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Pharmacology, Biochemistry and Behavior 78 (2004) 143–153
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Biochemical, behavioral and immunohistochemical alterations in MPTP-treated mouse model of Parkinson's disease

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Received 12 November 2003; received in revised form 11 February 2004; accepted 3 March 2004

Available online 6 May 2004

Abstract

The biochemical, behavioral and immunohistochemical manifestations were investigated in mice subjected to four experimental schedules with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride treatment. The mice were treated intraperitoneally with MPTP (20 mg/kg in saline) four times a day at 2-h intervals showed severe and persistent depletions of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum and behavioral deficits, as compared with those (1) treated with MPTP (15 mg/kg in saline ip) once a day for 14 consecutive days; (2) MPTP (30 mg/kg in saline ip) twice a day for five consecutive days; and (3) MPTP (10 mg/kg in saline ip) four times a day at 1-h intervals for two consecutive days. The immunohistochemical study has shown that the acute treatment with MPTP caused severe loss of tyrosine hydroxylase (TH)- and dopamine transporter (DAT)-immunoreactive dopaminergic neurons and marked increase in glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes in the striatum and the substantia nigra. Thus acute treatment of mice with MPTP was accompanied by sustained nigral degeneration and motor abnormalities. Furthermore, our results with Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) immunostainings suggest that altered capacity of free radicals quenching may play a key role in the development of the neurons and interneuron damage after MPTP neurotoxicity. Thus, our findings provide valuable information on age-related disease progression and mechanisms of neurodegeneration.

Keywords: MPTP; Behavior; Dopamine; Striatum; Substantia nigra; Immunohistochemistry; Free radicals; Mice

1. Introduction

Parkinson's disease is a progressive, age-related, neurodegenerative disease characterized by bradykinesia, resting tremor, rigidity and gait disturbance. This disease is also characterized by a massive progressive destruction of dopaminergic neurons in the substantia nigra. It has been proposed that parkinsonian clinical signs appear at the point when dopaminergic neuronal death exceeds a critical threshold: 70–80% of striatal nerve terminals and 50–60% of the substantia nigra pars compacta pericaryons (Agid, 1991; Bernheimer et al., 1973).

The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is also known to cause degeneration of mesencephalic dopaminergic neurons in several species, including monkeys, dogs, cats and mice. The neurotoxic effects of MPTP are thought to be initiated by 1-methyl-4-phenyl-pyridinium ion (MPP^+) which is a major metabolite of MPTP formed by the monoamine oxidase (MAO) B-mediated oxidation. MPP^+ is taken up by high-affinity dopamine and noradrenaline uptake systems and is subsequently accumulated within mitochondria of nigrostriatal dopaminergic cells (Turski et al., 1991; Tipton and Singer, 1993). This can lead to a number of deleterious effects on cellular function, resulting in neuronal cell death. Therefore, MPTP-treated animals, including nonhuman primates and C57BL/6 mice, are widely used as one of models for Parkinson's disease (Heikkila et al., 1989; Zuddas et al., 1994; Gerlach and Riederer, 1996; Tanji et al., 1999).

Induction of parkinsonism by MPTP in mice has generated a wealth of neurochemical, pharmacological and anatomical findings. However, to deplete striatal dopamine in

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doi:10.1016/j.pbb.2004.03.006

mice, large doses of MPTP and frequent injections are required (Sonsalla and Heikkila, 1986). Although the magnitude of the striatal dopamine loss can be increased under some circumstances, progressive and persistent dopamine depletion over a relatively long period has yet to be demonstrated. Furthermore, little is known about the relationship between motor abnormalities and dopamine depletions in MPTP-treated mice.

In the present study, we investigated the relationship between motor deficit and dopamine depletion, in mice subjected to four experimental schedules with various doses, time intervals and consecutive days of MPTP treatment. Furthermore, the immunohistochemically changes of neurons and glial cells in the striatum and the substantia nigra of mice were examined after MPTP treatment.

2. Method

2.1. Animals and MPTP treatment

Male C57BL/6 mice (Nihon SLC, Shizuoka, Japan), 8 weeks of age, were used in this study. The animals were housed in a controlled environment (23 ± 1 °C, 50 ± 5% humidity) and were allowed food and tap water ad libitum. The room light was on between 0800 and 2000 h. Animal husbandry was provided by Tohoku University animal care facility, which operates in compliance with the National Institute of Health's *Guide for Care and Use of Laboratory Animals* (Publication No. 85-23, revised 1985). All experiments were performed in accordance with Guidelines for Animal Experiments of the Tohoku University School of Medicine.

For testing the neurotoxic effect of MPTP, the animals were divided into four groups: (1) the mice that received MPTP hydrochloride (15 mg/kg in saline ip) once a day for 14 consecutive days; (2) the mice that received MPTP hydrochloride (30 mg/kg in saline ip) twice a day for five consecutive days; (3) the mice that received MPTP hydrochloride (10 mg/kg in saline ip) four times a day at 1-h intervals for two consecutive days; (4) the mice that received MPTP hydrochloride (20 mg/kg in saline ip) four times at 2-h intervals within a day. In addition, saline-treated mice for four experimental schedules with MPTP treatment showed no significant changes in the values of the striatal dopamine content. Furthermore, we confirmed that there was no significant change in values of the striatal dopamine content between saline-treated and nontreated animals. In the present study, therefore, we used the values of the striatal dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations in nontreated animals for four experimental schedules as control values for dopamine, DOPAC and HVA contents. In the present study, in addition, no animals died after MPTP treatment.

