0026-895X/00/020271-08$3.00/0
MOLECULAR PHARMACOLOGY

MOL 58:271–278, 2000 /12990/842189

The Selective Toxicity of 1-Methyl-4-phenylpyridinium to Dopaminergic Neurons: The Role of Mitochondrial Complex I and Reactive Oxygen Species Revisited

KEN NAKAMURA, VYTAUTAS P. BINDOKAS, JEREMY D. MARKS, DAVID A. WRIGHT, DAVID M. FRIM, RICHARD J. MILLER, and UN JUNG KANG

Committee on Neurobiology (K.N., R.J.M., U.J.K.), Departments of Neurology (D.A.W., U.J.K.), Neurobiology, Pharmacology & Physiology (V.P.B., R.J.M., U.J.K.), Pediatrics (J.D.M., D.M.F.), and Surgery (D.M.F.), The University of Chicago, Chicago, Illinois

Received September 21, 1999; accepted May 4, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

1-Methyl-4-phenylpyridinium (MPP^+) is selectively toxic to dopaminergic neurons and has been studied extensively as an etiologic model of Parkinson's disease (PD) because mitochondrial dysfunction is implicated in both MPP^+ toxicity and the pathogenesis of PD. MPP^+ can inhibit mitochondrial complex I activity, and its toxicity has been attributed to the subsequent mitochondrial depolarization and generation of reactive oxygen species. However, MPP^+ toxicity has also been noted to be greater than predicted by its effect on complex I inhibition or reactive oxygen species generation. Therefore, we examined the effects of MPP^+ on survival, mitochondrial membrane potential ($\Delta\Psi m$), and superoxide and reduced glutathione levels in individual dopaminergic and nondopaminergic mesencephalic neurons. MPP^+ (5 μM) selectively induced death in fetal rat dopaminergic neurons and caused a small decrease in their $\Delta\Psi m$. In contrast, the specific complex I inhibitor rotenone, at a dose (20 nM) that was less toxic than MPP^+ to dopaminergic neurons, depolarized $\Delta\Psi m$ to a greater extent than MPP^+. In addition, neither rotenone nor MPP^+ increased superoxide in dopaminergic neurons, and MPP^+ failed to alter levels of reduced glutathione. Therefore, we conclude that increased superoxide and loss of $\Delta\Psi m$ may not represent primary events in MPP^+ toxicity, and complex I inhibition alone is not sufficient to explain the selective toxicity of MPP^+ to dopaminergic neurons. Clarifying the effects of MPP^+ on energy metabolism may provide insight into the mechanism of dopaminergic neuronal degeneration in PD.

A combination of mitochondrial dysfunction and increased oxidative stress is hypothesized to contribute to the selective degeneration of nigrostriatal dopaminergic neurons in Parkinson's disease (PD; Jenner and Olanow, 1996). The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is selectively toxic to the nigrostriatal dopaminergic system and has been studied extensively as an etiologic model for PD. MPTP is oxidized by monoamine oxidase B to 1-methyl-4-phenylpyridinium (MPP^+; Langston et al., 1984), which is imported into dopaminergic neurons via the dopamine transporter (Javitch et al., 1985). MPP^+ accumulates in mitochondria (Ramsay and Singer, 1986), and high concentrations of MPP^+ partially inhibit mitochondrial complex I activity, resulting in mitochondrial depolarization and decreased levels of ATP and glutathione in various nondopaminergic cell types (Nicklas et al., 1985; Di Monte et al., 1987; Mizuno et al., 1987a). High concentrations of MPP^+ also increase superoxide levels in isolated mitochondria (Hasegawa et al., 1990). Superoxide has been hypothesized to interact with nitric oxide (NO) to produce peroxynitrate and cell death (Przedborski et al., 1996). Superoxide dismutase (SOD) converts superoxide to H_2O_2, which is then metabolized to H_2O by glutathione peroxidase, or catalase. MPP^+ toxicity in vivo is reduced after overexpression of the cytosolic copper/zinc form of SOD (Cu/Zn-SOD; Przedborski et al., 1992). In addition, a significant role for the glutathione system in detoxifying MPP^+-induced oxidative stress is suggested by the findings that glutathione depletion synergistically increases

This research was supported by the Brain Research Foundation, Louis R. Block Fund, National Parkinson Foundation, Parkinson Disease Foundation, United Parkinson Foundation, and U.S. Public Health Service Grant NS07113.

ABBREVIATIONS: PD, Parkinson's disease; MPP^+, 1-methyl-4-phenylpyridinium; $\Delta\Psi m$, mitochondrial membrane potential; GSH, reduced glutathione; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NO, nitric oxide; SOD, superoxide dismutase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; NFP, neurofilament protein 200; TH, tyrosine hydroxylase; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; HEt, hydroethidine; Et, ethidium; MCB, monochlorobimane; BSO, L-buthionine-[*S*,*R*]sulfoximine; FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; DIC, differential interference contrast; ROS, reactive oxygen species; Cu/Zn-SOD, copper/zinc form of SOD.

MPP^+ toxicity both in vitro (Nakamura et al., 1997) and in vivo (Wullner et al., 1996).

On the other hand, other investigators have noted that MPP^+ toxicity may not be primarily due to complex I inhibition or reactive oxygen species (ROS) generation (Bates et al., 1994; Espino et al., 1994). MPP^+ typically produces 10^4- to 10^6-fold less complex I inhibition than rotenone (Mizuno et al., 1987b; Hasegawa et al., 1990; Ramsay et al., 1991), a specific and potent inhibitor of complex I (Hartley et al., 1994). The toxicity of MPP^+ and its effect on energy depletion are also out of proportion to its effect on complex I inhibition (Bates et al., 1994; Espino et al., 1994). The lack of significant protection by antioxidants or specific NO synthase inhibitors (Sanchez-Ramos et al., 1988, 1997; Choi et al., 1999; Di Monte et al., 1999; Lotharius et al., 1999; Royland et al., 1999) also casts doubt on the significance of oxidative stress as an instigator of MPP^+-induced cell death.

In this study, we sought direct evidence for the effects of MPP^+ on mitochondrial function and ROS production in individual living mesencephalic neurons. Changes in mitochondrial membrane potential ($\Delta\Psi m$) and superoxide and reduced glutathione levels (GSH) were examined in dopaminergic and nondopaminergic neurons identified by subsequent immunohistochemistry. We also compared the effects of MPP^+ with those of rotenone to determine whether the inhibition of mitochondrial complex I is sufficient to explain the selective dopaminergic neurotoxicity of MPP^+.

