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Transgenic Mice with Increased Cu/Zn-Superoxide Dismutase Activity Are Resistant to *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–induced Neurotoxicity

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Administration of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to mammals causes damage to the nigrostriatal dopaminergic pathway similar to that observed in Parkinson's disease. It has been suggested that the mechanism by which MPTP kills dopamine (DA) neurons involves an energy crisis due to the inhibition of mitochondrial complex I. In addition, superoxide radicals (O_2^-), generated subsequent to the blockade of mitochondrial complex I, may also be involved in MPTP-induced neurotoxicity. Superoxide dismutase (SOD) is a scavenger enzyme that protects cells from the hazard of O_2^- radicals. To evaluate further the role of O_2^- radical in MPTP-induced toxicity, we tested the effects of MPTP in transgenic mice with increased SOD activity. In nontransgenic littermates with normal SOD activity, MPTP injection causes a marked reduction in striatal levels of DA and its metabolites as well as in striatal and nigral ^{3}H-DA uptake; these findings are consistent with a loss in dopaminergic neurons. In contrast, in transgenic mice with increased SOD activity, MPTP injection does not cause any significant changes either in levels of DA and metabolites or in ^{3}H-DA uptake. We show that this lack of toxicity is not due to a lower delivery of MPTP to the brain following its intraperitoneal injection, to reduced brain biotransformation of MPTP to *N*-methyl-4-phenylpyridinium ion (MPP^+), to diminished striatal mitochondrial monoamine oxidase B activity, to decreased synaptosomal uptake of MPP^+, to lower potency of MPP^+ to inhibit the complex I of the mitochondrial electron transport chain, or to faster brain elimination of MPP^+. These results suggest that increased SOD activity is, most likely, the protective factor that confers resistance to transgenic mice against MPTP-induced neurotoxicity. Thus, this study provides further evidence that some of the deleterious effects of MPTP may be mediated by O_2^- radicals. The similarity between the MPTP model and Parkinson's disease further raises the possibility that oxy-radicals may play a significant role in the etiology of this neurodegenerative disorder.

N-methyl-4-phenyl,1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of the meperidine analog 1-methyl-4-propionoxypyridine, which has potent heroinlike effects (Langston et al., 1983). In human and nonhuman primates, MPTP produces an irreversible and severe Parkinsonian-like syndrome (Davis et al., 1979; Burns et al., 1983; Langston et al., 1983). In addition, its administration to humans, monkeys, and mice causes changes in the nigrostriatal dopaminergic pathway that are reminiscent of those observed in Parkinson's disease (reviewed in Langston and Irwin, 1986; Kopin and Markey, 1988). These include a marked reduction in the levels of striatal dopamine (DA) and of its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Burns et al., 1983; Heikkila et al., 1984a; Sundstrom et al., 1987). There are also significant decreases in tyrosine hydroxylase activity (Mayer et al., 1986; Sundstrom et al., 1987), in ^{3}H-DA uptake (Heikkila et al., 1984a), and in ^{3}H-mazindol binding (Javitch et al., 1985b) in the striatum, all of which indicate a loss of dopaminergic nerve terminals. Moreover, histochemical analyses of the brains of MPTP-treated animals have revealed a marked loss of dopaminergic cell bodies within the substantia nigra pars compacta (Burns et al., 1983; Heikkila et al., 1984a; Langston et al., 1984a).

Thus far, the actual mechanism by which MPTP destroys dopaminergic neurons has remained uncertain. The toxic effects of MPTP depend on its biotransformation to *N*-methyl-4-phenylpyridinium (MPP^+) by the enzyme monoamine oxidase B (MAO-B) (Heikkila et al., 1984b). Since MAO-B is found predominantly in astrocytes and serotonergic neurons (Levitt et al., 1982; Westlund et al., 1985), it has been proposed that MPP^+ is generated within these cells, released, and then taken up by dopaminergic neurons (Javitch et al., 1985a). Once inside dopaminergic neurons, MPP^+ is actively concentrated within the mitochondria (Ramsey and Singer, 1986), where it inhibits complex I of the mitochondrial respiratory chain (Vays et al., 1986; Mizuno et al., 1987; Nicklas et al., 1987). This action is thought

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to be responsible for its toxic effects (Vays et al., 1986; Mizuno et al., 1987; Nicklas et al., 1987). Nevertheless, a number of studies have also suggested a possible role for oxygen-based free radicals in this toxic process (Cohen, 1984; Corsini et al., 1985; Johannessen et al., 1986; Poirier and Barbeau, 1985; Rossetti et al., 1988; Yurek et al., 1989). For instance, production of superoxide (O_2^-) radicals has been documented during the *in vitro* biotransformation of MPTP (Poirier and Barbeau, 1985; Rossetti et al., 1988). Moreover, pretreatment with diethyl dithiocarbamate, which inhibits superoxide dismutase (SOD), a scavenger enzyme involved in the protection of cells against O_2^- radicals (Fridovich, 1986), enhances MPTP-induced toxicity in mice (Corsini et al., 1985; Yurek et al., 1989). More recently, MPP^+ has actually been shown to increase the production of O_2^- radicals in mitochondrial preparations (Hasegawa et al., 1990). Therefore, in order to evaluate further the possible involvement of O_2^- radicals in MPTP-induced toxicity, the effects of this compound were studied *in vivo* in transgenic mice with increased SOD activity (Epstein et al., 1987).

Materials and Methods

Transgenic mice. Mice of strain HS/SF-218 (C57-B1/6J × SJL/J from C.J.E.'s laboratory) carrying the human copper/zinc-SOD (Cu/Zn-SOD) gene were used (Epstein et al., 1987). The genome of these transgenic animals carries several copies of the human Cu/Zn-SOD gene, presumably in tandem array. The founder mice have been bred to produce transgenic offspring expressing the human Cu/Zn-SOD gene. Transgenic animals were identified by Northern blot analysis for the presence of human Cu/Zn-SOD gene in red cell extracts as previously described (Epstein et al., 1987). Detection of human Cu/Zn-SOD enzymatic activity in brain tissues using nondenaturating gel electrophoresis followed by nitroblue tetrazolium staining was performed in a single SOD-transgenic mouse and its nontransgenic littermate. The gel was scanned on a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA) and quantitated using ImageQuant version 3.0. Cu/Zn-SOD activity in brain tissue homogenates was measured in the caudate–putamen of SOD-transgenic mice ($n = 3$) and in their nontransgenic littermates ($n = 3$) according to the method previously described (Przedborski et al., 1991).

MPTP administration. White female (25–30 gm) SOD-transgenic mice HS/SF-218 ($n = 10$) and their nontransgenic littermates ($n = 10$) received three injections of 30 mg/kg MPTP-HCl intraperitoneally in 0.2 ml of saline at 24 hr intervals. Control SOD-transgenic ($n = 10$) and nontransgenic mice ($n = 10$) received vehicle only. Five and twenty-one days after the last injection, five mice per group were decapitated, and their brains were used to determine biochemical and morphological changes in the dopaminergic system. Animal care was in accordance with institutional guidelines. This protocol of MPTP administration, based on our preliminary experiments on nontransgenic mice, caused approximately a 60% reduction in striatal DA levels with less than a 15% death rate.