2.2. Dopamine and its metabolite content analysis

The mice were killed by cervical dislocation at 1, 3, 7 and 14 days after MPTP treatment with each schedule. The striata were rapidly dissected and were then sonicated in ice-cold 0.2 M perchloric acid containing 100 ng/ml isoproterenol as an internal standard. Homogenates were centrifuged at 20,000 × *g* for 20 min at 4 °C. The supernatant was filtered (pore size, 0.45 μm, Millipore, MA, USA) and a 30-μl aliquot of the supernatant was used for determine the content of dopamine, DOPAC and HVA by high-performance liquid chromatography (HPLC) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). Concentrations of dopamine and its metabolites are expressed as μg/g tissue weight, as described previously (Araki et al., 2001a,b; Kurosaki et al., 2002; Muramatsu et al., 2002). Each group contained five to seven mice. All values were expressed as means ± S.E.M. and statistical significance was evaluated using a one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

2.3. Behavioral test

To measure cataleptic symptoms, bar-test catalepsy was evaluated by placing both forepaws of the mouse over a horizontal bar (diameter 0.2 cm), elevated 15 cm from floor, as described previously (Araki et al., 2001c; Kurosaki et al., 2003). The time during which the animals maintained this position was recorded. In this study, the test was performed at 7 and 14 days after MPTP treatments with each experimental schedule. Each group contained 7–10 mice. All values were expressed as means ± S.E.M. and statistical significance was evaluated using Student's *t* test.

To determine the degree of bradykinesia, pole test was performed according to the method of Ogawa et al. (1987) with minor modifications (Araki et al., 2001c; Kobayashi et al., 1997; Kurosaki et al., 2003). The mouse was placed head upward on the top of a rough-surfaced pole (8 mm in diameter and 50 cm in height) which was wrapped doubly with gauze to prevent slipping: the time until it turned completely downward (Tturn) and the time until it climbed down to the floor (TLA) were examined. In this study, the test was performed at 7 and 14 days after MPTP treatments with each experimental schedule. Each group contained 7–10 mice. All values were expressed as means ± S.E.M. and statistical significance was evaluated using Student's *t* test.

2.4. Immunohistochemistry

For an immunohistochemical study, the mice were anesthetized with sodium pentobarbital (50 mg/kg ip) 1, 3 and 7 days after MPTP treatment, and the brains were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) following a heparinized saline flush. The brains were removed 1 h after perfusion fixation at 4 °C and were immersed overnight in the same fixative at 4 °C until they

were embedded in paraffin. Coronal paraffin sections, 5 μm in thickness, of the striatum (bregma 0.98 mm, interaural 4.78 mm) and the substantia nigra (bregma 3.16 mm, interaural 0.64 mm) were used for immunohistochemistry according to the Atlas of Mouse brain (Paxinos and Franklin, 2001). Each group contained five animals.

For tyrosine hydroxylase (TH) , dopamine transporter (DAT), glial fibrillary acidic protein (GFAP) and manganese superoxide dismutase (Mn-SOD) immunostaining, a rabbit anti-TH polyclonal antibody (Chemicon International, Temecula, CA, USA), a rat anti-DAT polyclonal antibody (Alpha Diagnostic International, San Antonio, TX, USA), a mouse anti-GFAP monoclonal antibody (Chemicon International), a rabbit anti-Mn-SOD polyclonal antibody (Stressgen Biotechnologies, Victoria, BC, Canada) and a Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA, USA) were used. The paraffin sections were washed for 5 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and treated with 0.3% hydrogen peroxidase in 10% methanol. The paraffin sections were then washed three times for 3 min in 0.01 M PBS, followed by 30 min of preincubation with 10% normal horse serum. The brain sections were then incubated overnight with anti-TH antibody (1:200), anti-DAT antibody (1:200), anti-GFAP antibody (1:200) and anti-Mn-SOD (1:400), including 0.3% Triton X-100 at 4 °C. After a 15-min rinse in 0.01 M PBS, the sections were incubated with biotinylated secondary antibody for 1 h and then with avidin–biotin peroxidase complex for 30 min at room temperature. Immunoreactions were visualized using 0.05% diaminobenzidine and 0.01% hydrogen peroxide in 0.05% Tris–HCl buffer (pH 7.6). Negative control sections were treated in the same way as described above except that the antibody against anti-TH, anti-DAT, anti-GFAP or anti-Mn-SOD was omitted. In addition, the sections were counterstained with hematoxylin for GFAP immunostaining, as described previously (Kurosaki et al., 2002).

For Cu/Zn-superoxide dismutase (Cu/Zn-SOD) immunostaining, a mouse anti-Cu/Zn-SOD monoclonal antibody (Biogenesis, England, UK) and MOM immunodetection kit (Vector Laboratories) were used. Briefly, the paraffin sections were washed for 5 min in 0.01 M PBS and treated with 3% hydrogen peroxide in 0.01 M PBS. The sections were then washed twice for 2 min each in 0.01 M PBS, followed by 50 min of preincubation with mouse Ig G blocking reagent. The brain sections were then incubated with anti-Cu/Zn-SOD (1:100), including MOM diluent overnight at 4 °C. After a 5-min rinse in 0.01 M PBS, the sections were incubated with biotinylated secondary Ig G antibody for 10 min and then avidin–biotin peroxidase complex for 30 min at room temperature. Immunoreactions were visualized using an enzyme substrate kit (Vector Laboratories). Negative control sections were treated in the same way as described above except that the antibody against anti-Cu/Zn-SOD was omitted.

Microglial cells were stained histochemically with alpha-D-galactosyl-specific isolectin B_4 conjugated with horseradish peroxidase derived from Griffonia simplication seeds (GSA I-B_4-HRP, isolectin B_4; Sigma, St Louis, MO, USA). The paraffin sections were immersed in 0.01 M PBS containing 0.1 mM of $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.1% Triton X-100 for 20 min, then the sections were incubated for 2 h with the isolectin B_4 diluted to 20 μg/ml using 0.01 M PBS containing cations and 0.1% Triton X-100. After three washes in 0.01 M PBS, immunoreactions were visualized using enzyme substrate kit (Vector Laboratories). Negative control sections were treated in the same way as described above except that the isolectin B_4 was omitted.