Experimental Procedures

Materials. Timed pregnant Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Madison, WI). Dulbecco's modified Eagle's medium (DMEM) and Hanks' balanced salt solution (HBSS) were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum was obtained from Intergen (Purchase, NY). Trypsin was purchased from Worthington Biochemical Corporation (Freehold, NJ). Dimethyl sulfoxide (DMSO) and Hemo-De were obtained from Fisher (Itasca, IL). MPP^+ iodide was obtained from Research Biochemical Internationals (Natick, MA). Glass coverslips were purchased from Carolina Biological (Burlington, NC). DNase, L-buthionine-[*S*,*R*]sulfoximine (BSO), benzidine dihydrochloride, 3,3′-diaminobenzidine tetrahydrochloride (DAB), 5-fluoro-2-deoxyuridine, Triton X-100, sodium nitroprusside dihydrate, rotenone, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), BSA, and mouse monoclonal antibody against neurofilament protein 200 (NFP) were purchased from Sigma Chemical Co. (St. Louis, MO). Monochlorobimane (MCB), hydroethidine (HEt), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), and ProLong Antifade Kit were obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal antibody against tyrosine hydroxylase (TH) was obtained from Pel-Freez (Rogers, AR). Mouse monoclonal antibody against neuron-specific nuclear protein was obtained from Chemicon (Temecula, CA). Cy2-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Biotinylated anti-rabbit IgG and Vectastain ABC kit were obtained from Vector Laboratories (Burlingame, CA).

Cell Cultures. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago and were conducted in accordance with National Institutes of Health guidelines for care and use of experimental animals. Rodents were maintained in standard housing. Pieces of ventral mesencephalon, dissected from embryonic gestation day 14 rat embryos, were incubated for 20 min at 37°C in 0.4% trypsin in calcium- and magnesium-free HBSS and triturated 8 to 10 times in 0.015% DNase using flame-polished Pasteur pipettes. Cell number and viability were determined with trypan blue staining using a hemocytometer. Cells were plated onto poly(L-lysine)-coated glass coverslips at a density of 200,000 viable cells/cm^2. Cells were grown in high glucose DMEM supplemented with 10% fetal bovine serum. After 48 h, medium was changed to DMEM containing 10% fetal bovine serum and 10 μg/ml 5-fluoro-2-deoxyuridine to inhibit glial growth, and cells were grown an additional 4 to 6 days before study. Glia represented less than 15% of the total cells in the culture, based on the number of cells that lacked immunoreactivity to NFP or neuron-specific nuclear protein antibodies.

Toxicity Studies. Cells were exposed to experimental compounds (added as 3× stocks) for 24 or 48 h, at which time cell survival was quantified. Rotenone and staurosporine were prepared in DMSO, before dilution in medium. The final concentration of DMSO was 28 μM, and this concentration of DMSO did not affect any of the parameters examined (data not shown). In experiments examining the time course of dopaminergic cell death, cells were incubated in the presence or absence of MPP^+ (5 μM) for 24 h, at which time media were changed and cells were treated for an additional 24 h with or without MPP^+. To control for conditioning of the media by the cells and degradation of MPP^+ during the incubation, media used for the second 24-h incubation period were taken from parallel cultures that had already been incubated with the media for 24 h.

Immunohistochemistry. TH immunoreactivity was used as an unambiguous way of identifying dopaminergic neurons, by use of post hoc matching of fluorescent microscopic images (see later) with immunohistochemical data. Cells were fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 15 min. Endogenous peroxidase activity was removed by exposing cells to 0.6% H_2O_2 in 0.1 M PBS for 15 min. Cells were permeabilized, and nonspecific antibody binding was blocked by incubation in PBS containing 1% BSA and 0.2% Triton X-100 for 30 min. Cells were then incubated at room temperature overnight in either primary polyclonal anti-TH antiserum (1:1000) or primary monoclonal anti-NFP antibody (1:200). Cultures were next incubated for 1 h with the appropriate biotinylated secondary antibody (1:200). This was followed by a 1-h incubation with an avidin-biotin conjugate of peroxidase (Vectastain ABC kit). Benzidine dihydrochloride or DAB was used as chromogens for immunoperoxidase staining. Coverslips were dehydrated with graded ethanol washes followed by Hemo-De.

Quantification of Toxin-Induced Death. After treatment with MPP^+, rotenone, or control media, the number of surviving dopaminergic and nondopaminergic neurons were counted based on morphologic criteria using a protocol similar to that described previously (Nakamura et al., 1997). Briefly, a grid containing 12 squares was placed under the coverslip, and surviving cells were counted from a defined field in the center of each of the 12 squares. Cells were defined as surviving if they had intact cell bodies and neurites. Cells exhibiting fragmented or atrophic cell bodies, or loss or disruption of neurites, were not counted. The number of surviving neurons in each field was recorded in both experimental and control groups. Survival in a particular condition was calculated by dividing the number of surviving cells in each field by the mean of the control group, expressed as a percent. We sampled approximately 1% of the total coverslip area when counting NFP-immunoreactive, TH-negative nondopaminergic neurons. Because dopaminergic neurons make up approximately 5 to 10% of total neurons in the culture, we sampled approximately 10% of the total coverslip area when counting TH-immunoreactive dopaminergic neurons. All counting was blind to the incubation condition and made at a magnification of 400×.

Measurement of $\Delta\Psi m$. Changes in $\Delta\Psi m$ were estimated using the dual emission dye JC-1 (Salvioli et al., 1997). As a monomer, JC-1 emits green light, but the emission changes to red when JC-1 aggregates form as JC-1 concentrates within the mitochondria. JC-1 stock (5 mM) in DMSO was diluted in media to a final concentration of 10 μM, sonicated for 30 s, and filtered through a 0.2-μm filter into a glass container. Cells were loaded for 60 min in JC-1 at 37°C in the dark and then rinsed before being placed in a chamber on the stage

of an inverted microscope at room temperature. Within a given field, regions of interest were drawn over all cells with intact somata, regardless of their neurite morphology. Neurons growing on top of glia were excluded from physiology measurements. Neurons were alternately subjected to Xe epifluorescence illumination (attenuated with a 2.0 neutral density filter) for fluorescence imaging and halogen transmitted illumination for differential interference contrast (DIC) imaging. Images were formed with a 40×, 1.25 NA objective and collected with a cooled CCD camera (Photometrics, Tucson, AZ). Neurons were excited with 488-nm light, and the emitted fluorescence was sequentially imaged at 530 and 620 nm using a triple-bandpass polychroic mirror and single-wavelength emission filters (83000 series; Chroma Technology, Brattleboro, VT) and a computer-controlled filter wheel located in front of the camera. The DIC analyzer was located in the emission filter wheel. Images were acquired using Metafluor software (Universal Imaging Corp., West Chester, PA) and stored in digital format on a random access device. Within each region of interest, ratios of fluorescence from aggregates to that from monomers (JC-1 ratio) were calculated on a pixel-by-pixel basis, and the average of the JC-1 ratios over the entire region was used as an estimate of $\Delta\Psi m$ for the neuron.