Determination of monoamine levels. Striatal levels of DA, HVA, DOPAC, and serotonin (5-HT) were determined by HPLC as previously described (Jackson-Lewis et al., 1991). Briefly, left striata were dissected out, weighed, and sonicated in 10 vol of 0.1 M perchloric acid containing 0.01 M $NaHSO_3$, 0.001% ascorbic acid, and 1 μg/ml dihydroxybenzylamine (Sigma) as internal standard. After centrifugation (12,000 × *g*, 10 min), the supernatants were filtered (0.45 μm nylon filter; Schleicher & Schuell) and 20 μl were injected onto a C18 reverse-phase column (Phenomex). The mobile phase consisted of 0.15 M monochloroacetic acid, 0.1 M NaOH, 0.6 mM EDTA, 0.2 mM sodium octylsulfate, and 9% methanol, final pH 2.9. Monoamines were detected by electrochemical oxidation against a glassy carbon electrode (+0.77 V).

^{3}H-DA and ^{3}H-MPP$^+$ uptake assays. To assess MPTP-induced neuronal loss, the uptake of ^{3}H-DA was measured in the caudate–putamen complex and substantia nigra of MPTP- and saline-treated SOD-transgenic mice and nontransgenic littermates ($n = 5$ per group) according to the method described by Javitch et al. (1985a). The tissues were homogenized in 20 vol of 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 11 mM glucose, pH 7.4. For the assays, 25 μl of a fresh crude synaptosomal preparation was added to 475 μl of 0.015 μM ^{3}H-DA [17.9 Ci/mmol; New England Nuclear (NEN). The reactions were carried out at 37°C. Nonspecific uptake was defined in the presence of 10 μM unlabeled mazindol (Sandoz). The uptake was terminated after 8 min by the addition of 4 ml of ice-cold buffer and rapid filtration of the mixture through glass-fiber filters (Schleider & Schuell no. 32). Filters were washed with two consecutive 2 ml volumes of ice-cold buffer. Radioactivity remaining on the filters was measured by liquid scintillation spectrometry.

The uptake of ^{3}H-MPP$^+$ (70 Ci/mmol; NEN) was measured according to the same method (Javitch et al., 1985a). To define ^{3}H-MPP$^+$ uptake characteristics, caudate–putamen complexes were dissected out from the brains of SOD-transgenic mice and of their nontransgenic littermates (three mice per group), while for the regional distribution of ^{3}H-MPP$^+$ uptake, frontal cerebral cortex, caudate–putamen complex, substantia nigra, hippocampus, and cerebellum were dissected out from the brains of four animals of both groups. The tissues were processed as described above. For the assays, 25 μl of a fresh crude synaptosomal preparation were added to 475 μl of different concentrations (0.006–0.9 μM for saturation and 0.015 μM for regional distribution) of ^{3}H-MPP$^+$. Nonspecific uptake was also defined in the presence of 10 μM unlabeled mazindol. Preliminary experiments showed that striatal synaptosomal ^{3}H-MPP$^+$ accumulation was linear with time up to 6–8 min, after which it began to plateau in both SOD-transgenic and in nontransgenic mice (data not shown). In subsequent experiments, the uptake of ^{3}H-MPP$^+$ was terminated after 8 min and the mixture was processed as described for ^{3}H-DA uptake. The Michaelis constant (K_m) and the maximal velocity (V_{max}) were calculated from saturation data using an iterative curve-fitting program (ENZFITTER, Biosoft, UK).

Brain levels of MPTP and MPP$^+$. Levels of MPTP and MPP+ were determined by the method of Markey et al. (1984). SOD-transgenic mice ($n = 3$) and nontransgenic littermates ($n = 3$) received a single intraperitoneal injection of 10 mg/kg MPTP-HCl and 10 μCi ^{3}H-MPTP (72.6 Ci/mmol; NEN) in 0.2 ml of saline and were decapitated 10 min after injection. Brains were quickly removed and homogenized in 5 vol of distilled water to which was added the same volume of 100% ethanol. Homogenates were centrifuged at 15,000 × *g* for 10 min at 4°C. Supernatants were filtered (0.45 μm nylon filter; Scheicher & Schuell), and an aliquot of 100 μl was injected onto an HPLC system with a C18 reverse-phase column (Phenomex). The mobile phase consisted of acetonitrile/100 mM sodium acetate (60:40, v/v) containing 0.1% triethylamine (final pH 5.6). The flow rate was 1 ml/min, and 35 eluate fractions of 0.25 ml were collected and counted by liquid scintillation spectrometry. Before and after sample analyses, the elution profiles of ^{3}H-MPTP and ^{3}H-MPP$^+$ were determined. Selection of the 10 min time point was based on Markey's work (Markey et al., 1984) as well as on our own preliminary experiments and provides good reproducibility as well as high levels of MPTP and MPP$^+$.

Enzymatic MAO-B assay. MAO-B activity was assayed radiochemically as described by Youdim (1975). Caudate–putamen complexes were dissected out from the brain of SOD-transgenic mice ($n = 3$) and their nontransgenic littermates ($n = 3$) and were homogenized in 20 vol of 0.01 M potassium phosphate buffer (K-PB), pH 7.8. For the assay, 90 μl of the fresh crude mitochondria preparation were added to 10 μl of different concentrations (1–20 μM) of the substrate ^{14}C-β-phenylethylamine (50 mCi/mmol; NEN). The reaction was carried out at 30°C and terminated after 15 min in a bath of iced water. To each reaction tube, 100 μl of 1 M HCl were added followed by 1 ml of the extraction mixture (toluene-ethyl acetate, 1:1 v/v, saturated in water) (Fowler et al., 1979). After centrifugation for 3 min at 4000 × *g*, 0.8 ml of the organic phase was transferred and mixed with 10 ml of scintillation liquid (Econofluor, NEN). Radioactivity was counted by scintillation spectrometry. For each concentration, blanks were prepared in the same manner except for the addition of 100 μl of 1 M HCl at the beginning of the reaction. K_m and V_{max} values were calculated as described above.