The immunostaining for TH, DAT, GFAP and isolectin B_4 was semiquantatively graded as intense (Grade 3), moderate (Grade 2), weak (Grade 1) and undetectable (Grade 0) under a light microscope at a magnification of

Table 1
Time-course effects of MPTP (15 mg/kg ip) treatment once a day for 14 consecutive days on the striatal dopamine, DOPAC and HVA levels in mice

Days after MPTP treatment	Dopamine (μg/g tissue)	DOPAC (μg/g tissue)	HVA (μg/g tissue)
Control	16.22 ± 0.57	2.99 ± 0.26	1.73 ± 0.14
1 day	13.55 ± 0.77*	2.77 ± 0.12	1.31 ± 0.05*
3 days	16.38 ± 0.42	2.13 ± 0.24*	1.65 ± 0.07
7 days	15.51 ± 0.94	2.23 ± 0.27	1.32 ± 0.14*
14 days	15.59 ± 0.64	1.77 ± 0.14**	1.39 ± 0.09*

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 5–7$ mice.
* $P < .05$, compared with control (Dunnett's Multiple Range Test).
** $P < .01$, compared with control (Dunnett's Multiple Range Test).

Table 2
Time-course effects of MPTP (30 mg/kg ip) treatment twice a day for five consecutive days on the striatal dopamine, DOPAC and HVA levels in mice

Days after MPTP treatment	Dopamine (μg/g tissue)	DOPAC (μg/g tissue)	HVA (μg/g tissue)
Control	16.22 ± 0.57	2.99 ± 0.26	1.73 ± 0.14
1 day	3.96 ± 0.43*	0.94 ± 0.13*	0.51 ± 0.03*
3 days	5.09 ± 0.14*	0.80 ± 0.08*	0.64 ± 0.03*
7 days	6.10 ± 0.23*	0.81 ± 0.03*	0.99 ± 0.06*
14 days	8.56 ± 0.42*	1.64 ± 0.08*	1.24 ± 0.03*

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 5–6$ mice.
* $P < .01$, compared with control (Dunnett's Multiple Range Test).

Table 3
Time-course effects of MPTP (10 mg/kg ip) treatment four times a day at 1-h intervals for two consecutive days on the striatal dopamine, DOPAC and HVA levels in mice

Days after MPTP treatment	Dopamine (μg/g tissue)	DOPAC (μg/g tissue)	HVA (μg/g tissue)
Control	16.22 ± 0.57	2.99 ± 0.26	1.73 ± 0.14
1 day	7.76 ± 1.03*	2.10 ± 0.15	0.84 ± 0.06*
3 days	8.11 ± 1.07*	2.27 ± 0.17	0.94 ± 0.05*
7 days	7.31 ± 0.94*	3.38 ± 0.39	1.23 ± 0.06*
14 days	7.94 ± 1.15*	4.32 ± 0.49*	1.34 ± 0.08*

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 5–7$ mice.
* $P < .01$, compared with control (Dunnett's Multiple Range Test).

Table 4
Time-course effects of MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals on the striatal dopamine, DOPAC and HVA levels in mice

Days after MPTP treatment	Dopamine (μg/g tissue)	DOPAC (μg/g tissue)	HVA (μg/g tissue)
Control	16.22 ± 0.57	2.99 ± 0.26	1.73 ± 0.14
1 day	2.63 ± 0.53*	1.21 ± 0.25*	0.82 ± 0.17*
3 days	3.03 ± 0.62*	1.24 ± 0.23*	0.56 ± 0.06*
7 days	2.91 ± 0.37*	1.39 ± 0.18*	0.63 ± 0.06*
14 days	3.46 ± 0.32*	3.20 ± 0.20	1.11 ± 0.02*

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 5–6$ mice.
* $P < .01$, compared with control (Dunnett's Multiple Range Test).

× 400 without the examiner knowing the experimental protocol, as described previously (Kato et al., 1995). The mean values of the right and the left cells were expressed as means ± S.E.M. and statistical significance was evaluated using nonparametric Dunnett's Multiple Comparison Test.

3. Results

3.1. Effect of MPTP on the striatal dopamine, DOPAC and HVA levels

The mice that received MPTP (15 mg/kg ip) once a day for 14 consecutive days showed a significant reduction in striatal dopamine (15% loss) and HVA (24% loss) content 1 day posttreatment. Thereafter, the significant reduction in the striatal dopamine content was not found in the striatum up to 14 days after the MPTP treatment, although DOPAC and HVA showed a significant decline in this area (Table 1).

The mice that received MPTP (30 mg/kg ip) twice a day for five consecutive days showed a significant reduction in dopamine, DOPAC and HVA content in the striatum up to 14 days posttreatment. The depletion in the striatal dopamine content reached maximal levels (76% loss) 1 day after MPTP treatment. Thereafter, the striatal dopamine levels showed a tendency to recover gradually up to 14 days after MPTP treatment. However, the striatal dopamine content (33% loss) was significantly reduced even 14 days after MPTP treatment. The striatal DOPAC and HVA also showed a significant reduction throughout the experiment (Table 2).

Table 5
Effects of MPTP (15 mg/kg ip) treatment once a day for 14 consecutive days on the motor activity in mice

Days after MPTP treatment	Pole test (s)		Catalepsy test (s)
	Tturn	TLA	
Control	2.11 ± 0.23	5.14 ± 0.45	3.73 ± 0.43
7 days	2.19 ± 0.15	6.03 ± 0.71	5.25 ± 1.01
Control	1.92 ± 0.17	4.95 ± 0.52	3.87 ± 0.89
14 days	1.91 ± 0.13	5.32 ± 0.40	4.26 ± 0.76

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 7$ mice.

Table 6
Effects of MPTP (30 mg/kg ip) treatment twice a day for five consecutive days on the motor activity in mice

Days after MPTP treatment	Pole test (s)		Catalepsy test (s)
	Tturn	TLA	
Control	2.70 ± 0.15	6.14 ± 0.32	2.48 ± 0.49
7 days	2.49 ± 0.11	5.81 ± 0.26	3.25 ± 0.37
Control	2.00 ± 0.09	4.65 ± 0.15	2.83 ± 0.49
14 days	2.15 ± 0.14	4.62 ± 0.27	2.61 ± 0.41

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 9–10$ mice.