Measurement of Superoxide. The rate of increase in ethidium (Et) fluorescence when HEt is oxidized provides a relatively specific measure of superoxide (Bindokas et al., 1996). Fluorescence measurements were made with a Nikon Diaphot epifluorescence microscope with illumination from a 150-W Xe arc (attenuated by a neutral density 1.5, ultraviolet-grade filter; Omega Optical, Brattleboro, VT). Et imaging used standard rhodamine optics (excitation 510–560 nm; dichroic mirror 580 nm; emission >590 nm; Nikon, Melville, NY). Images were formed using a 40× Fluor NA 0.85 objective (Nikon) and collected on a Hamamatsu ICCD (sensitivity set at 7.0). Images were 8-bit (256 intensity levels), and 16 frames were averaged every 10 s. Background subtraction was made using the first image obtained when HEt solution was added and, in addition, from a cell-free region of the field to track subsequent changes in background. Data acquisition was controlled by MetaFluor or MetaMorph software (Universal Imaging Corp.), and average intensity over identified regions of interest was logged to hard disk and displayed in real time. The relative superoxide level for each cell was determined from the slope of the increase in Et fluorescence over time, fit by linear regression. HEt was freshly prepared and used at a final concentration of 3.2 μM.

Measurement of GSH. Levels of GSH were determined using MCB fluorescence. GSH is specifically conjugated with MCB to form a fluorescent bimane-GSH adduct, in a reaction catalyzed by glutathione *S*-transferases (Shrieve et al., 1988). This conjugation reaction proceeds according to a first-order kinetic reaction and is followed by a second kinetic process that is many orders of magnitude slower and is thought to reflect the nonenzymatic reaction of MCB with other intracellular thiols (Shrieve et al., 1988; Young et al., 1994). As has been described previously (Young et al., 1994), the concentration of the bimane-GSH adduct increased during the initial 10 to 12 min period with first order kinetics, before leveling off. By 15 min, BSO treatments no longer affected the slope of increase in fluorescence (data not shown), confirming that the first order kinetic phase was finished. Therefore, the fluorescence at 15 min was used as an indication of the intracellular GSH content, as has been described previously in similar paradigms (Shrieve et al., 1988). Imaging parameters with MCB were the same as described for Et above, except for the filters used (excitation <400 nm; dichroic mirror 410–430 nm; emission 440–500 nm). MCB stock (100 mM) was made in DMSO and diluted to 100 μM before imaging studies. Neurons growing on top of glia were excluded, because the high fluorescence of GSH in glia overwhelmed the neuronal signal.

Post Hoc Identification of Dopaminergic Neurons. After each study, the location of the field of cells from which fluorescence measurements were made was marked. Coverslips were then processed for TH immunohistochemistry, and the original field was relocated. Bright field images of the TH staining were obtained with a cooled CCD camera and displayed on a computer monitor overlaid with the original DIC images from that same field. The orientation and location of stained cells were brought into register with the DIC images, then neurons were identified as dopaminergic or nondopaminergic based on immunostaining.

Statistical Analysis. Statistical analyses were performed using the GB-STAT statistical package. Standard errors were calculated for each mean, and statistical differences between groups were determined by ANOVA followed by Newman-Keuls post hoc tests. One-way randomized ANOVA was used for cell counts and imaging studies performed 24 h after treatment, whereas one-way ANOVA with repeated measures was used for superoxide studies examining acute drug effects.

Results

MPP+ Is Selectively Toxic to Dopaminergic Neurons and Rotenone Is Not. We first established a paradigm in which MPP+ is selectively toxic to dopaminergic mesencephalic neurons by examining the toxicity of MPP+ at doses of 1, 5, and 10 μM. Treatment with 5 μM MPP+ for 48 h resulted in the death of more than 60% of dopaminergic neurons without significantly affecting the number of nondopaminergic mesencephalic neurons (Fig. 1). Thus, this dose of MPP+ was selectively toxic to dopaminergic neurons. We next compared the toxicity of MPP+ with that induced by rotenone, a specific and potent complex 1 inhibitor. We tested rotenone at doses of 10, 15, 20, 30, 50, and 100 nM to find a dose that is comparably toxic to dopaminergic neurons. Treatment with rotenone (20 nM) for 48 h resulted in the nonselective loss of approximately 40% of dopaminergic and nondopaminergic neurons (Fig. 1).

Time Course of Cell Death Induced by MPP+. To establish a time point for subsequent imaging studies when active processes leading to MPP+-induced cell death were present, we examined whether there were dopaminergic neurons that were still living at 24 h but were already committed to die by 48 h. We incubated cultures in the presence or absence of MPP+ (5 μM) for two consecutive 24-h periods

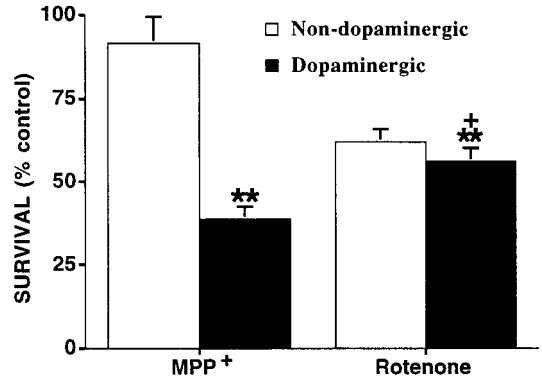

Fig. 1. Differential toxicity of MPP+ and rotenone to dopaminergic and nondopaminergic mesencephalic neurons. Ventral mesencephalic neurons were incubated for 48 h with either 5 μM MPP+ or 20 nM rotenone, and the number of surviving neurons was counted from TH and NFP double-immunostained cultures. TH-immunoreactive (dopaminergic) neurons are represented by the dark pattern, and TH-negative, NFP-immunoreactive (nondopaminergic) neurons are represented by the light pattern. The number of surviving neurons is expressed as a percentage of untreated dopaminergic or nondopaminergic control groups. Data show mean ± S.E. (error bars). $^{**}P < 0.01$ versus respective controls. $^{+}P < 0.05$ versus dopaminergic neurons treated with MPP+ (n = 8–13 separate cultures per group with 12 fields counted in each culture).