Assays of mitochondrial electron transport chain enzymes and MPP$^+$ inhibition of complex I. Purified brain mitochondria were prepared as described by Clark and Nicklas (1970). Preliminary experiments showed that contamination of the final mitochondrial fraction with either synaptosomes (<2% of original homogenate) or myelin (<4% of original homogenate) was low, based on the activities of lactate dehydrogenase (Clark and Nicklas, 1970) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (Sims and Carnegie, 1976). In addition, the mitochondrial fraction showed more than an 11-fold greater ratio of succinate dehydrogenase (King, 1967a) to lactate dehydrogenase then the original homogenate. Fresh brain mitochondrial enzymes were prepared from

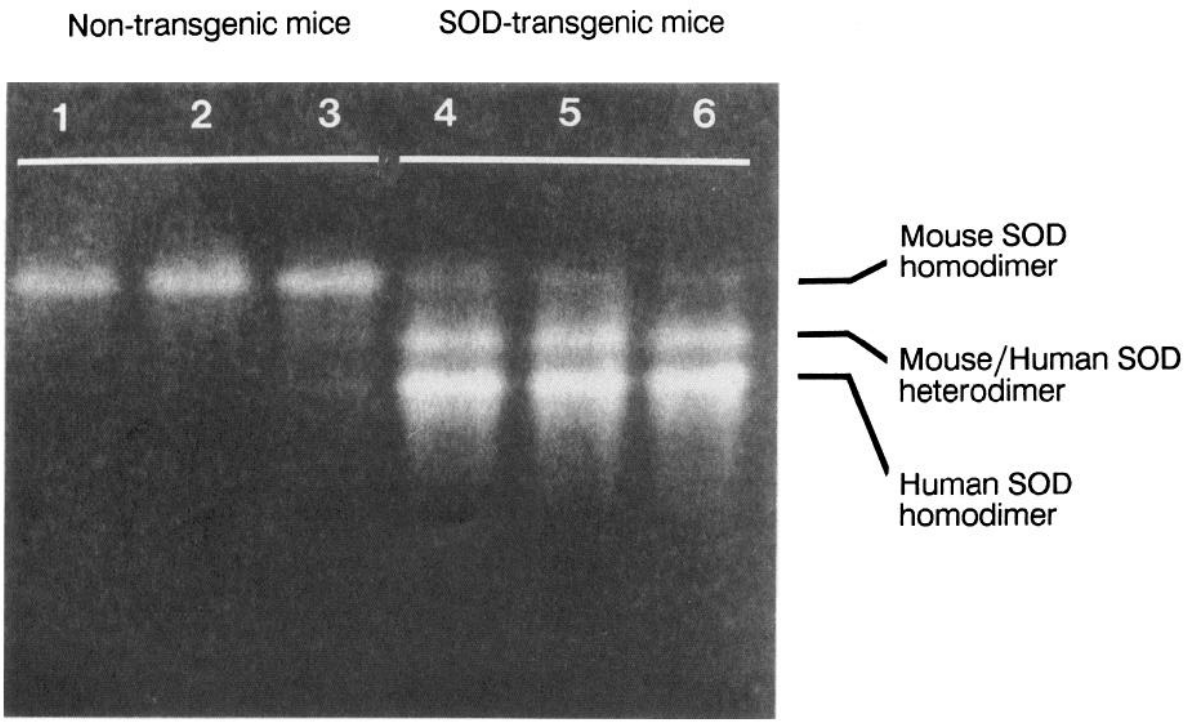


Figure 1. Expression of human Cu/Zn-SOD enzymatic activity in cerebral cortex (*lanes 1* and *4*), caudate–putamen (*lanes 2* and *5*), and cerebellum (*lanes 3* and *6*) of a SOD-transgenic mouse and its nontransgenic littermate. In the nontransgenic mouse, only mouse Cu/Zn-SOD activity was expressed, whereas in the SOD-transgenic animal there were mouse and human homodimers as well as human/mouse heterodimers. The ratios of human homodimer and human/mouse heterodimer in the SOD-transgenic mouse to mouse homodimer in the nontransgenic littermate were 4.61 and 4.28, respectively.

SOD-transgenic mice ($n = 3$) and nontransgenic littermates ($n = 3$) and were assayed in a final volume of 1 ml using a Shimadzu UV-160 recording spectrophotometer. Citrate synthase was assayed according to the method of Srere (1969). The reaction mixture contained 0.1 mM 5-5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in 1 M Tris-HCl (pH 8.1), 0.3 mM acetyl-CoA, 0.5 mM oxaloacetate in 0.1 M Tris-HCl (pH 8.1). NADH-ubiquinone-1 reductase (NADH-Q reductase) was assayed according to the method of Birch-Machin et al. (1989); the rotenone-sensitive rate was taken to constitute complex I activity. The reaction mixture contained 35 mM K-PB (pH 7.2), 5 mM $MgCl_2$, 2 mM potassium cyanide (KCN), 2.5 mg/ml defatted BSA, 2 μg/ml antimycin, 65 μM ubiquinone-1, and 0.13 mM NADH. NADH cytochrome C reductase activity was assayed according to the method of Hatefi and Rieske (1967); the rotenone-sensitive rate was taken to be the activity of the mitochondrial respiratory chain. Reaction mixture contained 0.2 mM K-PB (pH 7.5), 25 mM NADH, 5 mM KCN, and 100 mM cytochrome C. Succinate cytochrome C activity was assayed according to the method of King (1967b). Reaction mixture contained 50 mM K-PB (pH 7.5), 3 mM succinate (pH 7.4), 0.5 mM KCN, and 0.1 mM cytochrome C. Cytochrome C oxidase activity was assayed according to the method described by Wharton and Tzagoloff (1967). The reaction mixture contained 1 mM K-PB (pH 7.0) and 1% reduced cytochrome C. In all of the assays, enzyme activities were determined at 30°C and the reaction started with 20–40 μg mitochondrial protein. For the inhibition of NADH-Q reductase activity (complex I) by MPP^+, fresh mitochondria were preincubated 10 min at 25°C with or without various concentrations of MPP^+ before the assay was started (Ramsey and Singer, 1986; Mizuno et al., 1987).

Brain elimination of MPP^+. SOD-transgenic mice ($n = 16$) and their nontransgenic littermates ($n = 16$) received a single intraperitoneal injection of 30 mg/kg MPTP-HCl. Four SOD-transgenic and four nontransgenic mice were killed 2, 4, 8, and 10 hr after injection; these time points were chosen based on previous studies showing that concentrations of MPP^+ in the mouse brain decline to undetectable concentrations during this time period (Irwin et al., 1989). The brains were rapidly removed, and the frontal cerebral cortex, the caudate–putamen complex, and the ventral mesencephalon were dissected out on ice. Tissue samples were sonicated in 5 vol of 5% trichloroacetic acid containing 5 μg/ml 4-phenylpyridine (Sigma) as internal standard. The homogenates were centrifuged at 15,000 × *g* for 10 min at 4°C, and the concentrations of MPTP, 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$), and MPP^+ in the resulting supernatants were quantified by HPLC with UV detection, according to the method described by Shinka et al. (1987). A cation-exchange column (Altex Ultrasil-CX, 4.6 mm × 25 cm, 10 μm particle size) was used with a mobile phase consisting of 0.1 M acetic acid and 0.075 M triethylamine-HCl (pH 2.35, adjusted with formic acid) and acetonitrile (90:10, v/v). The compounds were monitored at the following wavelengths: MPTP, 245 nm; $MPDP^+$, 345 nm; MPP^+, 295 nm. The flow rate was set at 1.5 ml/min. Quantification was made by comparison of peak height ratios in the samples with those of the standards and corrections were made for recovery. The lower limits of detection for column injection of standards (MPP^+, $MPDP^+$, and MPTP) were found to be 3 ng. Retention times were as follows: MPTP, 2.6 min; $MPDP^+$, 6.8 min; MPP^+, 6.8 min.