The mice that received MPTP (10 mg/kg ip) four times a day at 1-h intervals for two consecutive days showed a significant reduction in dopamine (50–55% loss) and HVA (23–51% loss) content from 1 to 14 days posttreatment. However, the striatal DOPAC content showed no significant reduction throughout the experiments except for a significant increase 14 days after MPTP treatment (Table 3).

The mice that received MPTP (20 mg/kg ip) four times a day at 2-h intervals showed a severe reduction in striatal dopamine (79–85% loss), DOPAC (54–60% loss) and HVA (36–64% loss) content from 1 to 14 days posttreatment except for the DOPAC level 14 days posttreatment (Table 4).

3.2. Effect of MPTP on motor activity

The mice that received MPTP (15 mg/kg ip) once a day for 14 consecutive days did not produce a significant change on the Tturn and TLA in pole test and bar-test catalepsy after 7 and 14 days of MPTP treatment (Table 5).

The mice that received MPTP (30 mg/kg ip) twice a day for five consecutive days also did not show a significant change on the Tturn and TLA in pole test and bar-test catalepsy after 7 and 14 days of MPTP treatment (Table 6).

The mice that received MPTP (10 mg/kg ip) four times a day at 1-h intervals for two consecutive days had a significant prolongation of Tturn and TLA and cataleptic effect after 7 days of MPTP treatment. After 14 days, a significant prolongation of Tturn and TLA was still observed in these mice.

Table 7
Effects of MPTP (10 mg/kg ip) treatment four times a day at 1-h intervals for two consecutive days on the motor activity in mice

Days after MPTP treatment	Pole test (s)		Catalepsy test (s)
	Tturn	TLA	
Control	1.74 ± 0.10	4.02 ± 0.29	2.03 ± 0.21
7 days	2.87 ± 0.29**	5.02 ± 0.46*	3.54 ± 0.46**
Control	1.77 ± 0.16	3.75 ± 0.33	2.17 ± 0.40
14 days	2.54 ± 0.15**	5.65 ± 0.88*	3.47 ± 0.62

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 8–10$ mice.
* $P < .05$, compared with control (Student's t test).
** $P < .01$, compared with control (Student's t test).

Table 8
Effects of MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals on the motor activity in mice

Days after MPTP treatment	Pole test (s)		Catalepsy test (s)
	Tturn	TLA	
Control	1.74 ± 0.10	4.02 ± 0.29	2.03 ± 0.21
7 days	2.69 ± 0.20**	5.80 ± 0.60**	4.07 ± 0.54**
Control	1.77 ± 0.16	3.75 ± 0.33	2.17 ± 0.40
14 days	2.13 ± 0.18	5.25 ± 0.65*	3.11 ± 0.34*

Values are expressed as means ± S.E.M. Drug treatment schedules are described in the experimental design section. $n = 8–10$ mice.
* $P < .05$, compared with control (Student's t test).
** $P < .01$, compared with control (Student's t test).

However, the cataleptic effect was not evident in these mice (Table 7).

The mice that received MPTP (20 mg/kg ip) four times at 2-h intervals within a day had a significant prolongation of Tturn and TLA and cataleptic effect 7 days after MPTP treatment. After 14 days, a significant prolongation of TLA and cataleptic effect was still observed in these mice. However, a significant prolongation of Tturn was not evident in these mice (Table 8).

3.3. Immunohistochemistry

3.3.1. TH staining

Representative photographs of TH immunostaining in the striatum and the substantia nigra are shown in Fig. 1. Dopaminergic neurons with the TH antibody were easily detectable in the substantia nigra of control mice. The bodies or fibers of dopaminergic cells were intensely stained with evident immunopositive processes in the striatum and the substantia nigra. One day after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals, TH immunopositive fibers and cell bodies were reduced in the striatum and the substantia nigra, respectively. Thereafter, the TH immunopositive fibers and cell bodies were markedly decreased in the striatum and the substantia nigra up to 7 days after MPTP treatment (Table 9).

3.3.2. DAT staining

Representative photographs of DAT immunostaining in the striatum and the substantia nigra are shown in Fig. 1. DAT was mainly localized in the plasma membranes of axons and terminals. In the substantia nigra, the cell bodies and fibers of DAT-positive cells were stained strongly with evident immunopositive processes. In the striatum, a decrease in the number of DAT-immunopositive fibers was observed from 1 day after MPTP (20 mg/kg ip) treatment given four times a day at 2-h intervals. Thereafter, marked reductions in the immunoreactivity of DAT-positive fibers were observed in mice after 3 and 7 days of MPTP treatment. In the substantia nigra, decreased number and immunoreactivity of DAT-positive neurons were found from 1 day after MPTP treatment. Thereafter, marked decreases in the num-

