Neuronal Nitric Oxide Synthase Inhibitor and Monoamine Oxidase B Inhibitor Attenuate MPTP Neurotoxicity

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Introduction

The loss of dopamine in the striatum as a result of neuronal degeneration in the substantia nigra pars compacta of Parkinson's disease (PD) has been considered to be the major pathological correlate of the motor symptoms such as akinesia and rigidity. So far, many studies have focused on neurochemical and neuropathological mechanisms in PD.

In humans and rodents, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is well known to produce clinical, biochemical and neurochemical changes similar to those which occur in PD[1,2]. This neurotoxin also leads to a marked decrease of dopamine contents in the striatum and a reduction in the number of nigrostriatal dopaminergic neurons in several species, including monkeys, dogs, cats and mice. In several experimental animals, especially the C57BL mouse strain, is known to be highly susceptible to the neurotoxic effects of MPTP and are useful as excellent models of PD[1,3].

The pathogenesis of neuronal degeneration following MPTP treatment has been extensively investigated. The neurotoxic effects of MPTP are thought to be mediated by its metabolite MPP^+(1-methyl-4-phenylpyridinium ion) which is caused by the oxidation of MPTP by monoamine oxidase B (MAO-B) in glial cells[4]. MPP^+ is selectively taken up by the high affinity dopamine uptake system and is subsequently accumulated within mitochondria of dopamine neurons. There it disrupts oxidative phosphorylation by inhibiting complex I of the electron transport chain in dopaminergic neurons[5]. This can lead to a number of deleterious effects on cellular function. These include impaired intracellular calcium buffering as well as generation of free radicals from mitochondria and

activation of neuronal nitric oxide synthase (nNOS), a calmodulin dependent enzyme[6]. Therefore, it is possible that the generation of the free radical nitric oxide followed by the peroxynitrate has been implicated in neuronal cell death[7-9].

Several studies previously reported that 7-nitroindazole, a relatively selective inhibitor of the nNOS, can protect against MPTP-induced neurotoxicity in experimental animals[10-12]. This was confirmed and extended by showing that mice deficient in the neuronal isoform of NOS are also resistant to MPTP neurotoxicity[13]. We recently reported that 7-nitroindazole can protect against MPTP-induced neurotoxicity in mice[14]. It protected against both dopamine depletions in the striatum and tyrosine hydroxylase positive neurons in mice. In the present study, we further examined whether 7-nitroindazole also can protect against tyrosine hydroxylase (TH)-, glial fibrillary acidic protein (GFAP)-, parvalbumin(PV)-, dopamine transporter (DAT)-, nNOS- or endothelial nitric oxide synthase (eNOS)- positive cells, in comparison with pargyline (a relatively selective inhibitor of the MAO-B).

Materials and Methods

2.1. Experimental animals

Male C57BL/6 mice (Nihon SLC Co.), 8 weeks of age, were used in the present study. The animals were housed in a controlled environment (23 ± 1^{0}C, $50 \pm 5\%$ humidity) and were allowed food and tap water *ad libitum*. The room lights were on between 8:00 and 20:00. All experiments were performed in accordance with the Guideline for Animal Experiments of the Tohoku University School of Medicine.

2.2. Experimental drugs

7-Nitroindazole (Research Biochemicals Int.), pargyline (Sigma) and MPTP (Sigma) were used in this study. 7-Nitroindazole (30 and 50 mg/kg) was suspended in peanut oil. Pargyline (5 and 15 mg/kg) or MPTP (10 mg/kg) was dissolved in saline. 7-Nitroindazole, pargyline or MPTP was given intraperitoneally (i.p.) in mice.

2.3. Experimental procedures

2.3.1. Analysis of contents of dopamine and its metabolites

The mice were injected i.p. four times with MPTP (10 mg/kg) at 1h intervals, the total dose per mouse being 40 mg/kg, as described previously[14,15]. The mice were killed

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by cerebral dislocation at 3 and 7 days after MPTP treatment. After cerebral dislocation, the striata were rapidly dissected out and sonicated in ice-cold 0.2M perchloric acid containing 100 ng/ml isoproterenol as an internal standard. Dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were quantified by high-performance liquid chromatography (HPLC) with an electrochemical detector (Eicom, Kyoto, Japan), as described previously[14,15]. Each group consisted of 6-9 mice. All values were expressed as mean±S.E. and statistical significance was evaluated using an analysis of variance (ANOVA) followed by Dunnett's multiple comparison test 2-side.

2.3.2. Effects of 7-nitroindazole or pargyline on the striatal dopamine and DOPAC content in MPTP-treated mice

Experiment 1

The animals were divided into 5 groups: (1) peanut oil-treated group (n=6); (2) 7-nitroindazole (50 mg/kg)-treated group (n=5); (3) MPTP- and peanut oil-treated group (n=6); (4) MPTP- and 7-nitroindazole (30 mg/kg)-treated group (n=6); (5) MPTP- and 7-nitroindazole (50 mg/kg)-treated group (n=6). The mice were injected i.p. with 7-nitroindazole or peanut oil 30 min before and 90 min after the first administration of MPTP. For groups (1) and (2), the peanut oil-treated or 7-nitroindazole-treated mice were injected i.p. in the same manner with saline instead of MPTP.

Experiment 2

The animals were divided into 5 groups: (6) saline-treated group (n=6); (7) pargyline (15 mg/kg)-treated group (n=5); (8) MPTP- and saline-treated group (n=6); (9) MPTP- and pargyline (5 mg/kg)-treated group (n=6); (10) MPTP- and pargyline (15 mg/kg)-treated group (n=6). The mice were injected i.p. with pargyline or saline 30 min before and 90 min after the first administration of MPTP. For groups (6) and (7), the saline-treated or pargyline-treated animals were injected i.p. in the same manner with saline instead of MPTP.