Protein concentration. For all the assays, protein concentration was determined by the method of Lowry et al. (1951) using BSA as standard.

Statistics. All assays were carried out in duplicates. Differences between the SOD-transgenic mice and their nontransgenic littermates were analyzed by a two-tailed Student's *t* test. Differences between the MPTP-injected and saline-injected SOD-transgenic mice and their nontransgenic littermates were analyzed by an one-way analysis of variance (ANOVA), while a two-way ANOVA was used to test differences in regional elimination of MPP^+ between the two groups of mice; all ANOVAs were followed by a Scheffé post hoc test. In all cases, the null hypothesis was rejected at the level 0.05. All values are expressed as means ± SEM.

Results

SOD enzymatic activity in the transgenic and nontransgenic animals

The presence of human Cu/Zn-SOD enzymatic activity was found in all the regions of the brain examined resulting from the expression of human Cu/Zn-SOD transgene in mice (Fig. 1). In nontransgenic mice, only mouse Cu/Zn-SOD activity was expressed (Fig. 1). In contrast, in SOD-transgenic mice HS/SF-

218, mouse homodimer as well as human Cu/Zn-SOD homodimer and human/mouse heterodimer Cu/Zn-SOD enzymatic activities were detected (Fig. 1). Gel computerized analysis showed substantially higher expression of human homodimer and human/mouse heterodimer in SOD-transgenic HS/SF-218 mice compared to mouse homodimer in nontransgenic littermates (Fig. 1). This finding is in good correlation with the high amount of specific human Cu/Zn-SOD mRNA found in SOD-transgenic HS/SF-218 mice (Epstein et al., 1987). In addition, we found that Cu/Zn-SOD activity in the caudate–putamen of SOD-transgenic HS/SF-218 mice (14.26 ± 0.32 U/mg protein) was 3.21 times higher than in their nontransgenic littermates (4.33 ± 0.32 U/mg protein); the values are in good agreement with the ones we previously measured in other brain regions of these transgenic and nontransgenic mice (Przedborski et al., 1991). Taken together, these data indicate that the increased Cu/Zn-SOD activity measured in SOD-transgenic mice is related to the overexpression of the human Cu/Zn-SOD transgene.

Effects of MPTP on striatal levels of DA and metabolites

Five days after the last injections of MPTP, we observed marked decreases in striatal DA levels in nontransgenic mice (4.73 ± 1.28 ng/mg tissue) in comparison to saline-injected controls (12.45 ± 1.32 ng/mg tissue) (Fig. 2*A*). The striatal content of the two major DA metabolites, DOPAC (MPTP, 0.58 ± 0.11, vs. saline, 1.26 ± 0.10 ng/mg tissue) (Fig. 2*B*) and HVA (MPTP, 0.57 ± 0.10, vs. saline, 1.10 ± 0.08 ng/mg tissue) (Fig. 2*C*) were also decreased. In contrast, in SOD-transgenic mice, MPTP administration did not cause any significant changes in striatal DA (MPTP, 11.64 ± 1.31, vs. saline, 12.98 ± 1.16 ng/mg tissue), DOPAC (MPTP, 1.04 ± 0.10, vs. saline, 1.15 ± 0.09 ng/mg tissue), or HVA (MPTP, 0.99 ± 0.09, vs. saline, 1.09 ± 0.12 ng/mg tissue) levels (Fig. 2*A–C*). MPTP did not cause any significant changes in striatal 5-HT levels in any of the animals studied (data not shown).

Three weeks after the last injections, striatal levels of DA, DOPAC, and HVA were still markedly reduced in nontransgenic mice treated with MPTP (DA, 5.81 ± 1.35 ng/mg tissue; DOPAC, 0.67 ± 0.14 ng/mg tissue; HVA, 0.76 ± 0.12 ng/mg tissue) compared to the saline-treated animals (DA, 11.94 ± 1.18 ng/mg tissue; DOPAC, 1.11 ± 0.10 ng/mg tissue; HVA, 1.19 ± 0.09 ng/mg tissue). Results 3 weeks after the last injections of MPTP were similar to those observed 5 d after the last injections in that no significant reductions in DA or in its metabolites were found in SOD-transgenic mice (DA, 11.23 ± 1.10 ng/mg tissue; DOPAC, 1.03 ± 0.15 ng/mg tissue; HVA, 1.21 ± 0.12 ng/mg tissue) in comparison to the saline-injected controls (DA, 11.75 ± 1.21 ng/mg tissue; DOPAC, 1.17 ± 0.12 ng/mg tissue; HVA, 1.23 ± 0.10 ng/mg tissue).

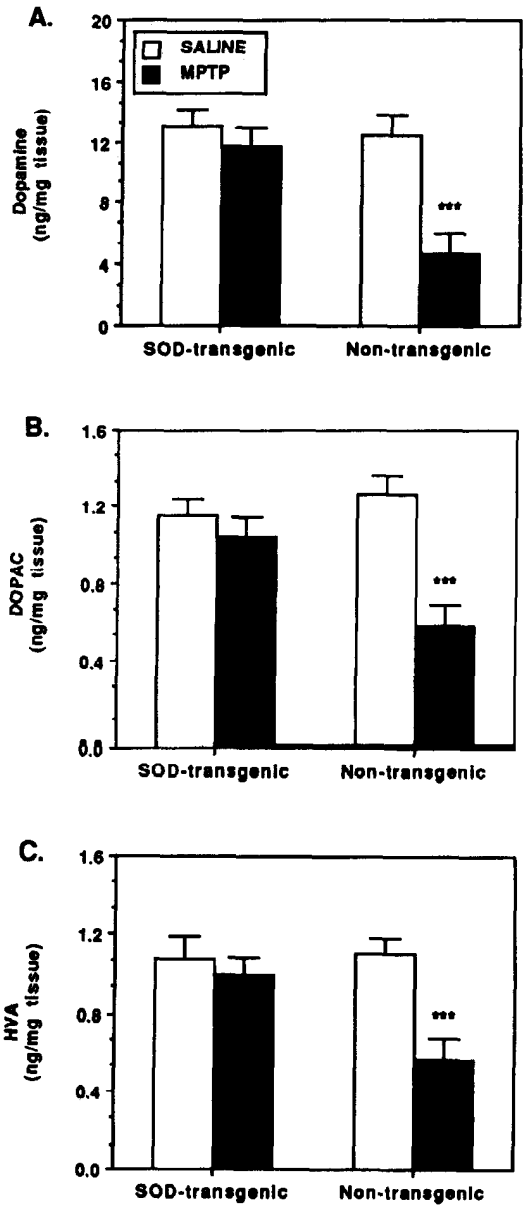


Figure 2. The effects of MPTP on the content of DA (*A*), DOPAC (*B*), and HVA (*C*) in the striatum of SOD-transgenic mice and their nontransgenic littermates 5 d after the last injections. MPTP causes significant decreases in DA, DOPAC, and HVA in nontransgenic mice. It does not affect significantly these levels in SOD-transgenic mice. Values represent means ± SEM of five animals per group. Differences between MPTP-treated nontransgenic mice and all other groups: **, $p < 0.01$; ***, $p < 0.001$ (Scheffé post hoc test).