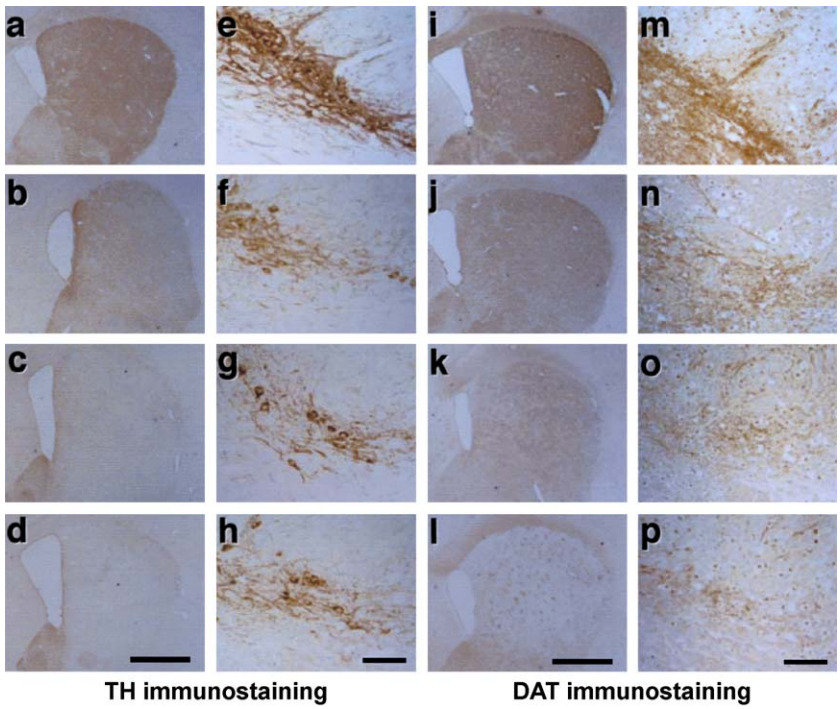

Fig. 1. Representative microphotographs of TH and DAT immunostainings in the striatum and the substantia nigra of mice after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals within a day. (a,e,i,m) Control. (b,f,j,n) One day after MPTP treatment. (c,g,k,o) Three days after MPTP treatment. (d,h,l,p) Seven days after MPTP treatment. Left half: striatum (a–d), bar = 100 μm; right half: substantia nigra (e–h), bar = 100 μm for TH immunostaining. Left half: striatum (i–l), bar = 100 μm; right half: substantia nigra (m–p), bar = 100 μm for DAT immunostaining. $n = 5$ mice.

Table 9
Intensity of immunoreactivity against TH and DAT in the striatum and the substantia nigra of mice after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals

	Striatum	Substantia nigra
TH immunostaining		
Control	2.9 ± 0.1	2.6 ± 0.2
1 day	1.3 ± 0.3*	1.4 ± 0.3
3 days	1.0 ± 0.3**	1.1 ± 0.2**
7 days	0.6 ± 0.1**	0.9 ± 0.2**
DAT immunostaining		
Control	2.5 ± 0.2	2.4 ± 0.3
1 day	1.7 ± 0.3	1.1 ± 0.2
3 days	1.4 ± 0.1**	0.9 ± 0.2*
7 days	1.3 ± 0.1**	0.6 ± 0.1**

The TH and DAT immunoreactivity was graded semiquantitatively using a scoring with 0 = no staining, 1 = weak, 2 = moderate and 3 = intense. Values are expressed as means ± S.E.M. $n = 5$ mice.

* $P < .05$, compared with control (nonparametric Dunnett's Multiple Comparison Test).

** $P < .01$, compared with control (nonparametric Dunnett's Multiple Comparison Test).

ber and immunoreactivity of DAT-positive neurons after 3 and 7 days of MPTP treatment were observed (Table 9).

3.3.3. GFAP staining

Representative photographs of GFAP immunostaining in the striatum and the substantia nigra are shown in Fig. 2. GFAP positive astrocytes were evident in the substantia nigra. One day after the MPTP (20 mg/kg ip) treatment given four times a day at 2-h intervals, GFAP immunopositive astrocytes were increased in the striatum and the substantia nigra. Thereafter, GFAP positive astrocytes were markedly increased in the striatum and the substantia nigra after 3 and 7 days of MPTP treatment (Table 10).

3.3.4. Isolectin B_4 staining

Representative photographs of isolectin B_4 staining in the striatum and the substantia nigra are shown in Fig. 2. Isolecton B_4 positive cells were evident in the striatum and the substantia nigra. One day after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals, isolectin B_4 positive cells were increased in the striatum and the substantia nigra. Thereafter, isolectin B_4 positive cells were markedly increased in the striatum and the substantia nigra after 3 and 7 days of MPTP treatment (Table 10).

3.3.5. Mn-SOD staining

Representative photographs of Mn-SOD immunostaining in the striatum and the substantia nigra are shown in Fig. 3. Mn-SOD immunoreactivity was evident in glial cells and interneurons of the striatum and the substantia nigra. These findings were consistent with the our previous report (Kato et al., 1995). In contrast, weak Mn-SOD immunoreactiviy was observed in neurons of the striatum

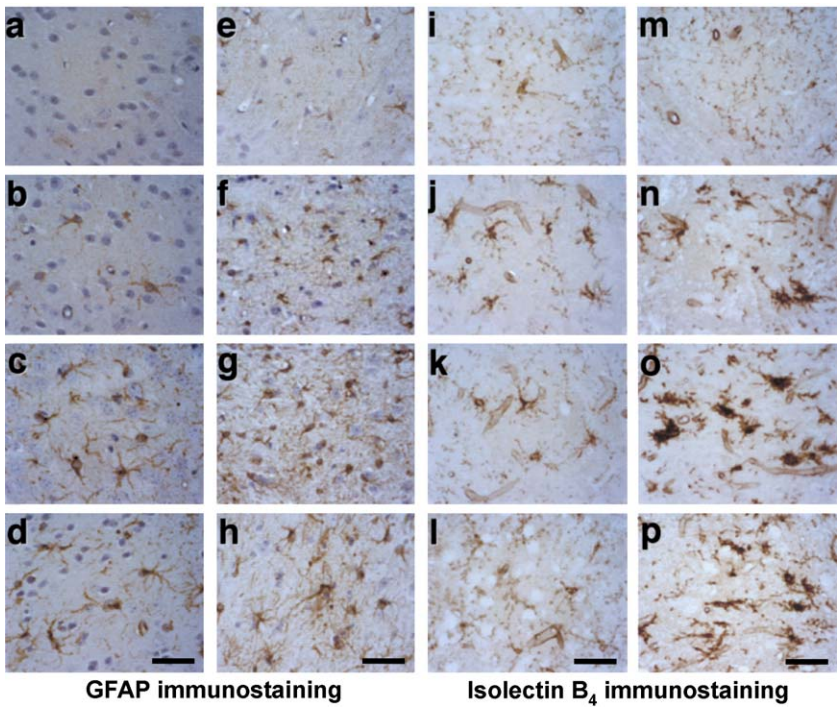

Fig. 2. Representative microphotographs of glial fibrillary acidic protein (GFAP) and isolectin B_4 immunostainings in the striatum and the substantia nigra of mice after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals within a day. (a,e,i,m) Control. (b,f,j,n) One day after MPTP treatment. (c,g,k,o) Three days after MPTP treatment. (d,h,l,p) Seven days after MPTP treatment. Left half: striatum (a–d), bar = 100 μm; right half: substantia nigra (e–h), bar = 100 μm for GFAP immunostaining. Left half: striatum (i–l), bar = 100 μm; right half: substantia nigra (m–p), bar = 100 μm for Isolectin B_4 immunostaining. $n = 5$ mice.