The mice were killed by cerebral dislocation 3 days after MPTP treatment. Dopamine and DOPAC were quantified by HPLC with an electrochemical detector, as described above. Each group consisted of 5-6 mice. All values were expressed as mean±S.E. and statistical significance was evaluated using an analysis of variance (ANOVA) followed by Dunnett's multiple comparison test 2-side.

2.3.3. Immunohistochemistry

The mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) 3 and 7 days after MPTP treatment, and the brains were perfusion-fixed with 4% paraformaldehyde in 0.1M 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 in the same fixative until they were embedded in paraffin. Paraffin sections, 5 μm, of the striatum and substantia nigra were used for immunohistochemistry. In addition, the mice that received treatment with MPTP and 7-nitroindazole (50 mg/kg, i.p.) or pargyline (15 mg/kg, i.p.) were also treated in the same way, as described above. Each group consisted of 5 animals.

For immunohistochemical studies, a Vectastain *elite* ABC kit (Vector Labs.), M.O.M. immunodetection kit (Vector Labs.), rabbit anti-TH polyclonal antibody (Chemicon International, Inc.), mouse anti-GFAP monoclonal antibody (Chemicon International, Inc.), mouse anti-PV monoclonal antibody (Chemicon International, Inc.), rabbit anti-DAT polyclonal antibody (Alpha Diagnositic international.), rabbit anti-nNOS polyclonal antibody (Zymed Laboratories Inc.) and rabbit anti-eNOS polyclonal antibody (Affinity BioReagents Inc.) were used.

Briefly, the paraffin sections were washed for 5 min in 0.01M phosphate-buffered saline (PBS, pH 7.4) and treated with 3% hydrogen peroxide in 0.01M PBS. The paraffin sections were then washed twice for 2 min each time in 0.01M PBS, followed by 60 min of pre-incubation with mouse IgG blocking reagent. The brain sections were then incubated with or anti-PV (1:500) antibody including M.O.M. diluent overnight at 4°C. After a 5 min rinse in 0.01M PBS, the sections were incubated with biotinylated secondary IgG antibody for 10 min and then with avidin-biotin peroxidase complex for 30 min at room temperature. Immunoreactivity was visualized using enzyme substrate kits (Vector Lab. Burlingame, CA, USA). Negative control sections were treated in the same way, except that the antibodies against anti-PV were omitted. For TH, GFAP, DAT, nNOS and eNOS immunostaining, a Vectastain *elite* ABC kit was used. Immunohistochemical staining with anti-TH (1:200), anti-GFAP(1:200), anti-nNOS (1:100) and anti-eNOS (1:300) antibody was performed as described previously[14,15].

Results

3.1. Effects of 7-nitroindazole or pargyline on the MPTP-induced decreases in the striatal dopamine and DOPAC content in mice

As shown in Tables 1 and 2, four administrations of MPTP to mice produced a marked depletion in the dopamine, and DOPAC content of the striatum. Striatal dopamine and DOPAC levels were significantly decreased 3 and 7 days after MPTP treatment in mice. The depletion in the striatal dopamine and DOPAC concentrations reached a maximal levels 3 days after MPTP treatment. In contrast, 7-nitroindazole dose-dependently prevented the significant reduction in the striatal dopamine and DOPAC levels 3 days after MPTP treatment. Pargyline also prevented the significant reduction in the striatal dopamine levels 3 days after MPTP treatment in a dose-dependent manner . However, pargyline showed no significant changes in the striatal DOPAC levels after MPTP treatment. In addition, 7-nitroindazole showed no significant changes in the striatal dopamine and DOPAC contents in mice. However, pargyline had marked increases in the striatal dopamine content and decreases in DOPAC content in mice.

3.2. Immunohistological changes of 7-nitroindazole or pargyline on the striatum and substantia nigra of MPTP-treated mice

TH immunostaining

Representative photomicrographs of TH immunostaining in the striatum and substantia nigra are shown in Fig. 1. Striatal TH-immunoreactive fibers and nigral TH-immunoreactive neurons were easily detectable in vehicle-treated mice, respectively. The bodies and fibers of dopaminergic cells were intensely stained with evident immunopositive processes. In the striatum, a decrease in the number of TH-immunopositive fibers was observed 3 and 7 days after MPTP treatment. In the substantia nigra, a decrease in the number of TH-immunopositive neurons was also observed 3 and 7 days after MPTP treatment. In contrast, 7-nitroindazole and pargyline prevented the decreases in number of the striatum TH-immunopositive fibers and nigral TH-immunopositive neurons 3 and 7 days after MPTP treatment in mice.

GFAP immunostaining

Representative photomicrographs of GFAP immunostaining in the striatum and substantia nigra are shown in Fig. 2. GFAP-immunoreactive astrocytes were absent in the

striatum of vehicle-treated mice. In the substantia nigra, however, GFAP-immunoreactive astrocytes had a ramified form with many fine processes in control mice. In the striatum, the number of GFAP-immunopositive astrocytes increased 3 days after MPTP treatment. Seven days after MPTP treatment, GFAP-immunopositive astrocytes exhibited a ramified form with many fine processes in the striatum, which were markedly increased in this region. In the substantia nigra, marked increases in the number of GFAP-immunopositive astrocytes were noted in mice 3 and 7 days after MPTP treatment. In contrast, 7-nitroindazole and pargyline reduced the increases in number of the striatal and nigral astrocytes 3 and 7 days after MPTP treatment.