Effects of MPTP on striatal and nigral ^{3}H-DA uptake

Five days after the last injections of MPTP, we observed marked decreases in striatal uptake of ^{3}H-DA in nontransgenic mice (0.023 ± 0.003 nmol/gm tissue per min) in comparison to saline-injected controls (0.054 ± 0.004 nmol/gm tissue per min) (Fig. 3*A*). A significant reduction in the uptake of ^{3}H-DA was also found in the substantia nigra (pars compacta plus reticulata) of MPTP-injected nontransgenic mice (0.022 ± 0.001 nmol/gm tissue/min) in comparison to saline-injected controls (0.030 ± 0.002 nmol/gm tissue/min) (Fig. 3*B*). In contrast, in SOD-transgenic mice, MPTP did not induce any significant changes in ^{3}H-DA uptake in either the striatum (MPTP, 0.046 ± 0.004, vs. saline, 0.052 ± 0.004 nmol/gm tissue/min) (Fig. 3*A*) or the substantia nigra (MPTP, 0.028 ± 0.002, vs. saline, 0.031 ± 0.002 nmol/gm tissue/min) (Fig. 3*B*).

Brain levels of MPTP

In order to evaluate whether entry of MPTP into the brain was abnormal in SOD-transgenic mice, brain MPTP levels were measured 10 min after a single intraperitoneal injection of this compound. These experiments showed that the brain levels of MPTP were not significantly different in SOD-transgenic mice (3.05 ± 0.66 μg/gm tissue; $n = 3$) in comparison to their nontransgenic littermates (3.19 ± 0.43 μg/gm tissue; $n = 3$).

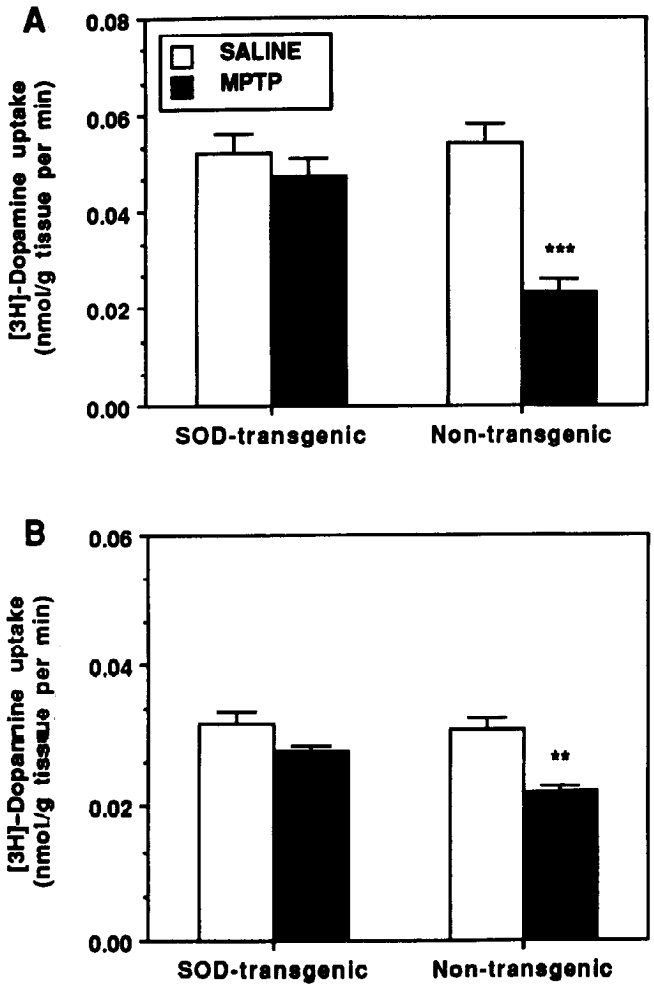


Figure 3. The effects of MPTP on the uptake of ^{3}H-DA in the striatum and the substantia nigra (pars compacta and reticulata) of SOD-transgenic mice and their nontransgenic littermates 5 d after the last injections. MPTP causes significant decreases in ^{3}H-DA uptake in the striatum (*A*) and the substantia nigra (pars compacta and reticulata) (*B*) of nontransgenic mice but does not affect significantly this uptake in SOD-transgenic mice. Values represent means ± SEM of five animals per group. Differences between MPTP-treated nontransgenic mice and all other groups: **, $p < 0.01$; ***, $p < 0.001$ (Scheffé post hoc test).

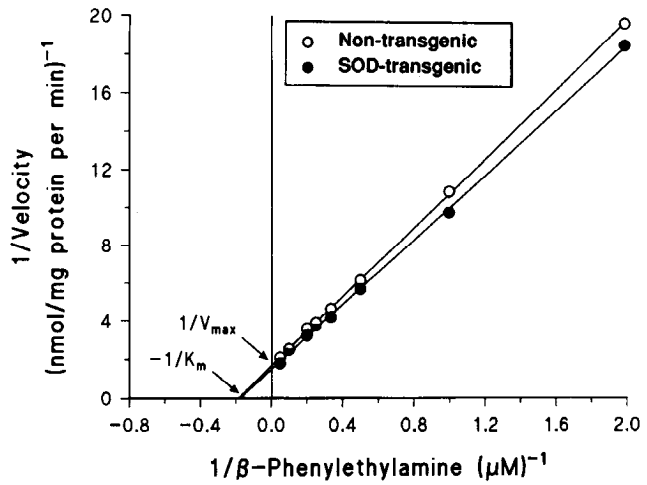


Figure 4. Striatal MAO-B activity in SOD-transgenic mice and their nontransgenic littermates. Lineweaver–Burk plot shows no significant differences in oxidation of β-phenylethylamine by striatal mitochondrial MAO-B enzyme preparation between SOD-transgenic mice and their nontransgenic littermates (V_{max}: SOD-transgenic, 0.69 ± 0.13, vs. nontransgenic, 0.62 ± 0.09 nmol/mg protein/min; K_m: SOD-transgenic, 5.78 ± 0.15, vs. nontransgenic, 5.57 ± 0.34 μM). Values represent the means ± SEM of three mice per group and were tested for difference by two-tailed Student's *t* test.

Brain formation of MPP⁺ and MAO-B activity

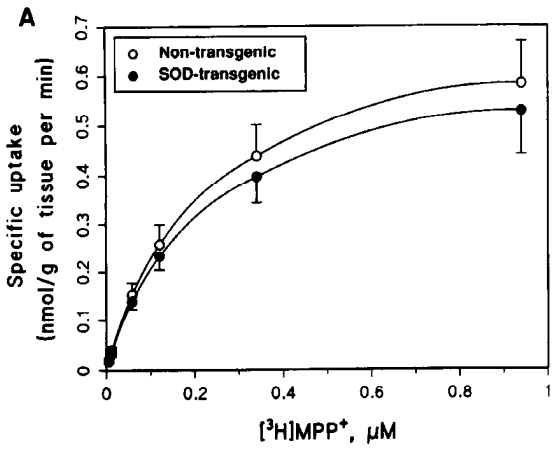
In order to determine if there were differences in the biotransformation of MPTP to MPP⁺ in brain between the SOD-transgenic mice and their nontransgenic littermates, we compared the levels of MPP⁺ formed after intraperitoneal injection of MPTP. No significant differences were observed in the levels of MPP⁺ produced in the brains of SOD-transgenic mice (2.65 ± 0.54 μg/gm tissue; $n = 3$) in comparison to their nontransgenic littermates (2.52 ± 0.32 μg/gm tissue; $n = 3$) using the same experimental protocol and time point described above for MPTP levels determination.