(Table 1). All three groups incurred significantly more cell death than treatment with control media during both 24-h periods. Treatment with MPP^+ for both 24-h periods (MPP^+/MPP^+) induced significantly more toxicity than incubation with MPP^+ during only one of the 24-h periods (MPP^+/control or control/MPP^+). In addition, toxicity after treatment with MPP^+/control was not significantly different from control/MPP^+. Thus, we infer that most cells surviving the initial 24 h of MPP^+ treatment continue to survive for an additional 24 h if MPP^+ is removed from the media. These data suggest that MPP^+-mediated toxicity is an active process that depends on the continuous presence of MPP^+ and indicate that such active processes are likely to be present 24 h after incubation with MPP^+. Therefore, most of the following physiologic studies comparing the effects of MPP^+ on $\Delta\Psi m$ and ROS in individual dopaminergic versus nondopaminergic neurons were performed 24 h after treatment. In addition, to include cells that are in the process of cell death, all cells with intact somata were used for imaging studies regardless of their neurite morphology.

MPP^+ Has Minimal Effect on $\Delta\Psi m$, Whereas Rotenone Significantly Decreases $\Delta\Psi m$. We next examined whether MPP^+ mediates its selective toxicity to dopaminergic neurons by decreasing $\Delta\Psi m$. We compared the effect of MPP^+ on $\Delta\Psi m$ with that of 20 nM rotenone, a dose that induced significantly less toxicity to dopaminergic neurons than 5 μM MPP^+. We hypothesized that if MPP^+ exerted its toxicity primarily by inhibiting complex I, then MPP^+ would depolarize $\Delta\Psi m$ to a greater extent than rotenone. To estimate $\Delta\Psi m$, we used JC-1 fluorescence, which is sensitive to changes in $\Delta\Psi m$ over a physiologic range (Reers et al., 1995). In addition, JC-1 resists quenching, and its ratiometric properties minimize artifacts encountered using single-wavelength dyes. The mean JC-1 ratios were lower in control dopaminergic neurons than in control nondopaminergic neurons by 13% (Fig. 2, A versus D). The JC-1 ratios in each treatment group were normalized to the ratios in the untreated control group to appreciate the relative effects of treatment on $\Delta\Psi m$ in each cell type (Fig. 2J). After 24 h of MPP^+ exposure, the JC-1 ratio in dopaminergic neurons decreased by 12% but did not change significantly in nondopaminergic neurons. In contrast, rotenone significantly decreased the JC-1 ratio in both dopaminergic and nondopaminergic neurons by 26 and 25%, respectively. As a control, 1 μM FCCP was used to depolarize the mitochondria and decreased the JC-1 ratios by 52% in dopaminergic and 58% in nondopaminergic neurons. These data suggest that although 5 μM MPP^+ is more toxic to dopaminergic neurons than 20 nM rotenone, it has less effect on complex I activity.

MPP^+ Does Not Increase Superoxide Levels Selectively in Dopaminergic Neurons. To determine whether MPP^+ increased superoxide, we used real-time Et fluorescence measurements in living dopaminergic and nondopaminergic neurons (Bindokas et al., 1996). HEt has been shown to be oxidized rapidly to Et by reacting with superoxide but not with O_2, H_2O_2, HOCl, or peroxynitrite (Bindokas et al., 1996). Incubation with MPP^+ (5 μM) had no effect on superoxide in either dopaminergic or nondopaminergic neurons when Et fluorescence was monitored acutely 3 to 6 min and 4 h after the addition of MPP^+ (Fig. 3A). After 24 h, a slight increase in superoxide was noted in nondopaminergic neurons (30%), which was a significantly different effect from that on dopaminergic neurons. We also compared the effects of MPP^+ on superoxide with those of rotenone, a potent complex I inhibitor. Rotenone (20 nM) increased superoxide in nondopaminergic neurons but did not affect superoxide in dopaminergic neurons acutely (Fig. 3B). After 24 h, no persistent effect of rotenone on the superoxide level were noted in either dopaminergic or nondopaminergic neurons. Therefore, at low doses that are still sufficient to kill dopaminergic neurons, neither MPP^+ nor rotenone increased superoxide in dopaminergic neurons.

To compare these data with previous literature showing that high doses of MPP^+ increase superoxide in isolated mitochondria, we also examined the effects of 100 μM MPP^+. This dose of MPP^+ roughly doubled superoxide in both nondopaminergic and dopaminergic neurons (Fig. 3A).

MPP^+ Has No Detectable Effect on GSH. To provide additional evidence that MPP^+ selectively kills dopaminergic neurons independent of oxidative stress, we examined the effects of MPP^+ on GSH levels. MPP^+-induced mitochondrial dysfunction can also inhibit GSH synthesis by decreasing ATP levels (Di Monte et al., 1987; Mithofer et al., 1992). Nevertheless, MPP^+ (5 μM) did not change GSH levels in either dopaminergic or nondopaminergic neurons after 24 h (Fig. 4), as assessed from MCB fluorescence. Although MPP^+ may selectively deplete mitochondrial GSH, the mitochondrial pool is usually maintained at the expense of cytosolic levels (Meredith and Reed, 1982). Therefore, a significant loss of GSH from the mitochondria should lead to decreased cytosolic levels and would be reflected in the total cellular levels that we measured. BSO, a specific inhibitor of the rate-limiting enzyme in glutathione synthesis, γ-glutamylcysteine synthetase, was used as a positive control for decreased GSH levels. BSO (10 μM) decreased GSH levels in dopaminergic and nondopaminergic neurons to 54 and 30% of control values, respectively by 24 h. Glia were noted to have comparatively higher levels of GSH than neurons (data not shown), which is consistent with previous observations (Rice and Russo-Menna, 1998).

TABLE 1

Time course of MPP^+-induced dopaminergic cell death

Mesencephalic neurons were incubated in the presence or absence of MPP^+ (5 μM) for two consecutive 24-h periods before fixation. The number of surviving dopaminergic neurons was counted from TH-immunostained cultures and expressed as a percentage of controls. Data show mean ± S.E.

Treatment		
First 24 h	Second 24 h	Survival of Dopaminergic Neurons
		% control
MPP^+	MPP^+	17.3 ± 2.12*,**
MPP^+	Control	57.6 ± 5.64**
Control	MPP^+	46.5 ± 5.51**

* $P < .01$ versus MPP^+/control and control/MPP^+.
** $P < .01$ versus untreated control group. Four separate cultures per group, with 12 fields counted in each culture.