PV immunostaining

Representative photomicrographs of PV immunostaining in the striatum and substantia nigra are shown in Fig. 3. Striatal PV-immunoreactive cells were easily detectable in vehicle-treated mice. The bodies and fibers of PV-immunoreactive cells were stained intensely with evident immunopositive processes. In the striatum, PV-immunopositive cells were unchanged throughout the experiment. In the substantia nigra, marked decreases of the immunostaining of PV-positive cells were noted in the substantia nigra 3 and 7 days after MPTP treatment. In contrast, 7-nitroindazole and pargyline did not affect the striatal PV-immunopositive neurons 3 and 7 days after MPTP treatment. However, both compounds prevented the decrease of immunoreactivity of PV-immunopositive neurons in the substantia nigra of mice 3 and 7 days after MPTP treatment.

DAT immunostaining

Representative photomicrographs of DAT immunostaining in the striatum and substantia nigra are shown in Fig. 4. DAT was localized in the plasma membranes of axons and terminals. In the substantia nigra, the bodies and fibers of dopaminergic cells were stained intensely with evident immunopositive processes. In the striatum, a decrease in the number of DAT-immunopositive fibers was observed in MPTP-treated mice 3 days after MPTP treatment. Seven days after MPTP treatment, marked reductions in the number of the DAT-immunopositive fibers were noted in mice 3 and 7 days after MPTP treatment. In the substantia nigra, marked reductions in the number of the DAT-immunopositive neurons were found in mice 3 and 7 days after MPTP treatment. In contrast, 7-nitroindazole showed mild reductions in the striatal DAT- immunopositive fibers

3 days after MPTP treatment, although this compound prevented marked decreases in the number of nigral DAT-immunopositive neurons. On the other hand, pargyline prevented decreases of the striatal DAT-immunopositive fibers and nigral DAT-immunopositive neurons 3 days after MPTP treatment. Seven days after MPTP treatment, both compounds prevented marked decreases in the striatal DAT-immunopositive fibers and nigral DAT-immunopositive neurons after MPTP treatment.

nNOS immunostaining

Representative photomicrographs of nNOS immunostaining in the striatum and substantia nigra are shown in Fig. 5. nNOS-immunoreactive cells were detectable in the striatum and substantia nigra of vehicle-treated mice. The bodies and fibers of nNOS-immunoreactive cells were stained intensely with evident immunopositive processes. In the striatum, nNOS-immunopositive cells were unchanged throughout the experiment. In the substantia nigra, marked decreases of the immunostaining of nNOS-positive cells were noted 3 and 7 days after MPTP treatment. In contrast, 7-nitroindazole and pargyline did not affect the striatal eNOS-immunopositive neurons 3 and 7 days after MPTP treatment. However, both compounds prevented the decrease of immunoreactivity of nNOS-immunopositive neurons in the substantia nigra of mice 3 and 7 days after MPTP treatment.

eNOS immunostaining

Representative photomicrographs of eNOS immunostaining in the striatum and substantia nigra are shown in Fig. 6. NNOS-immunoreactive cells were detectable in the striatum and substantia nigra of vehicle-treated mice. The bodies and fibers of eNOS-immunoreactive cells were stained intensely with evident immunopositive processes. In the striatum and substantia nigra, eNOS-immunopositive cells were unchanged in MPTP-treated mice throughout the experiment. 7-Nitroindazole and pargyline did not affect the striatal and nigral eNOS-immunopositive neurons 3 and 7 days after MPTP treatment.

Discussion

MPTP causes the biochemical, neuropathological and clinical features of PD[16,17]. The pathogenesis of the lesions appears to be inhibition of complex I of the electron transport chain by MPP^+ as well as free radical production[4]. A role of NO in the pathogenesis of the lesions has been supported by many studies. Previous studies

demonstrated that MPTP neurotoxicity was markedly attenuated by 7-nitroindazole in mice[10,13]. Furthermore, MPTP neurotoxicity was attenuated in mice deficient in nNOS[13]. In primates, 7-nitroindazole is known to exert profound neuroprotective effects against MPTP neurotoxicity[11]. A interesting previous study suggested that both striatal lesion volume and substantia nigra degeneration caused by injection of MPP^+ were significantly attenuated in the nNOS mutant mice but not in the eNOS mutant mice[18]. Based on these observations, it is conceivable that neuronally derived NO and peroxynitrite play a key role in the pathogenesis of MPTP neurotoxicity. However, there are a few reports about the immunohistochemical examination for the effect of 7-nitroindazole against MPTP neurotoxicity. In the present study, therefore, we examined whether 7-nitroindazole also can protect against TH-, GFAP-, PV-, DAT-, nNOS- and eNOS-positive cells, in comparison with pargyline as a relatively selective inhibitor of the MAO-B.

The present study showed that four administrations of MPTP to mice caused marked depletions in the dopamine and DOPAC content of the striatum 3 and 7 days after MPTP treatment. The depletion in the striatal dopamine and DOPAC concentrations reached a maximal levels 3 days after MPTP treatment. Therefore, we investigated the effect of 7-nitroindazole and pargyline on the striatal dopamine and DOPAC content 3 days after MPTP treatment. The present study showed that 7-nitroindazole dose-dependently prevented the significant reduction in the striatal dopamine and DOPAC levels 3 days after MPTP treatment. Pargyline also prevented the significant reduction in the striatal dopamine levels 3 days after MPTP treatment in a dose-dependent manner. However, pargyline showed no significant changes in the striatal DOPAC levels after MPTP treatment. Therefore, our results indicate that 7-nitroindazole and pargyline can protect against MPTP-induced striatal dopamine depletion in mice. The findings are consistent with previous reports[10,13,14]. These findings demonstrate that nNOS inhibitor or MAO-B inhibitor may be useful in the treatment of PD.