We also measured MAO-B activity in the brains of these two groups of animals. Again, the enzymatic assay showed no significant differences in striatal MAO-B activity between the two groups of mice (Fig. 4). Analysis of the data of the oxidation of β-phenylethylamine showed V_{max} values of 0.69 ± 0.13 nmol/mg protein/min and K_m values of 5.78 ± 0.15 μM in SOD-transgenic mice. In nontransgenic mice, the values were 0.62 ± 0.09 nmol/mg protein/min and 5.57 ± 0.34 μM for V_{max} and K_m, respectively.

Uptake of MPP⁺ by brain synaptosomes

We have previously shown similar binding characteristics (i.e., B_{max} and K_d) and distribution of ^{3}H-mazindol–labeled DA uptake sites in the brains of SOD-transgenic mice and their nontransgenic littermates (Cadet et al., 1990). Since impaired uptake of 5-HT in platelets of SOD-transgenic mice has been reported (Schickler et al., 1989), we tested the possibility that the uptake of MPP⁺ might be abnormal even in the absence of any alteration in brain ^{3}H-mazindol binding (Cadet et al., 1990) or ^{3}H-DA uptake (present study). This experiment showed that ^{3}H-MPP⁺ accumulation in striatal synaptosomes of SOD-transgenic mice and their nontransgenic littermates was saturable without any significant differences in uptake characteristics (V_{max}: SOD-transgenic, 0.72 ± 0.20, vs. nontransgenic, 0.65 ± 0.24 nmol/gm tissue/min; K_m: SOD-transgenic, 220.0 ± 42.0, vs. nontransgenic, 210.0 ± 40.0 nM) between the two groups (Fig. 5*A*).

In addition, the regional study of [^{3}H]-MPP⁺ uptake showed no significant differences in the amount of MPP⁺ accumulated in SOD-transgenic compared to nontransgenic mice (Fig. 5*B*). Maximal MPP⁺ uptake values were observed in the caudate–putamen complex (Fig. 5*B*). In contrast, substantia nigra (reticulata plus compacta) accumulated 1.7 times less MPP⁺ than caudate–putamen (Fig. 5*B*). Other brain regions examined accumulated between 4 and 11 times less MPP⁺ compared to the caudate–putamen (Fig. 5*B*). In a parallel experiment, we measured the uptake of ^{3}H-DA and found good correlations between the amount of DA and MPP⁺ taken up after 8 min by the different brain regions examined (data not shown). However, no specific uptake of ^{3}H-DA was measured in the cerebellum, contrasting with its rather high uptake of ^{3}H-MPP⁺ (see Fig. 5*B*). This latter observation is consistent with the recent study



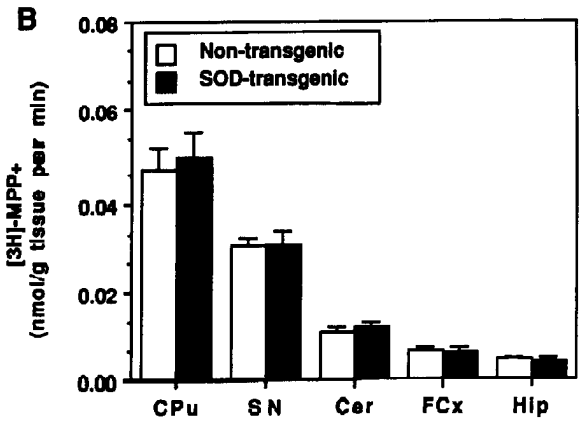


Figure 5. Striatal uptake of MPP^+ in SOD-transgenic mice and their nontransgenic littermates. *A*, Saturation studies did not show any significant differences in ^{3}H-MPP^+ uptake by striatal synaptosomes between SOD-transgenic mice and their nontransgenic littermates (V_{max}: SOD-transgenic, 0.72 ± 0.20, vs. nontransgenic, 0.65 ± 0.24 nmol/gm tissue/min; K_m: SOD-transgenic, 220 ± 42, vs. nontransgenic, 210 ± 40 nM). *B*, Brain regional studies of ^{3}H-MPP^+ uptake did not reveal any significant differences between the two groups of mice. Maximal values were found in the caudate–putamen (*CPu*), followed by the substantia nigra (*SN*) (reticulata plus compacta) and the cerebellum (*Cer*); the lowest values were measured in the frontal cerebral cortex (*FCx*) and the hippocampus (*Hip*). Values represent the means ± SEM of three or four mice per group and were tested for differences by two-tailed Student's *t* test or ANOVA followed by a Sheffé post hoc test.

of Marini et al. (1989), who provided evidence that cerebellar uptake of MPP^+ occurs mainly through glutamate uptake sites.

Inhibition of the mitochondrial respiratory chain by MPP^+

We tested the possibility that the activities of the different complexes of the mitochondrial electron transport chain might be different between SOD-transgenic mice and their nontransgenic littermates. We found no such differences (Table 1). We also tested the possibility that MPP^+ would be less effective in inhibiting complex I activity of SOD-transgenic mice. These experiments did not reveal any significant differences in the magnitude of complex I inhibition caused by different concentrations (1 μM to 10 mM) of MPP^+ used in mitochondrial preparations obtained from SOD-transgenic mice and their nontransgenic littermates (Fig. 6).

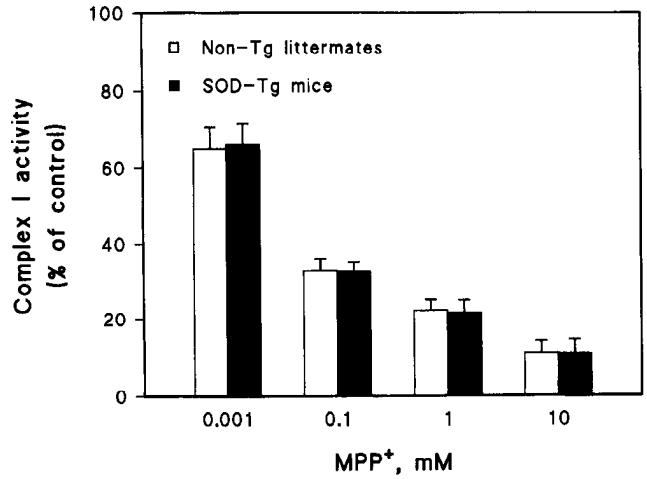


Figure 6. MPP^+ inhibition of complex I activity (NADH-Q reductase) in SOD-transgenic (*SOD-Tg*) mice and their nontransgenic (*Non-Tg*) littermates. No significant differences in the magnitudes of inhibition are observed between the two groups of mice for the different concentrations of MPP^+ used. Values represent the means ± SEM of three mice per group and are expressed as percentage of control (complex I activity in absence of MPP^+; see Table 1 for actual values). Values were tested for differences by two-tailed Student's *t* test.