Discussion

Role of Complex I Inhibition in MPP^+ Toxicity: Effects on $\Delta\Psi m$, ROS, and GSH. Our data show that MPP^+ caused minimal dissipation of $\Delta\Psi m$ in dopaminergic neurons at a dose (5 μM) that produced selective toxicity (Fig. 2). This finding is consistent with Lotharius et al. (1999), who found that low doses of MPP^+ produced only a late transient

change in ΔΨm in dopaminergic neurons, as measured by rhodamine 123. In addition, a less toxic dose of the specific complex I inhibitor rotenone (20 nM) decreased ΔΨm to a greater extent than MPP^+. Therefore, depolarization of ΔΨm is not the primary mechanism of MPP^+ toxicity.

A toxic dose of MPP^+ also failed to increase superoxide levels in dopaminergic neurons (Fig. 3). In an attempt to reconcile this finding with previous observations that MPP^+ increases superoxide, we also examined the acute effects of a high dose of MPP^+ (100 μM) on ROS levels. This increased ROS levels in both dopaminergic and nondopaminergic neurons (Fig. 3A), confirming our ability to detect elevated superoxide and suggesting that high doses of MPP^+ increase ROS production nonselectively in mesencephalic neurons.

To provide additional evidence that MPP^+ selectively kills dopaminergic neurons independent of mitochondrial inhibition and oxidative stress, we examined the effects of MPP^+ on GSH in dopaminergic neurons. If binding of MPP^+ to complex I resulted in greater superoxide production in mitochondria, a resulting increase in the formation of H_2O_2 through dismutation by manganese SOD and possibly Cu/Zn-SOD would also be expected. Such an increased formation of H_2O_2 might stress the glutathione system, because catalase is not present in mitochondria. Alternatively, because ATP is required for the synthesis of GSH, high concentrations of MPP^+ and other mitochondrial inhibitors can decrease GSH content independent of oxidative stress (Di Monte et al., 1987; Mithofer et al., 1992).

Nevertheless, we have previously noted that toxic doses of MPP^+ do not affect the total glutathione content (i.e., GSH plus oxidized glutathione) of primary mesencephalic cultures or of the dopaminergic cell line MN9D (Nakamura et al., 1997). We now report that MPP^+ also does not alter levels of GSH in primary dopaminergic neurons (Fig. 4). Although MPTP has been shown to decrease brain levels of glutathione in vivo (Yong et al., 1986; Sriram et al., 1997), this may result from differences between in vivo and in vitro conditions. Reduction of total glutathione in dopaminergic neurons potentiates their susceptibility to MPP^+ toxicity both in vitro (Nakamura et al., 1997) and in vivo (Wullner et al., 1996), suggesting that glutathione depletion and MPP^+ toxicity may interact downstream in the cell death pathway.