Table 10
Intensity of immunoreactivity against GFAP and isolectin B_4 in the striatum and the substantia nigra of mice after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals

	Striatum	Substantia nigra
GFAP immunostaining		
Control	0.5 ± 0.2	1.2 ± 0.1
1 day	1.2 ± 0.3	2.0 ± 0.2
3 days	2.9 ± 0.1**	2.9 ± 0.1**
7 days	2.9 ± 0.1**	2.5 ± 0.2**
Isolectin B_4 immunostaining		
Control	0.1 ± 0.1	0.7 ± 0.1
1 day	1.5 ± 0.3**	1.2 ± 0.3
3 days	1.6 ± 0.2**	2.8 ± 0.1**
7 days	1.4 ± 0.2*	2.3 ± 0.2*

The GFAP and isolectin B_4 immunoreactivity was graded semiquantitatively using a scoring with 0 = no staining, 1 = weak, 2 = moderate and 3 = intense. Values are expressed as means ± S.E.M. $n = 5$ mice.

* $P < .05$, compared with control (nonparametric Dunnett's Multiple Comparison Test).

** $P < .01$, compared with control (nonparametric Dunnett's Multiple Comparison Test).

and the substantia nigra. One day after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals, Mn-SOD immunoreactivty was increased in the glial cells of the striatum. However, striatal neurons showed no changes in Mn-SOD immunoreactivity throughout the experiments. In contrast, nigral interneurons showed intense Mn-SOD immunoreactivity 1 day after MPTP treatment. However, Mn-SOD immunoreactivity was reduced in nigral neurons 1 day after MPTP treatment. Thereafter, Mn-SOD immunoreactive glial cells had a ramifid form with fine processes in the striatum after 3 and 7 days of MPTP treatment. In the substantia nigra, Mn-SOD immunoreactivity increased in interneurons after 3 days and decreased after 7 days of MPTP treatment. In addition, glial cells in the substantia nigra showed no changes in Mn-SOD immunoreactivity throughout the experiments.

3.3.6. Cu/Zn-SOD staining

Representative photographs of Cu/Zn-SOD immunostaining in the striatum and the substantia nigra are shown in Fig. 3. Cu/Zn-SOD immunoreactivity was evident in neurons and glial cells of the striatum and the substantia nigra. One day after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals, Cu/Zn-SOD immunoreactivity was increased in the glial cells of the striatum and the substantia nigra. However, striatal neurons showed no changes in Cu/Zn-SOD immunoreactivity throughout the experiments. In contrast, nigral neurons showed a decrease in Cu/Zn-SOD immunoreactivity 1 day after MPTP treatment. Thereafter, Cu/Zn-SOD immunoreactivity was markedly increased in the glial cells of the striatum and the substantia nigra after 3 and 7 days of MPTP treatment. In the substantia nigra, however, Cu/Zn-SOD immunoreactivity was reduced in neurons after 3 and 7 days of MPTP treatment.

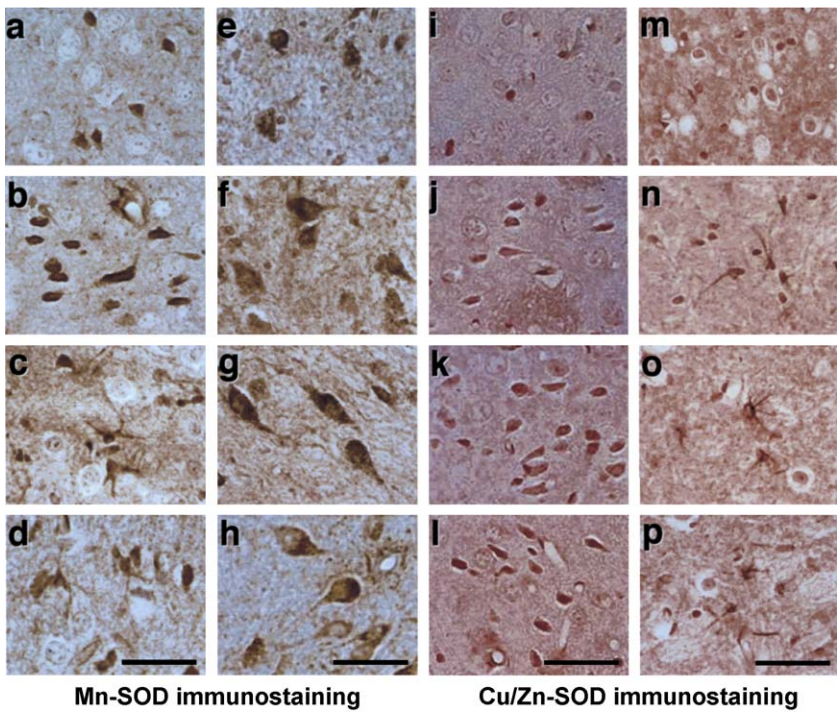

Fig. 3. Representative microphotographs of Mn-SOD and Cu/Zn-SOD immunostainings in the striatum and the substantia nigra of mice after MPTP (20 mg/kg ip) treatment four times a day at 2-h intervals within a day. (a,e,i,m) Control. (b,f,j,n) One day after MPTP treatment. (c,g,k,o) Three days after MPTP treatment. (d,h,l,p) Seven days after MPTP treatment. Left half: striatum (a–d), bar = 100 μm; right half: substantia nigra (e–h), bar = 100 μm for Mn-SOD immunostaining. Left half: striatum (i–l), bar = 100 μm; right half: substantia nigra (m–p), bar = 100 μm for Cu/Zn-SOD immunostaining. $n = 5$ mice.