To examine exactly the effects of 7-nitroindazole against MPTP neurotoxicity, we investigated the immunohistochemical changes of TH-, GFAP-, PV-, DAT-, nNOS- and eNOS-positive cells, in comparison with pargyline. The present study showed that severe reductions in levels of TH and DAT immunoreactivity were observed in the striatum and substantia nigra 3 and 7 days after MPTP treatment. These results suggest that MPTP can cause severe damage in the striatum and substantia nigra as well as the reduction of the striatal dopamine and DOPAC content. 7-Nitroindazole and pargyline showed a protective

effect against the severe reductions in TH immunoreactivity in the striatum and substantia nigra 3 and 7 days after MPTP treatment. However, 7-nitroindazole showed mild decreases in the striatal DAT-immunopositive fibers 3 days after MPTP treatment, although this compound prevented marked decreases in the number of nigral DAT-immunopositive neurons. On the other hand, pargyline prevented decreases of the striatal DAT-immunopositive fibers and nigral DAT-immunopositive neurons 3 days after MPTP treatment. Seven days after MPTP treatment, both compounds prevented marked decreases in the striatal DAT-immunopositive fibers and nigral DAT-immunopositive neurons after MPTP treatment.

DAT terminates dopaminergic neurotransmission by accumulating neurotransmitter from synaptic cleft into the presynaptic terminal. DAT is also known to accumulate the neurotoxic MPTP or MPP$^+$, into dopaminergic neurons, results in dopaminergic cell death and parkinsonian syndrome that is similar to idiopathic PD. In the present study, we observed that DAT immunoreactivity especially recognized in the striatum and substantia nigra. Therefore, the present study demonstrates that MPTP can damage selectively the striatal DAT-immunopositive fibers and nigral DAT-immunopositive neurons. However, 7-nitroindazole showed mild decreases in the striatal DAT-immunopositive fibers 3 days after MPTP treatment, whereas this compound prevented marked decreases in the number of nigral DAT-immunopositive neurons. The reason for this phenomenon is presently unclear. For this reason, we speculate that 7-nitroindazole may attenuate the neurotoxic effect of MPTP against the remaining DAT-immunopositive fibers in the striatum. However, the precise mechanisms for our findings remain to be elucidated in further studies.

GFAP is well known to be a good marker for reactive astrocytes in the response to the CNS injury, due to specificity it in astrocytes[19,20]. Stromberg et al.[21] and Francis et al.[22] have found astroglial reaction in the striatum, which started 48 h after MPTP treatment and was still observed 6 weeks following the treatment. In the present study, marked increases in the number of GFAP-immunopositive astrocytes were found in the striatum and substantia nigra 3 and 7 days after MPTP treatment. In contrast, the present study showed that 7-nitroindazole and pargyline can prevent the increases in the number of GFAP-immunopositive astrocytes 3 and 7 days after MPTP treatment. From these observations, we suggest that increases in GFAP-immunopositive astrocytes may reflect compensatory action against neuronal cell damage after MPTP treatment. Therefore, it is conceivable

that reactive astrocytes may play a key role in the maintenance of injury areas caused by MPTP neurotoxicity.

PV belongs to a family of homologous calcium binding proteins and is widely distributed throughout the (CNS)[23,24]. PV has also been regarded as a superior marker for a subpopulation of GABA (γ-aminobutyric acid) ergic interneurons throughout the brain[25]. Furthermore, a previous study demonstrated that less than 1% of the TH-immunopositive neurons were immunoreactive for the calcium binding proteins PV and calbindin in the primate striatum[26]. From these observations, it is possible that PV immunoreactivity is mainly expressed in the interneurons in the brain. In the present study, PV-immunopositive cells were unchanged in the striatum throughout the experiments. In the substantia nigra, however, marked decreases of the immunostaining of PV-positive cells were noted 3 and 7 days after MPTP treatment. The present results suggest a functional damage in PV-immunopositive interneurons in the substantia nigra after MPTP treatment. However, our study indicates that the PV-immunopositive interneurons are unchanged in the striatum after MPTP treatment. A previous study suggested that the amount of PV immunoreactivity within the nigral interneurons is mildly reduced by end-stages of PD[27]. These observations are, at least in part, consistent with our findings, suggesting a functional damage in PV-immunopositive interneurons in the substantia nigra of mice after MPTP treatment. In the present study, 7-nitroindazole and pargyline showed no significant changes in the striatum throughout the experiments. In contrast, both compounds prevented marked decreases of the immunostaining of PV-positive cells 3 and 7 days after MPTP treatment. These results suggest that 7-nitroindazole and pargyline can protect the functional damage of interneurons in the substantia nigra after MPTP treatment. However, further studies are needed to investigate the precise mechanism for our interesting findings.