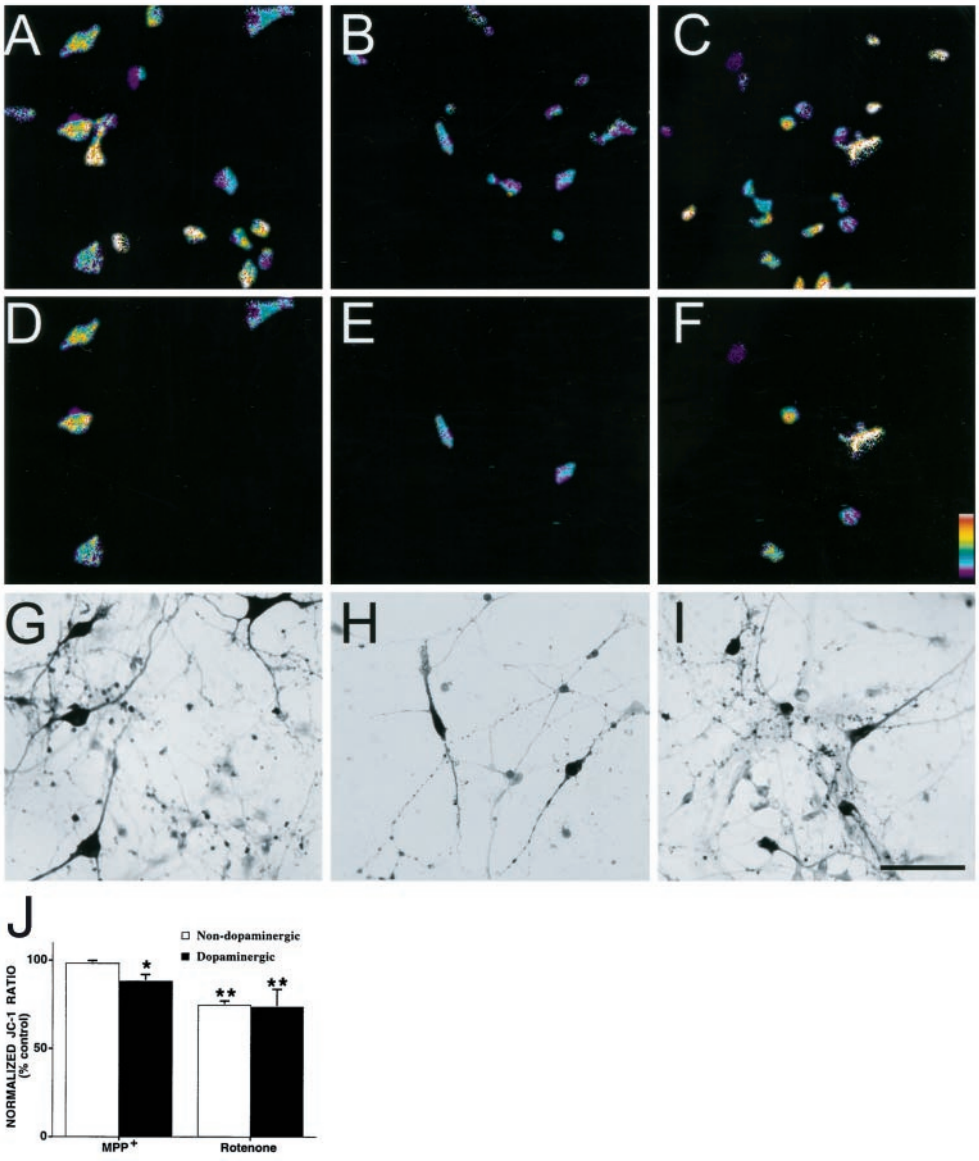

Fig. 2. Rotenone dissipates ΔΨm more than MPP^+. A–I, color-coded ratio images of aggregate to monomer fluorescence (JC-1 ratio) in mesencephalic cultures treated with either MPP^+ (5 μM) or rotenone (20 nM) for 24 h. The color scale codes the highest JC-1 ratio as red/white (top of the scale; 2.8), to indicate higher ΔΨm and the lowest ratio as purple/black to indicate lower ΔΨm (bottom of the scale, 0.6), as shown in I. A–C, JC-1 ratios in mesencephalic cultures containing both nondopaminergic and dopaminergic cells. D–F, only the dopaminergic neurons in those fields for clarity. Cultures treated with MPP^+ (C and F) show JC-1 ratios that are similar to controls, whereas cultures treated with rotenone (20 nM) show significantly lower JC-1 ratios (B and E). G–I, corresponding TH immunohistochemistry of the same fields of cells. The scale bar is 50 μm. J, aggregate data from several experiments for normalized mean JC-1 fluorescent intensity ratios in dopaminergic versus nondopaminergic neurons treated with MPP, rotenone, or FCCP (1 μM). The JC-1 ratios in each treatment group are normalized to ratios in untreated control group. The data from dopaminergic neurons are represented by the dark fill pattern, whereas those from nondopaminergic neurons are represented by the light fill pattern. Data show mean ± S.E. (error bars). $^*P < .05$, $^{**}P < .01$ versus respective controls (n = 18–29 dopaminergic and 156–457 nondopaminergic neurons).

There are several potential pitfalls that could complicate the interpretation of our findings. First, if changes in $\Delta\Psi m$, superoxide, or GSH occurred transiently in any given cell but triggered an irreversible cascade of events that subsequently led to cell death, they might have been difficult to detect at any given point. However, the time course of cell death induced by MPP^+ (Table 1) indicates that the continued presence of MPP^+ is necessary for cells to reach the point of our morphological criteria of cell death, and hence intracellular events that are critical for committing cells to the death process must also be present up until this point. Second, our observation that a significant proportion of dopaminergic neurons at 24 h already showed changes in nuclear morphologies but still had intact cell bodies (data not shown) indicates that our sample population included a large number of dopaminergic neurons that were in the process of dying. Consistent with this, the analysis of data from individual dopaminergic neurons did not show bimodal distribution of $\Delta\Psi m$, superoxide, and GSH in both controls and dopaminergic neurons treated with MPP^+ (data not shown). Bimodal distribution would be expected if there were populations of cells undergoing MPP^+-induced changes and those that were not. Third, Budd et al. (1997) noted that a false-positive increase in Et signal could occur when mitochondria are incubated with high concentrations of HEt. These mitochondria can release unbound Et into the cytoplasm after depolarization. This sort of a false-positive increase would not confound our main results because we did not observe any changes in Et fluorescence signal or any significant depolarization in dopaminergic neurons using 5 μM MPP^+. Although a potential contribution of such an artifact to the observed increase in Et fluorescence after treatment with a high concentration (100 μM) of MPP^+ cannot be completely ruled out, there are several data presented here that indicate the validity of Et fluorescence as a measure of ROS in our system independent of changes in $\Delta\Psi m$. Et fluorescence did not increase in dopaminergic neurons (Fig. 3B) despite mitochondrial depolarization by rotenone (Fig. 2J), demonstrating the absence of a false-positive increase in Et fluorescence from the mitochondrial depolarization in our system. Second, Et fluorescence increased in nondopaminergic neurons after 24 h of 5 μM MPP^+ treatment (Fig. 3A) without any change in $\Delta\Psi m$ (Fig. 2J), suggesting that Et fluorescence changes independent of mitochondrial depolarization.

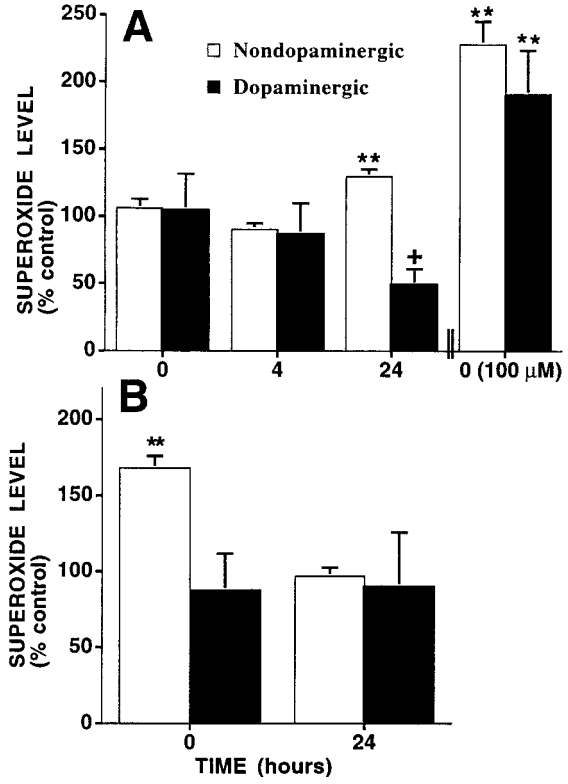

Fig. 3. MPP^+ does not preferentially increase superoxide in dopaminergic neurons. Effects of MPP^+ (A) and rotenone (B) on superoxide levels in mesencephalic neurons. Cultures were treated with either MPP^+ (5 or 100 μM) or rotenone (20 nM) and then examined for Et fluorescence either acutely (noted as 0) or after 4 or 24 h. Superoxide levels are expressed as a normalized percentage of the Et fluorescence in dopaminergic and nondopaminergic neurons from control cultures. Data show mean ± S.E. (error bars). $^{**}P < 0.01$ versus respective controls. $^{+}P < 0.01$ versus nondopaminergic neurons in the same treatment group (n = 9–18 dopaminergic and 61–341 nondopaminergic neurons).

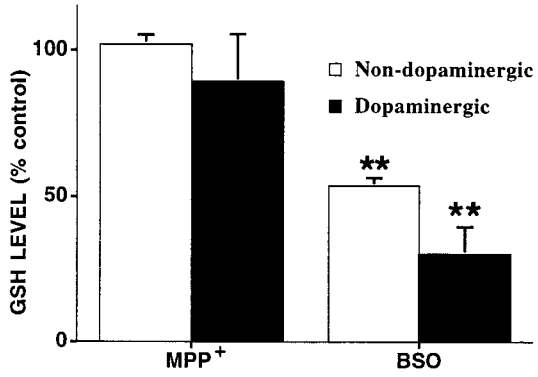

Fig. 4. MPP^+ does not decrease GSH content. Mesencephalic cultures were examined for MCB fluorescence after 24-h treatment with either MPP^+ (5 μM) or BSO (10 μM). GSH levels were estimated from MCB fluorescence as described under *Experimental Procedures*. The values were expressed as a percentage of the MCB fluorescence from dopaminergic or nondopaminergic neurons in control cultures. Data show mean ± S.E. (error bars). $^{**}P < .01$ versus respective controls (n = 12–19 dopaminergic and 168–460 nondopaminergic neurons).