4. Discussion

To clarify the pathological mechanisms of Parkinson's disease, first we investigated a close correlation between neurochemical and behavioral manifestation using mice with several experimental studies with MPTP treatment. We provide further evidence that mice subjected to acute MPTP (20 mg/kg ip) can cause severe and persistent depletions of dopamine, DOPAC and HVA levels in the striatum and behavioral deficits, as compared with other experimental studies. This is the first report using mice in several experimental studies to test the close correlation between neurochemical and behavioral manifestation.

Since the discovery that MPTP selectively destroys nigrostriatal dopaminergic neurons in humans, a wide range of animal models for parkinsonism have been extensively studied. Primate models closely resemble human Parkinson's disease patients and the use of this species may be useful for exploring neurological and pathological mechanisms of Parkinson's disease. However, the use of primates is very expensive for exploring neurological and pathological mechanisms of Parkinson's disease. Therefore, the development of a comparable model in small animals is desirable. Mice are susceptible to MPTP neurotoxicity and the animals make excellent conventional models for Parkinson's disease. In general, MPTP is usually administered in mice either by an acute or a subacute regimen (Heikkila et al., 1984; Sonsalla and Heikkila, 1986). In the acute and subacute mice models, MPTP can produce depletions in the striatal level of dopamine and its metabolites (DOPAC and HVA) along with a reduction in the striatal synaptosomal dopamine uptake (Ricaurte et al., 1986). However, when survival time in mice was extended, the neurotoxic effect of MPTP was reversible (Hallman et al., 1985). Furthermore, despite evidence of dopamine reductions, animals that receive MPTP acutely or subacutely do not always exhibit motor dysfunctions or motor abnormalities (Heikkila et al., 1989; Gerlach et al., 1994). Therefore, we examined the exact neurotoxic effects of acute or consecutive treatment of MPTP in mice.

In the present study, the level of the striatal dopamine, DOPAC and HVA levels after 1–14 days of acute MPTP treatment was investigated. As shown in Tables 1 and 5, MPTP treatment (15 mg/kg ip) for 14 consecutive days showed no persistent decrease in the striatal dopamine levels or motor abnormalities. In contrast, MPTP treatment (30 mg/kg ip) for five consecutive days showed a significant decrease in the striatal dopamine, DOPAC and HVA levels from 1 day. However, the depletion in the striatal dopamine level showed a tendency to recover gradually over 14 days (Table 2). Furthermore, the treatment did not cause motor abnormalities in mice throughout the experiments (Table 6). From these results, we speculate that the continuous treatment with MPTP for 5 or 14 days only produces transient neurotoxic changes to the nigrostriatal neurons, which reverse spontaneously.

However, the acute MPTP treatment (10 mg/kg ip) caused significant reductions in the striatal dopamine and HVA levels from 1 day posttreatment. Thereafter, the persistent reductions were observed up to 14 days after MPTP treatment (Table 3). In a behavioral study, the acute MPTP treatment (10 mg/kg ip) caused significant motor abnormalities in mice after 7 and 14 days of MPTP treatment (Table 7). Furthermore, the acute MPTP treatment (20 mg/kg ip) showed a severe reduction in the striatal dopamine, DOPAC and HVA contents in mice from 1 to 14 days posttreatment (Table 4). In our behavioral studies, the acute MPTP treatment (20 mg/kg ip) caused severe motor deficits in mice after 7 and 14 days of posttreatment (Table 8). From the present findings, we suggest that the model with acute MPTP treatment can cause a severe dopamine depletion and motor deficiency in mice, as compared to the model with the continuous treatment with MPTP for 5 or 14 days. Based on these results, we speculate that the acute treatment with MPTP is a very useful model of Parkinson's disease, as compared to the models with the continuous treatment with MPTP.

Our study also suggests that continuous and excessive production of endogenous MPTP-like substrates, such as 1,2,3,4-tetrahydroisoquinoline and 1-benzyl-tetrahydroisoquinoline, in the brain may be one of the mechanisms in the development of Parkinson's disease (Tasaki et al., 1991; Kotake et al., 1995). However, numerous studies suggest that MPTP is able to induce apoptosis in various cell types, such as primary mesencephalic dopaminergic cells and catecholaminergic cells (Hartley et al., 1994; Itano and Nomura, 1995; Leist et al., 1998). Therefore, MPTP-induced apoptosis of nigrostriatal dopaminergic cells may also be involved in the neurodegenerative processes causing Parkinson's disease, although further studies will be necessary for determining the exact mechanisms of MPTP-mediated apoptosis.

The immunohistological study has shown the loss of TH and DAT immunoreactivity in the striatal fibers and nigral cells from 1 day after the acute MPTP treatment (20 mg/kg ip) and the loss of severity up to 7 days posttreatment (Fig. 1). The patterns of the intensity of TH immunorecative fibers and cell bodies were nearly all similar to those of the intensity of DAT immunoreactive fibers and cell bodies in the striatum and the substantia nigra of mice after MPTP treatment. A previous study demonstrated that nearly all TH immunopositive cells were double labeled with DAT, suggesting they contain the machinery to be functional dopaminergic neurons in the primate striatum (Betarbe et al., 1997). Furthermore, a recent study suggested that DAT immunoreactivity was distributed in a pattern similar to that of TH immunoreactivity in human brains and demonstrated that DAT may be a selective marker for dopaminergic neurons and their processes (Ciliax et al., 1999). In contrast, after 1 day of acute MPTP (20 mg/kg ip) treatment, GFAP immunopositive astrocytes were increased in the striatum and the substantia nigra. Thereafter, GFAP positive astro-