The present study demonstrated that striatal nNOS-immunopositive cells were unchanged in MPTP-treated mice throughout the experiments. In the substantia nigra, in contrast, marked decreases of the immunoreactivity of nNOS-positive cells were noted 3 and 7 days after MPTP treatment. The nNOS-immunoreactive cells are well known to be interneurons in the hippocampus. The present study showed that 7-nitroindazole and pargyline prevented marked decreases of the immunostaining of nNOS-positive cells in the substantia nigra 3 and 7 days after MPTP treatment. These results demonstrate that 7-nitroindazole and pargyline can protect against the functional damage of nNOS-immunoreactive interneurons in the substantia nigra after MPTP treatment, although further

studies are needed to investigate the precise mechanism for our findings. In the striatum and substantia nigra, in contrast, eNOS-immunopositive cells were unchanged in MPTP-treated mice throughout the experiments. Furthermore, 7-nitroindazole and pargyline showed no significant changes of eNOS-immunopositive cells in the striatum and substantia nigra throughout the experiments. From these results, we speculate that nNOS play a key role in the pathogenesis of MPTP neurotoxicity, in comparison with eNOS. Matthews et al.[18] reported previously that MPP[+]-induced neuronal degeneration was significantly attenuated in the nNOS-deficient mice but not in eNOS-deficient mice. Furthermore, a previous report demonstrated that in nNOS-deficient mice, infarct volumes decreased significantly after middle cerebral artery occlusion[28]. These observations are, at least in part, consistent with our findings. Base on these observations, our data support that neuronal NO production contributes to the development of MPTP- or MPP[+]-induced neurodegeneration of nigral neurons.

In the present study, it is interestingly that the changes of nNOS-immunopositive cells in the striatum and substantia nigra were similar to these of PV-immunopositive neurons after MPTP treatment. Both nNOS and PV-immunopositive cells are known to be interneurons in the brain. Therefore, we speculate that the functional changes of interneurons also may play a key role in the pathogenesis of MPTP-induced neurotoxicity, although further studies should be performed to investigate the precise mechanisms responsible for the present findings.

In conclusion, our results show that nNOS inhibitor as well as MAO-B inhibitor has a dose-dependent protective effect against MPTP-induced striatal dopamine and DOPAC depletion in mice. These protective effects may be , at least in part, produced by the reduction of neuronally derived NO and peroxynitrite caused by MPTP. Furthermore, our results demonstrate that MPTP can cause the functional damage of interneurons in the substantia nigra. These results suggest possibility that nNOS inhibitors as well as MAO-B inhibitors may be therapeutically useful in neurodegenerative diseases such as PD, although further studies will be needed to elucidate our findings.

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Table 1. Striatal contents of dopamine and DOPAC in mice after MPTP treatment.

	Dopamine	DOPAC
	(μg/g tissue)	
Control	10.16±0.37	2.74±0.14
3 days	2.28±0.26*	1.76±0.29*
7 days	5.67±0.56*	2.29±0.24*

Values were expressed as mean±S.E. of 6-9 mice. The mice received four intraperitoneal (i.p.) injections of MPTP (10 mg/kg) at 1h intervals. *$P<0.01$ vs. control group (Dunnett's multiple comparison test).

Table 2. Neuroprotective effects of 7-nitroindazole and pargyline on the striatal dopamine and DOPAC contents in mice after MPTP treatment.

Treatment	Dopamine	DOPAC
	(μg/g tissue)	
Experiment 1 (Effect of 7-nitroindazole)		
Vehicle (peanut oil)	8.99±0.49**	3.64±0.50**
7-Nitroindazole (50 mg/kg)	9.80±0.31**	5.31±0.38**
MPTP+vehicle (peanut oil)	3.36±0.31	1.78±0.15
MPTP+7-nitroindazole (30 mg/kg)	6.94±0.81**	3.00±0.21*
MPTP+7-nitroindazole (50 mg/kg)	8.64±0.58**	3.54±0.20**
Experiment 2 (Effect of pargyline)		
Vehicle (saline)	9.69±0.70**	4.62±0.58**
Pargyline (15 mg/kg)	16.32±0.67**	1.56±0.29
MPTP+vehicle (saline)	2.36±0.59	2.44±0.50
MPTP+pargyline (5 mg/kg)	15.02±0.59**	2.50±0.25
MPTP+pargyline (15 mg/kg)	16.63±0.91**	1.05±0.34

