *Neuroscience* **59,** 429–445.
46. Bredt, D. S., Glatt, C. E., Huang, P. L., Fotuhi, M., Dawson, T. M. & Snyder, S. H. (1991) *Neuron* **7,** 615–624.
47. Dawson, T. M. & Snyder, S. H. (1994) *J. Neurosci.* **14,** 5147–5159.
48. Herkenham, M., Little, M. D., Bankiewicz, K., Yang, S. C., Markey, S. P. & Johannessen, J. N. (1991) *Neuroscience* **40,** 133–158.
49. Przedborski, S., Levivier, M., Jiang, H., Ferreira, M., Jackson-Lewis, V., Donaldson, D. & Togasaki, D. M. (1995) *Neuroscience* **67,** 631–647.
50. Dawson, V. L., Kizushi, V. M., Huang, P. L., Snyder, S. H. & Dawson, T. M. (1996) *J. Neurosci.*, in press.
51. Beckman, J. S. (1991) *J. Dev. Physiol.* **15,** 53–59.
52. Beckman, J. S. (1994) *Ann. N.Y. Acad. Sci.* **738,** 69–75.
53. Walker, M. J., Jenner, P. & Marsden, C. D. (1991) *Biochem. Pharmacol.* **42,** 913–919.
54. Beckman, J. S., Chen, J., Crow, J. P. & Ye, Y. Z. (1994) *Prog. Brain Res.* **103,** 371–380.
55. Beckman, J. S., Ischiropoulos, H., Zhu, L., Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C. & Tsai, M. (1992) *Arch. Biochem. Biophys.* **298,** 438–445.
56. Zhang, J., Dawson, V. L. & Dawson, T. M. (1994) *Science* **263,** 687–689.
57. Berger, N. A. (1985) *Radiat. Res.* **101,** 4–15.