cytes were markedly increased in the striatum and the substantia nigra after 3 and 7 days of MPTP treatment (Fig. 2). These results suggest that an increase in GFAP immunostaining produced by MPTP in the striatum and the substantia nigra is linked to decrements in TH immunostaining, suggesting that factors originating in the damaged dopamine neurons initiated the astrocyte reaction to MPTP. For histochemical staining of isolectin B_4, which specifically combines with terminal alpha-D-galactose residues located on the cell surface of microglia (Streit, 1990; Morioka et al., 1991), isolectin B_4 staining was weak in the striatum and the substantia nigra of control mice. One day after acute MPTP (20 mg/kg ip) treatment, isolectin B_4-positive microglia were increased markedly in the striatum and the substantia nigra. Thereafter, the increase of isolectin B_4-positive microglia lasted up to 7 days after MPTP treatment. It is known that activated microglia exert cytotoxic effects in the brain through two different, yet complementary processes (Banati et al., 1993). First, activated microglia can act as phagocytes, which involve direct cell-to-cell contact. Second, they are capable of releasing a large variety of potentially neurotoxic substances (Banati et al., 1993). Therefore, it is believed that activated microglia may sometimes be associated with beneficial effects and often they may appear to be deleterious (Vila et al., 2001). A recent interesting study suggested that inhibition of microglial activation by minocycline can protect the nigrostriatal dopaminergic pathway against neurotoxic effects of MPTP in mice (Wu et al., 2002). These findings seem to suggest that activated microglia plays an important role in the pathogenesis of MPTP-induced degeneration of dopaminergic neurons. In the present study, the number of activated microglia appeared in the striatum and the substantia nigra earlier than that of reactive astrocytes, as shown in Fig. 2. These results were, at least in part, consistent with the previous report (Liberatore et al., 1999). Based on these observations, we also suggest that microglial activation may play a key role in the MPTP neurotoxic process.

The cytotoxic hydroxy radical has been implicated in dopamine neurotoxicity caused by MPTP and iron (Youdim et al., 1989; Chiueh et al., 1993). Oxidative stress may be involved in the pathogenesis of Parkinson's disease (Hirsch et al., 1988; Gerlach et al., 1994). The excessive formation of hydroxy peroxide and oxygen-derived free radicals can cause neuronal damage due to chain reactions of membrane lipid peroxidation and alterations in membrane fluidity (Halliwell, 1992). SOD, a scavenger of free radicals, plays a key role in the regulation of cellular antioxidant defenses by catalyzing the dismutation of free radicals into oxygen and hydrogen peroxidase (Kato et al., 1995). There are two intracellular SOD enzymes. The Cu/Zn-SOD (SOD1) is mainly located in cytoplasmic and nuclear compartments. The Mn-SOD (SOD2) is localized predominantly in the mitochondria (Weisiger and Fridovich, 1973; Beyer et al., 1991). These SOD enzymes are known to play a critical role in normal antioxidant defense. However, the alterations in the endogenous SOD activity after MPTP treatment remain to be elucidated.

The present study has shown the increased Cu/Zn-SOD immunoreactivity in the glial cells of the striatum and the substantia nigra on Days 1, 3 and 7 after the acute MPTP (20 mg/kg ip) treatment, but the treatment was marked on Days 3 and 7. However, striatal neurons has shown no changes in Cu/Zn-SOD immunoreactivity throughout the experiments. In contrast, nigral neurons have shown a decrease of Cu/Zn-SOD immunoreactivity from 1 to 7 days of MPTP treatment. These findings suggest that acute MPTP treatment can cause severe neuronal damage in the substantia nigra and the demand for Cu/Zn-SOD in glial cells may be greatly increased after MPTP treatment to quench the free radicals in surviving dopaminergic neurons. However, 1 day after the acute MPTP (20 mg/kg ip) treatment, Mn-SOD immunoreactivty was increased in the glial cells of the striatum. Thereafter, Mn-SOD-immunoreactive glial cells had a ramifid form with fine processes in the striatum after 3 and 7 days of MPTP treatment. In contrast, the striatal neurons showed no changes in Mn-SOD immunoreactivity throughout the experiments. Nigral interneurons showed intense Mn-SOD immunoreactivity after 1 and 3 days of MPTP treatment. Seven days after MPTP treatment, however, Mn-SOD immunoreactivity was reduced in interneurons of the substantia nigra. In addition, Mn-SOD immunoreactivity was reduced in nigral neurons from 1 to 7 days after MPTP treatment. Glial cells in the substantia nigra showed no changes in Mn-SOD immunoreactivity throughout the experiments. These findings suggest that acute MPTP treatment can cause severe neuronal damage in the nigral neurons and a functional damage in the nigral interneurons. Furthermore, the present study demonstrates that the intense Mn-SOD immunoreactivity as well as Cu/Zn-SOD in glial cells may be markedly increased after MPTP treatment to quench the free radicals in surviving neurons and interneurons of the substantia nigra.

We previously reported that reactive glial cells had intense immunoreactivies to both Cu/Zn-SOD and Mn-SOD after cerebral ischemia in gerbils (Kato et al., 1995). Furthermore, we confirmed that these reactive glial cells were both astrocytes and micloglia by means of comparison with GFAP immunostaining and isolectin B_4 staining. In the present study, however, we suggest that Cu/Zn-SOD may be expressed mainly in reactive astrocytes after the acute MPTP treatment. In contrast, Mn-SOD may be expressed predominantly in interneurons, microglia and astrocytes after the acute MPTP treatment by means of comparison with GFAP immunostaining and isolectin B_4 staining. Therefore, we speculate that Cu/Zn-SOD and Mn-SOD immunoreactivities in neurons, glial cells and interneurons after the acute MPTP treatment are expressed with different patterns and the glial SODs possibly help the surviving neurons and interneurons to resist attack from superoxide radicals produced by MPTP treatment. However, further detailed investigation is required to clarify our findings.

In conclusion, we have shown that the acute treatment of mice with MPTP is accompanied by sustained nigral degeneration and motor abnormalities. Furthermore, our immunohistochemical study suggests that altered capacity of free radical quenching may play a key role in the development of the damage to neurons and interneurons after MPTP neurotoxicity. Thus, our findings provide valuable information on age-related disease progression and mechanisms of neurodegeneration.

Acknowledgements

This study was supported, in part, by a Grant-in-Aid for Scientific Research (C) 13670627 from the Japan Society for the Promotion of Science.

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