Values were expressed as mean±S.E. of 5-6 mice. Drug treatment schedules were described in Materials and methods section. *$P<0.05$, **$P<0.01$ vs. MPTP+vehicle group (Dunnett's multiple comparison test).
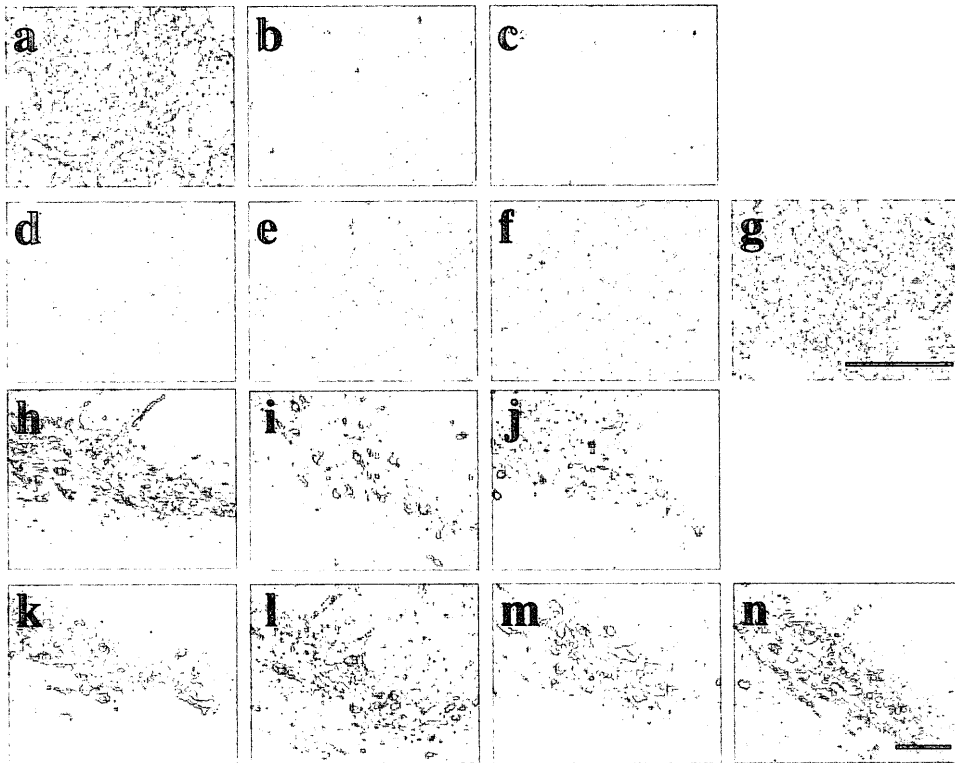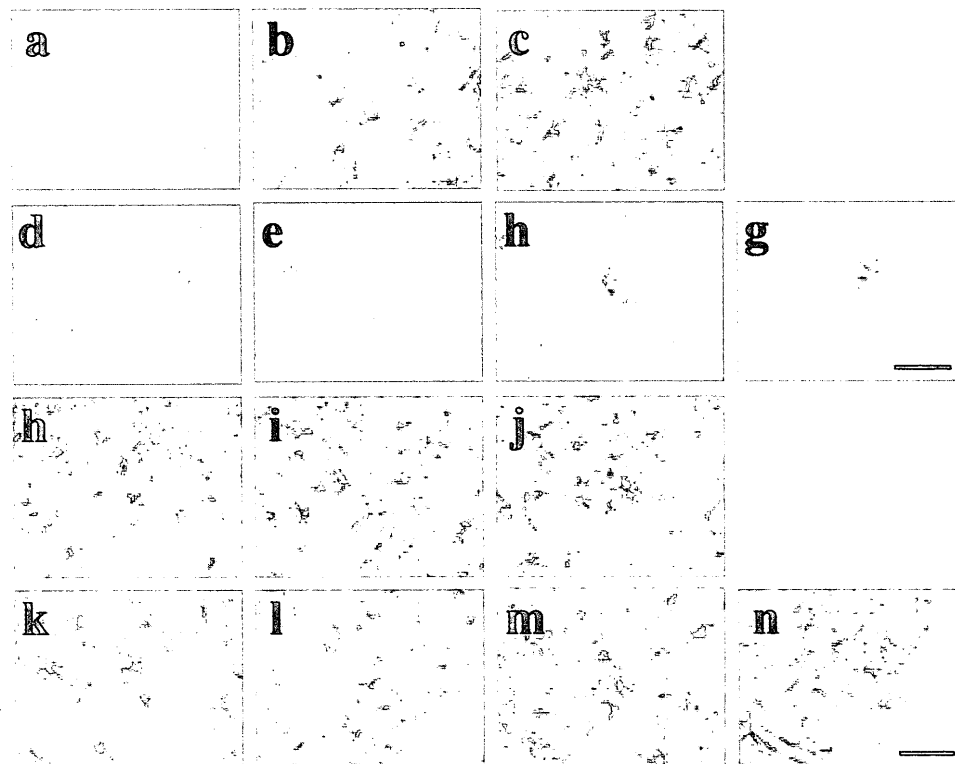
Fig. 1. Representative microphotographs of TH immunostaining in the mouse striatum (a-g) and substantia nigra (h-n) after MPTP treatment. (a, h) Control (vehicle) group. (b, i) 3 days after MPTP treatment. (c, j) 7 days after MPTP treatment. (d, k) 3 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (e, l) 7 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (f, m) 3 days after MPTP and pargyline (15 mg/kg) treatment. (g, n) 7 days after MPTP and pargyline (15 mg/kg) treatment. Bar (a-g)= 100 μm. Bar (h-n)= 100 μm. n=5.
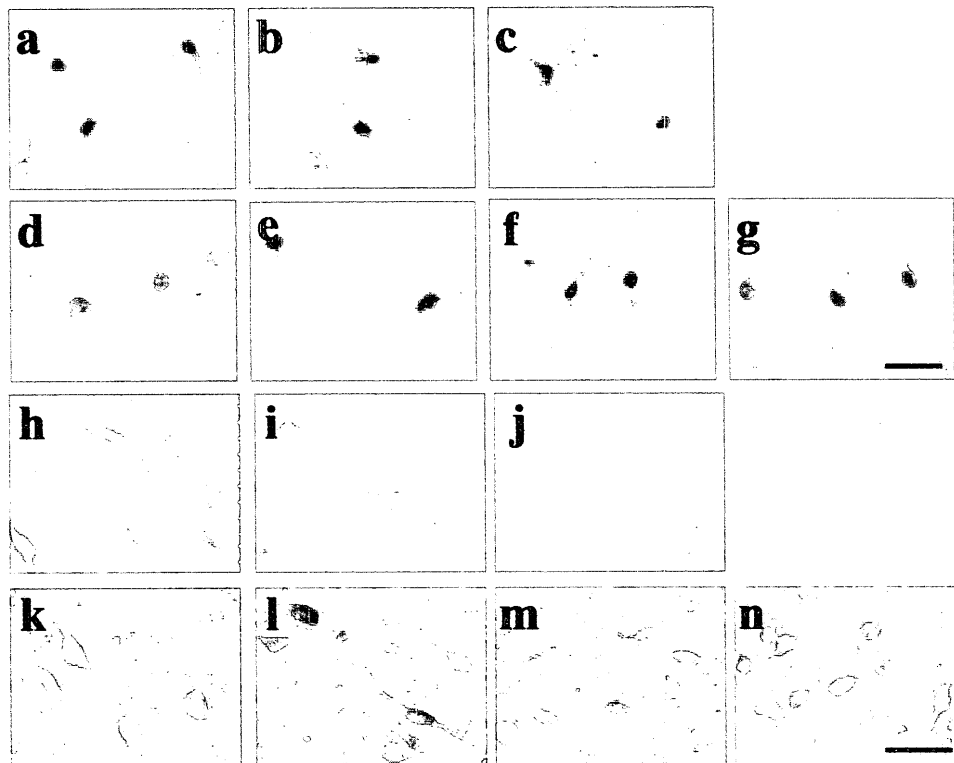