Brain elimination of MPP^+

It has been proposed that the rate of elimination of MPP^+ from the brain might account for the degree of susceptibility to MPTP-induced toxicity among species (Markey et al., 1984; Irwin et al., 1989). Thus, we quantified regional brain MPP^+ concentrations at different time points in SOD-transgenic mice and their nontransgenic littermates.

For all the time points examined, MPTP and $MPDP^+$ levels were below detectable limits; this is in accordance with previous observations (Giovanni et al., 1991). In contrast, MPP^+ levels were easily detected in all brain regions studied in both SOD-transgenic mice and their nontransgenic littermates. Over the 10 hr experiment, there were no significant differences in MPP^+ content between SOD-transgenic mice and their nontransgenic littermates in either the caudate–putamen, the ventral mesencephalon, or the frontal cerebral cortex (Table 2). In addition, regression analyses revealed that the data from these studies fit

Table 1. Enzymatic activities of the mitochondrial electron chain transport in SOD-transgenic mice and their nontransgenic littermates

	Non-transgenic	SOD-transgenic
Citrate synthase	1.20 ± 0.24	1.49 ± 0.22
Succinate dehydrogenase	0.11 ± 0.01	0.11 ± 0.01
NADH-Q reductase (complex I)	0.42 ± 0.06	0.43 ± 0.06
NADH-cytochrome C reductase (complex I–III)	1.23 ± 0.09	1.13 ± 0.12
Succinate-cytochrome C reductase (complex II–III)	0.39 ± 0.02	0.37 ± 0.03
Cytochrome C oxidase (complex IV)	3.18 ± 0.13	3.20 ± 0.31

Values are nmol/mg protein/min and represent the means ± SEM of three mice per group. Data were tested for differences by two-tailed Student's *t* test.

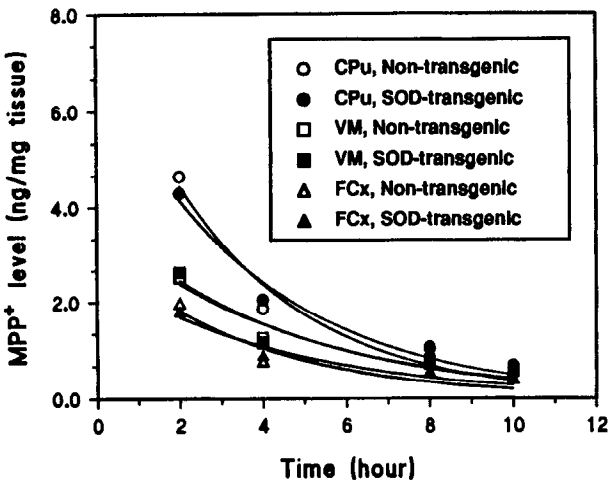


Figure 7. Kinetics of MPP^+ elimination of caudate–putamen (*CPu*), ventral mesencephalon (*VM*), and frontal cortex (*FCx*) of SOD-transgenic mice and their nontransgenic littermates. No significant differences were found between the two groups of animals for any of the brain regions examined. However, caudate–putamen has significantly lower ($p < 0.001$) half-life than ventral mesencephalon and frontal cortex.

an exponential function in all three brain regions (Fig. 7). MPP^+ half-life values obtained from the caudate-putamen (SOD-transgenic, 2.60 ± 0.04 hr; nontransgenic, 2.30 ± 0.06 hr) were significantly lower than those obtained from ventral mesencephalon (SOD-transgenic, 3.10 ± 0.12 hr; nontransgenic, 3.00 ± 0.09 hr) and frontal cortex (SOD-transgenic, 3.00 ± 0.09 hr, nontransgenic, 2.90 ± 0.07 hr).

Discussion

Our results indicate that the dopaminergic nigrostriatal pathway of SOD-transgenic mice, with 3.21 times higher striatal Cu/Zn-SOD activity, is resistant to MPTP-induced toxicity. In contrast, their nontransgenic littermates, which have Cu/Zn-SOD activity within the normal range (Epstein et al., 1987), show biochemical and morphological indices consistent with injury to the dopaminergic system after similar MPTP administration. The possible role of O_2^- radicals in MPTP-induced toxicity and the manner by which increased SOD activity might have attenuated the effects of this toxin are here discussed.

After systemic administration, MPTP rapidly enters the CNS (Markey et al., 1984; Langston et al., 1984b), where it is converted to MPP^+ by the enzyme MAO-B (Heikkila et al., 1984b; Markey et al., 1984). MPP^+ is thought to be the active neurotoxin since MAO-B inhibitors prevent MPTP-induced toxicity in the brain (Heikkila et al., 1984b). Subsequent to MPP^+ production in the brain, it accumulates in dopaminergic neurons through the specific reuptake mechanism for DA (Javitch et al., 1985b). The specificity of MPP^+ for the DA uptake sites was demonstrated by the finding that DA uptake blockers provide protection against MPTP-induced toxicity (Javitch et al., 1985b). Once inside the dopaminergic neurons, MPP^+ is concentrated within mitochondria (Ramsey and Singer, 1986), where it inhibits complex I of the electron transport chain (Vays et al., 1986; Mizuno et al., 1987; Nicklas et al., 1987). Alteration at any of these steps significantly attenuates MPTP-induced toxicity (see Kopin, 1987, for review). Increased SOD activity in transfected cells causes significant changes in the characteristics of the cell membrane as a consequence of increased lipid peroxidation (Elroy-Stein et al., 1986). Because MPTP is a very lipophilic compound, changes in the properties of the cell membrane of transgenic mice could have led to disturbances in its entry into the CNS (Langston et al., 1984b; Markey et al., 1984) or into astrocytes and serotonergic neurons (Levitt et al., 1982; Westlund et al., 1985) on which it depends for its metabolism (Kopin, 1987). There were, however, no differences in the brain levels of MPTP or MPP^+ after intraperitoneal administration of MPTP to SOD-transgenic mice and their nontransgenic littermates. There were also no changes in striatal MAO-B activity or impaired MPP^+ uptake into brain synaptosomes between the two groups of animals. As mentioned above, inhibition of MAO-B and blocking of MPP^+ uptake provide protection against MPTP-induced neurotoxicity (Heikkila et al., 1984b; Javitch et al., 1985a). Moreover, complex I of the electron transport chain of both SOD-transgenic mice and their nontransgenic littermates exhibited the same sensitivity to inhibition by MPP^+. Finally, we found no evidence for a different rate of brain elimination of MPP^+ between SOD-transgenic mice and nontransgenic littermates. Therefore, when taken together, these experiments, which assessed the different steps in the neurotoxic pathway of MPTP, indicate that SOD-transgenic mice metabolize MPTP in the same fashion as their littermates. The binding of MPP^+ to neuromelanin (D'Amato et al., 1986) has been related to the vulnerability of primate nigrostriatal neurons to MPTP (Burns et al., 1983). Thus, a lower amount of neuromelanin in SOD-transgenic mice nigrostriatal neurons might be relevant to their resistance to MPTP. However, to the best of our knowledge, neuromelanin has never been described in mouse nigrostriatal neurons (Marsden, 1983), even under electron microscopy (Reinhard and Gerhard, 1983). Thus, it is unlikely that the neuromelanin hypothesis might account for the differ-