Mechanisms of MPP^+ Toxicity to Dopaminergic Neurons. The apparently contradictory findings in the literature regarding the involvement of mitochondrial disruption and ROS generation in MPTP or MPP^+ toxicity may be due to differences between high and low doses and between MPTP and MPP^+, as well as differences among model systems. Most of the data supporting the role of ROS and mitochondrial dysfunction in MPP^+ toxicity come from experiments using high doses of MPP^+ in nondopaminergic, and often nonneuronal, cell types or from indirect evidence obtained in vivo. In vivo, the conversion of MPTP to MPP^+ may represent a significant source of ROS including H_2O_2 and superoxide (Lai et al., 1993). In addition, glutaminergic neurons projecting from cortex to striatum may contribute to the toxicity through excitotoxicity and oxidative stress (Sonsalla et al., 1998). Studies that specifically examined dopaminergic cells showed that low doses of MPP^+ produced minimal ROS generation (Choi et al., 1999). Dopaminergic neurons cultured from Cu/Zn-SOD transgenic mice are not resistant to MPP^+ toxicity compared with controls (Sanchez-Ramos et al., 1997). In addition, NO synthase inhibitors have little protective effect against MPP^+ toxicity in vitro (Lotharius et al., 1999; Choi et al., 1999). The potent ROS scavenger C_3 carboxyfullerene blocked the toxicity of 6-hydroxydopamine in mes-

encephalic cultures completely but offered only partial protection against MPP^+ (Lotharius et al., 1999). A variety of other antioxidants also failed to protect against MPP^+ in dopaminergic cells (Sanchez-Ramos et al., 1988; Choi et al., 1999). Taken together, these studies suggest that the selective toxicity of a low dose of MPP^+ to dopaminergic neurons in vitro is not mediated through complex I inhibition leading to depolarization of $\Delta\Psi m$ or the generation of ROS. Although superoxide may be elevated under some conditions, such elevation is not necessary for either MPP^+ or rotenone to induce dopaminergic neuronal death.

Despite the relative preservation of $\Delta\Psi m$, decreased ATP synthesis due to complex I inhibition may still contribute to MPP^+ toxicity. To maintain $\Delta\Psi m$, cells may decrease or even reverse flux through the ATP synthase (Budd and Nicholls, 1996). MPP^+ has also been shown to impair energy metabolism independently of NO (Royland et al., 1999) and ROS (Di Monte et al., 1999) and, in some cases, to a greater degree than predicted by MPP^+-induced complex I inhibition alone both in vitro and in vivo (Bates et al., 1994; Espino et al., 1994). Consistent with this, MPP^+ also impairs other aspects of energy metabolism, such as α-ketoglutarate dehydrogenase (Mizuno et al., 1987a). In addition, by inhibiting the replication of mitochondrial DNA, MPP^+ decreases the content of mitochondrial DNA, whereas rotenone increases mitochondrial DNA despite its inhibitory effects on complex I (Miyako et al., 1999). Low concentrations of MPP^+ have also been found to induce permeability transition and the release of cytochrome *c* from isolated mitochondria, and this effect was abolished by rotenone or higher concentrations of MPP^+ (Cassarino et al., 1999). Last, MPP^+ inhibits cell growth independent of complex I inhibition (Soldner et al., 1999). Therefore, the extent to which complex I inhibition contributes to MPP^+ toxicity, the commonly cited mechanism, remains to be established. MPP^+ may have effects on multiple steps in energy metabolism, and the relative contribution of each effect to cell death may vary depending on the concentration of the toxin and the experimental paradigms.

In summary, our data indicate that inhibition of mitochondrial complex I, with the subsequent loss of $\Delta\Psi m$ and generation of ROS, is not the primary mechanism by which MPP^+ preferentially kills dopaminergic neurons, although they may occur in certain situations as epiphenomena. Rather, further research into how low doses of MPP^+ induce the selective death of dopaminergic neurons independent of mitochondrial depolarization will be useful. Such studies may also provide insight into the mechanism of nigrostriatal degeneration in PD.

Acknowledgments

We thank Dr. B. Weir for use of his microscope for some of the experiments and Dr. J. Brorson for helpful comments.

References

Bates TE, Heales SJR, Davies SEC, Boakye P and Clark JB (1994) Effects of 1-methyl-4-phenylpyridinium on isolated rat brain mitochondria: Evidence for a primary involvement of energy depletion. *J Neurochem* **63:**640–648.

Bindokas VP, Jordan J, Lee CC and Miller RJ (1996) Superoxide production in rat hippocampal neurons: Selective imaging with hydroethidine. *J Neurosci* **16:**1324–1336.

Budd SL, Castilho RF and Nicholls DG (1997) Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. *FEBS Lett* **415:**21–24.

Budd SL and Nicholls DG (1996) Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurochem* **67:**2282–2291.

Cassarino DS, Parks JK, Parker WD Jr and Bennett JP Jr (1999) The parkinsonian neurotoxin MPP^+ opens the mitochondrial permeability transition pore and releases cytochrome *c* in isolated mitochondria via an oxidative mechanism. *Biochim Biophys Acta* **1453:**49–62.

Choi WS, Yoon SY, Oh TH, Choi EJ, O'Malley KL and Oh YJ (1999) Two distinct mechanisms are involved in 6-hydroxydopamine- and MPP^+-induced dopaminergic neuronal cell death: role of caspases, ROS, and JNK. *J Neurosci Res* **57:**86–94.

Di Monte D, Sandy MS and Smith MT (1987) Increased efflux rather than oxidation is the mechanism of glutathione depletion by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Biochem Biophys Res Commun* **148:**153–160.

Di Monte DA, Tokar I and Langston JW (1999) Impaired glutamate clearance as a consequence of energy failure caused by MPP(+) in astrocytic cultures. *Toxicol Appl Pharmacol* **158:**296–302.