Fig. 2. Representative microphotographs of GFAP immunostaining in the mouse striatum (a-g) and substantia nigra (h-n) after MPTP treatment. (a, h) Control (vehicle) group. (b, i) 3 days after MPTP treatment. (c, j) 7 days after MPTP treatment. (d, k) 3 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (e, l) 7 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (f, m) 3 days after MPTP and pargyline (15 mg/kg) treatment. (g, n) 7 days after MPTP and pargyline (15 mg/kg) treatment. Bar (a-g)= 50 μm. Bar (h-n)= 50 μm. n=5.

Fig. 3. Representative microphotographs of PV immunostaining in the mouse striatum (a-g) and substantia nigra (h-n) after MPTP treatment. (a, h) Control (vehicle) group. (b, i) 3 days after MPTP treatment. (c, j) 7 days after MPTP treatment. (d, k) 3 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (e, l) 7 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (f, m) 3 days after MPTP and pargyline (15 mg/kg) treatment. (g, n) 7 days after MPTP and pargyline (15 mg/kg) treatment. Bar (a-g)= 50 μm. Bar (h-n)= 50 μm. n=5.
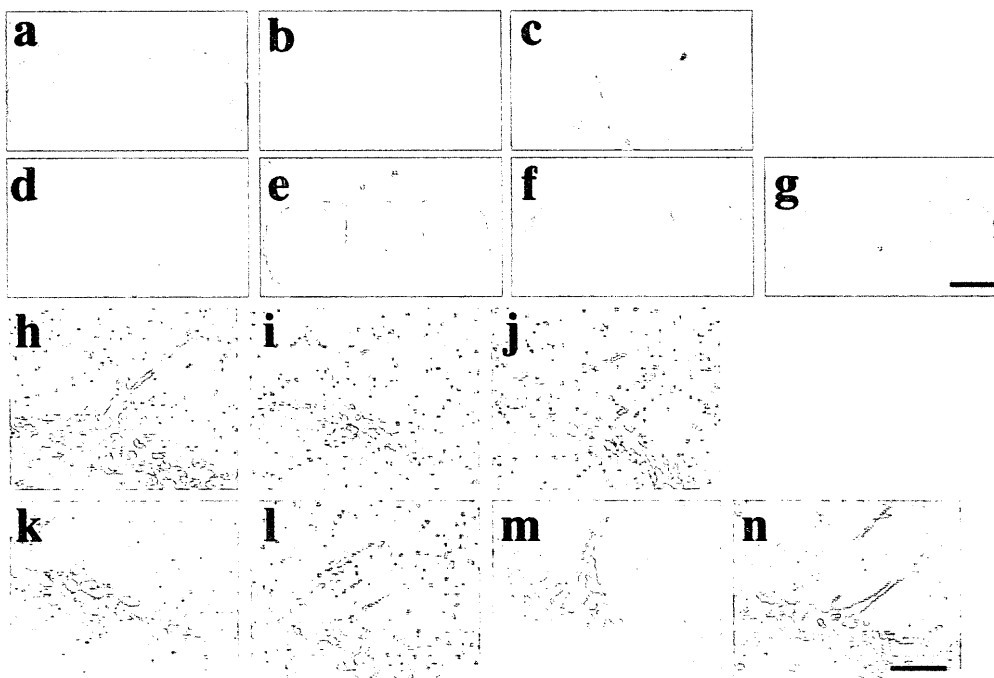