Table 2. Brain levels of MPP^+ in SOD-transgenic mice and their nontransgenic littermates

Time	Nontransgenic			SOD-transgenic		
(hr)	CPu	VM	FCx	CPu	VM	FCx
2	4.65 ± 0.04	2.52 ± 0.05	1.94 ± 0.02	4.30 ± 0.11	2.63 ± 0.09	1.78 ± 0.02
4	1.86 ± 0.09	1.24 ± 0.02	0.73 ± 0.04	2.05 ± 0.05	1.15 ± 0.03	0.86 ± 0.08
8	1.05 ± 0.06	0.72 ± 0.03	0.50 ± 0.01	1.02 ± 0.02	0.79 ± 0.02	0.52 ± 0.02
10	0.66 ± 0.05	0.57 ± 0.02	0.37 ± 0.02	0.68 ± 0.05	0.57 ± 0.02	0.37 ± 0.02

CPu, caudate–putamen complex; VM, ventral mesencephalon; FCx, frontal cerebral cortex. Values are ng/mg tissue and represent means ± SEM of four mice per group at each time point. Data were tested for differences by two-way ANOVA followed by Scheffé post hoc test. There were no significant differences between SOD-transgenic and nontransgenic animals in any of the three brain regions examined at all time points. In contrast, concentrations of MPP^+ were significantly different between the three brain regions ($p < 0.01$, ANOVA) at every time point in both groups of mice.

ence in MPTP sensitivity found between SOD-transgenic and nontransgenic mice.

In relation to the mechanism of MPTP-induced toxicity, we have thus far found only one significant difference between the two groups of mice, namely, the level of Cu/Zn-SOD. Therefore, the most parsimonious explanation is to assume that the high Cu/Zn-SOD activity measured in the SOD-transgenic mice is, most likely, the factor involved in the resistance of these animals to MPTP-induced neurotoxicity. This interpretation is consistent with the recent demonstration that mouse clonal cell lines that were transfected with the human Cu/Zn-SOD gene and that expressed 3.6 times higher Cu/Zn-SOD activity were resistant to the toxic effects of paraquat (Elroy-Stein et al., 1986). The herbicide paraquat increases the production of O_2^- radicals by a process involving redox cycling (Hassan and Fridovich, 1979). Structural similarity between MPP^+ and paraquat had earlier raised the possibility that MPTP-induced toxicity might be due to a similar process (Di Monte et al., 1986b, and reviewed in Langston and Irwin, 1986). In addition, like paraquat, when administered systematically, MPP^+ causes severe toxic damage in the lung and increases plasma levels of glutathione disulfide, an indication of oxidative stress (Johannessen et al., 1986). However, neither MPTP nor MPP^+ undergoes redox cycling (Sayre et al., 1986). On the other hand, because MPP^+ impairs mitochondrial respiration by inhibiting complex I of the electron transport chain (Vays et al., 1986; Mizuno et al., 1987; Nicklas et al., 1987), it was concluded that MPP^+ must induce nigrostriatal cell death by depleting the cellular levels of ATP through its inhibition of complex I. This suggestion is supported by the observations that MPP^+ does produce rapid ATP depletion in isolated hepatocytes (Di Monte et al., 1986a; Sayre et al., 1986) and in mouse brain synaptosomes (Schotcher et al., 1990). These findings do not, however, indicate that the depletion of ATP is the final pathway or the sole manner via which MPP^+ kills cells. Indeed, inhibition of complex I by MPP^+ has also been shown to be associated with the production of O_2^- radicals *in vitro* (Hasegawa et al., 1990). In addition, rotenone, a potent inhibitor of the mitochondrial respiration that blocks complex I at the same site as MPP^+ does (Ramsey et al., 1987), also increases the production of O_2^- radicals (Takeshige and Minakami, 1979) and causes similar striatal DA depletion to MPP^+ when injected stereotaxically into the median forebrain bundle in rat (Heikkila et al., 1985). Thus, O_2^- radicals generated in this fashion can be cytotoxic by themselves and/or participate with hydrogen peroxide (H_2O_2) in the iron-catalyzed Haber–Weiss reaction to yield the highly reactive hydroxyl radical ($\cdot OH$), which is known to cause lipid peroxidation, enzyme denaturation, damage to cell membrane, and subsequent cell death (Freeman and Crapo, 1982). Therefore, the lack of significant MPTP-induced neurotoxicity observed in SOD-transgenic mice suggests that the high level of SOD present in these animals may have dismutated any excess of O_2^- radicals generated in the nigrostriatal dopaminergic pathway as a consequence of MPTP administration. However, as for the correlation between human Cu/Zn-SOD overexpression in transfected mouse cells and their resistance to paraquat (Elroy-Stein et al., 1986), it should be emphasized that although SOD-transgenic mice HS/SF-218 were protected against a total dose of 90 mg/kg MPTP, higher MPTP dosages could exceed, in all likelihood, the protection provided by a 3.21-fold increase in Cu/Zn-SOD activity, resulting in a certain degree of toxicity in the dopaminergic pathway of these animals.

The present findings with MPTP may possibly be of relevance to the understanding of the pathogenesis of Parkinson disease. For instance, abnormalities in mitochondrial complex I in the substantia nigra of Parkinsonian patients have been recently identified (Mizuno et al., 1989; Schapira et al., 1990). Other observers have reported changes in lipid peroxidation (Dexter et al., 1989) and iron content (Dexter et al., 1987) and abnormalities in free radical scavenging systems (Ambani et al., 1975; Kish et al., 1986; Marttila et al., 1988; Saggu et al., 1989) in these patients. Although the primary or secondary nature of these changes is not clear, it is possible to draw some mechanistic comparisons between Parkinson's disease and the MPTP model. For example, if abnormalities of nigral complex I are primary, these changes could lead to an excess production of oxygen-based radicals and associated nigral cell death. Alternatively, if the production of endogenous oxygen radicals is primary, then these substances could also affect the mitochondrial genomes (Richter et al., 1988; Bandy and Davison, 1990) with subsequent production of deletionlike abnormalities in subpopulations of nigral mitochondria. This idea is consistent with recent demonstrations of gene deletion in the basal ganglia of some Parkinsonian patients (Ikebe et al., 1990). It is of interest that common deletions found in some neurodegenerative diseases often encompass sequences that code for various subunits of mitochondrial complex I (Schon et al., 1989; Nakase et al., 1990). In any case, these two scenarios would result in a deleterious cascade of events against which the brain scavenging system and the mitochondrial poor genetic repair mechanisms (Richter et al., 1988; Bandy and Davison, 1990) cannot protect.

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