Espino A, Tortosa A, Bendahan G, Bartrons R, Calopa M, Ferrer I and Ambrosio S (1994) Stereotaxic administration of 1-methyl-4-phenylpyridinium ion (MPP^+) decreases striatal fructose 2,6-bisphosphate in rats. *J Neurochem* **62:**1913–1920.

Hartley A, Stone JM, Heron C, Cooper JM and Schapira AHV (1994) Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: Relevance to Parkinson's disease. *J Neurochem* **63:**1987–1990.

Hasegawa E, Takeshige K, Oishi T, Murai Y and Minakami S (1990) 1-Methyl-4-phenylpyridinium (MPP^+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem Biophys Res Commun* **170:**1049–1055.

Javitch JA, D'Amato RJ, Strittmatter SM and Snyder SH (1985) Parkinsonism-inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: Uptake of the metabolite *N*-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc Natl Acad Sci USA* **82:**2173–2177.

Jenner P and Olanow CW (1996) Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* **47:**S161–S170.

Lai M, Griffiths H, Pall H, Williams A and Lunec J (1993) An investigation into the role of reactive oxygen species in the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity using neuronal cell lines. *Biochem Pharmacol* **45:**927–933.

Langston JW, Irwin I, Langston EB and Forno LS (1984) Pargyline prevents MPTP-induced parkinsonism in primates. *Science (Wash DC)* **225:**1480–1482.

Lotharius J, Dugan LL and O'Malley KL (1999) Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. *J Neurosci* **19:**1284–1293.

Meredith MJ and Reed DJ (1982) Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J Biol Chem* **257:**3747–3753.

Mithofer K, Sandy MS, Smith MT and Di Monte D (1992) Mitochondrial poisons cause depletion of reduced glutathione in hepatocytes. *Arch Biochem Biophys* **295:**132–136.

Miyako K, Irie T, Muta T, Umeda S, Kai Y, Fujiwara T, Takeshige K and Kang D (1999) 1-Methyl-4-phenylpyridinium ion (MPP^+) selectively inhibits the replication of mitochondrial DNA. *Eur J Biochem* **259:**412–418.

Mizuno Y, Saitoh T and Sone N (1987a) Inhibition of mitochondrial alpha-ketoglutarate dehydrogenase by 1-methyl-4-phenylpyridinium ion. *Biochem Biophys Res Commun* **143:**971–976.

Mizuno Y, Sone N and Saitoh T (1987b) Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on activities of the enzymes in the electron transport system in mouse brain. *J Neurochem* **48:**1787–1793.

Nakamura K, Wang WY and Kang UJ (1997) The role of glutathione in dopaminergic neuronal survival. *J Neurochem* **69:**1850–1858.

Nicklas WJ, Vyas I and Heikkila RE (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Life Sci* **36:**2503–2508.

Przedborski S, Jackson-Lewis V, Yokoyama R, Shibata T, Dawson VL and Dawson TM (1996) Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. *Proc Natl Acad Sci USA* **93:**4565–4571.

Przedborski S, Kostic V, Jackson-Lewis V, Naini AB, Simonetti S, Fahn S, Carlson E, Epstein CJ and Cadet JL (1992) Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J Neurosci* **12:**1658–1667.

Ramsay RR, Krueger MJ, Youngster SK, Gluck MR, Casida JE and Singer TP (1991) Interaction of 1-methyl-4-phenylpyridinium ion (MPP^+) and its analogs with the rotenone/piericidin binding site of NADH dehydrogenase. *J Neurochem* **56:**1184–1190.

Ramsay RR and Singer TP (1986) Energy-dependent uptake of *N*-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. *J Biol Chem* **261:**7585–7587.

Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M and Chen LB (1995) Mitochondrial membrane potential monitored by JC-1 dye. *Methods Enzymol* **260:**406–417.

Rice ME and Russo-Menna I (1998) Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. *Neuroscience* **82:**1213–1223.

Royland JE, Delfani K, Langston JW, Janson AM and Di Monte DA (1999) 7-Nitroindazole prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced ATP loss in the mouse striatum. *Brain Res* **839:**41–48.

Salvioli S, Ardizzoni A, Franceschi C and Cossarizza A (1997) JC-1, but not $DiOC_6(3)$ or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\Psi$ changes in intact cells: Implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett* **411:**77–82.

Sanchez-Ramos JR, Michel P, Weiner WJ and Hefti F (1988) Selective destruction of cultured dopaminergic neurons from fetal rat mesencephalon by 1-methyl-4-

phenylpyridinium: Cytochemical and morphological evidence. *J Neurochem* **50:** 1934–1944.
Sanchez-Ramos JR, Song S, Facca A, Basit A and Epstein CJ (1997) Transgenic murine dopaminergic neurons expressing human Cu/Zn superoxide dismutase exhibit increased density in culture, but no resistance to methylphenylpyridinium-induced degeneration. *J Neurochem* **68:**58–67.
Shrieve DC, Bump EA and Rice GC (1988) Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J Biol Chem* **263:**14107–14114.
Sonsalla PK, Albers DS and Zeevalk GD (1998) Role of glutamate in neurodegeneration of dopamine neurons in several animal models of parkinsonism. *Amino Acids* **14:**69–74.
Soldner F, Weller M, Haid S, Beinroth S, Miller SW, Wullner U, Davis RE, Dichgans J, Klockgether T and Schulz JB (1999) MPP^+ inhibits proliferation of PC12 cells by a p21(WAF1/Cip1)-dependent pathway and induces cell death in cells lacking p21(WAF1/Cip1). *Exp Cell Res* **250:**75–85.
Sriram K, Pai KS, Boyd MR and Ravindranath V (1997) Evidence for generation of oxidative stress in brain by MPTP: In vitro and in vivo studies in mice. *Brain Res* **749:**44–52.
Wullner U, Loschmann PA, Schulz JB, Schmid A, Dringen R, Eblen F, Turski L and Klockgether T (1996) Glutathione depletion potentiates MPTP and MPP^+ toxicity in nigral dopaminergic neurones. *Neuroreport* **7:**921–923.
Yong VW, Perry TL and Krisman AA (1986) Depletion of glutathione in brainstem of mice caused by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is prevented by antioxidant pretreatment. *Neurosci Lett* **63:**56–60.
Young PR, ConnorsWhite AL and Dzido GA (1994) Kinetic analysis of the intracellular conjugation of monochlorobimane by IC-21 murine macrophage glutathione-S-transferase. *Biochim Biophys Acta* **1201:**461–465.

Send reprint requests to: Un Jung Kang, M.D., MC 2030, S225B, Department of Neurology, The University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637. E-mail: u-kang@uchicago.edu