Fig. 4. Representative microphotographs of DAT immunostaining in the mouse striatum (a-g) and substantia nigra (h-n) after MPTP treatment. (a, h) Control (vehicle) group. (b, i) 3 days after MPTP treatment. (c, j) 7 days after MPTP treatment. (d, k) 3 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (e, l) 7 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (f, m) 3 days after MPTP and pargyline (15 mg/kg) treatment. (g, n) 7 days after MPTP and pargyline (15 mg/kg) treatment. Bar (a-g)= 1 mm. Bar (h-n)= 100 μm. n=5.
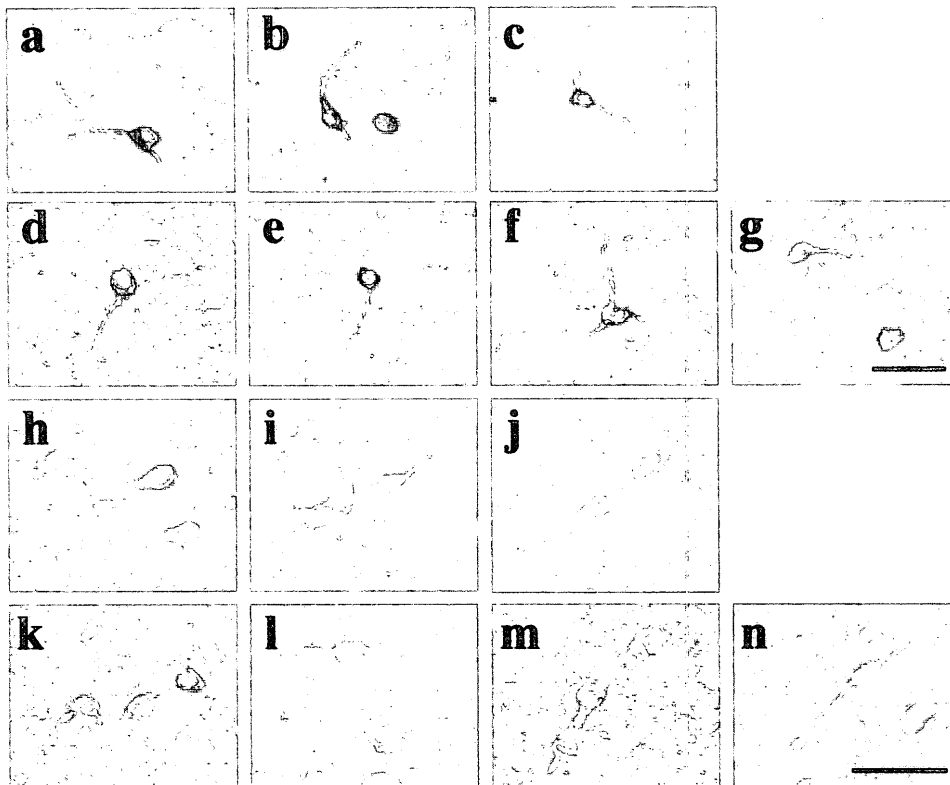
Fig. 5. Representative microphotographs of nNOS immunostaining in the mouse striatum (a-g) and substantia nigra (h-n) after MPTP treatment. (a, h) Control (vehicle) group. (b, i) 3 days after MPTP treatment. (c, j) 7 days after MPTP treatment. (d, k) 3 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (e, l) 7 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (f, m) 3 days after MPTP and pargyline (15 mg/kg) treatment. (g, n) 7 days after MPTP and pargyline (15 mg/kg) treatment. Bar (a-g)= 50 μm. Bar (h-n)= 50 μm. n=5.
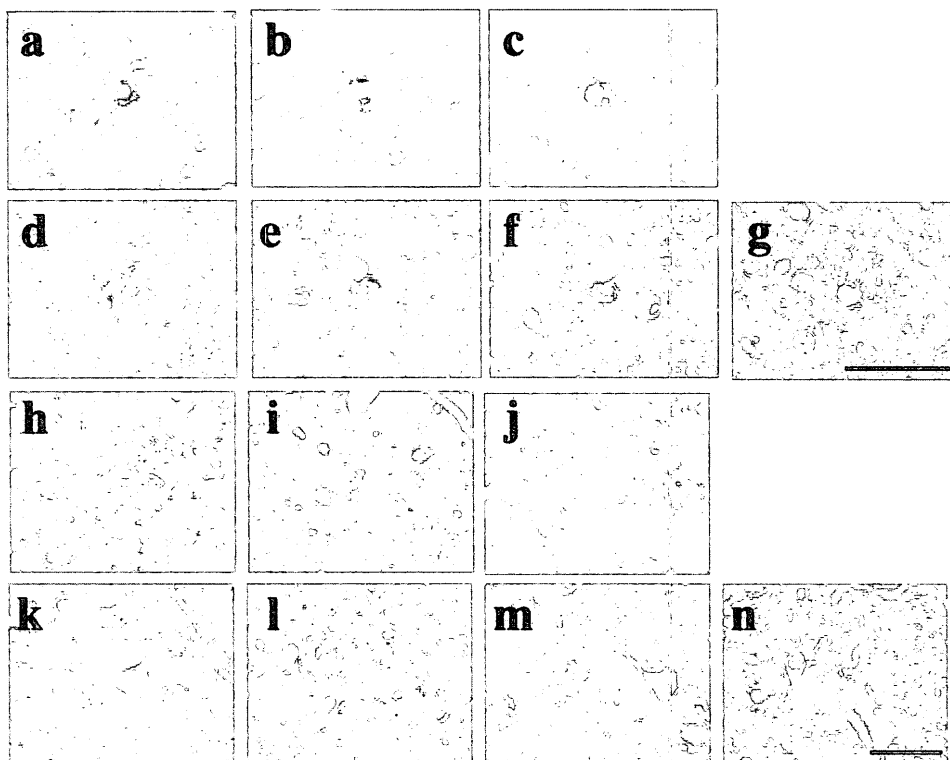

Fig. 6. Representative microphotographs of eNOS immunostaining in the mouse striatum (a-g) and substantia nigra (h-n) after MPTP treatment. (a, h) Control (vehicle) group. (b, i) 3 days after MPTP treatment. (c, j) 7 days after MPTP treatment. (d, k) 3 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (e, l) 7 days after MPTP and 7-nitroindazole (50 mg/kg) treatment. (f, m) 3 days after MPTP and pargyline (15 mg/kg) treatment. (g, n) 7 days after MPTP and pargyline (15 mg/kg) treatment. Bar (a-g)= 50 μm. Bar (h-n)= 50 μm. n=5.