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### III. 6. Biochemical changes in the brain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -treated mouse

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#### Introduction

Parkinson's disease is mainly characterized by the progressive loss of dopaminergic neurons in the substantia nigra that projects to the striatum. So far, many studies have focused on neurochemical and neuropathological mechanisms in this disease. The selective neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is well known to deplete striatal dopamine and cause neuronal degeneration of the nigrostriatal pathway when administered to humans, non-human primates and rodents. The resulting neurochemical and histopathological deficits are similar to those observed in idiopathic Parkinson's disease<sup>1-3</sup>). Some of these studies have demonstrated that mice, especially the C57BL mouse strain, are highly susceptible to the neurotoxic effects of MPTP and are useful as animal models of Parkinson's disease<sup>3,4</sup>).

The neurotoxic effects of MPTP are thought to be initiated by the 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>), which is metabolite formed by monoamine oxidase (MAO)-B mediated oxidation of MPTP. MPP<sup>+</sup> is known to be actively accumulated by dopaminergic neurons, where it is further concentrated within mitochondria by an energy-dependent mechanism. The inhibition of mitochondrial electron transport at Complex I (NADH-ubiquinone oxidoreductase) results in decreased oxygen consumption and ATP production and a disruption of ion homeostasis<sup>5-7</sup>). It is also suggested that the oxidative stress produced by MPP<sup>+</sup> may potentiate its toxicity to dopaminergic neurons<sup>8,9</sup>). Furthermore, recent studies suggest that the toxic effects of MPP<sup>+</sup> are mediated, in part, through an excessive production of nitric oxide (NO)<sup>10,11</sup>). Based on these observations, it is conceivable that MPTP-treated mouse model may be useful for evaluating brain functions in Parkinson's disease. However, little is known about the acute changes of brain functions in mice after MPTP treatment. In the present study, therefore, we conducted to examine the biochemical changes in the mouse brain after acute treatment with MPTP.

## Materials and Methods

Male C57BL/6 mice (22-28 g) were used in this study. The mice received intraperitoneal four injections of MPTP (10 mg/kg) at 1h intervals, the total dose per mouse being 40 mg/kg. In the present study, there were no died animals after MPTP treatment. The mice were sacrificed by cervical dislocation at 3 and 7 days after the last injection for biochemical studies as described below.

### *Receptor autoradiography*

The mice were sacrificed by cervical dislocation at 3 and 7 days after MPTP treatment, and the brains were quickly removed, frozen in powdered dry-ice and stored at -80 °C until receptor assay. Coronal sections, 12 µm in thickness, were cut at the level of the striatum and the substantia nigra of MPTP-treated and control mouse brains on a cryostat and thaw-mounted onto silane-coated cover glasses.

Autoradiographic localization of dopamine D<sub>1</sub> receptors was detected using [<sup>3</sup>H]SCH23390 according to the method of Dawson et al<sup>12)</sup> with minor modifications<sup>13)</sup>. The sections were incubated with 1 nM [<sup>3</sup>H]SCH23390 (specific activity 70.3 Ci/mmol, New England Nuclear) in 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> for 30 min at room temperature. After incubation, the sections were then dipped in fresh buffer at 4 °C, followed by 25-min rinses in fresh buffer at 4 °C. Non-specific binding was determined using 1 µM non-labeled SCH23390 (Research Biochemicals Int.).

Autoradiographic distribution of dopamine D<sub>2</sub> receptors was determined using [<sup>3</sup>H]raclopride according to the method of Köhler and Radesäter<sup>14)</sup> with minor modifications. The sections were incubated with 3 nM [<sup>3</sup>H]raclopride (specific activity 79.3 Ci/mmol, New England Nuclear) in 170 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> for 30 min at room temperature. After incubation, the sections were washed four times in fresh buffer for 1 min at 4 °C and dipped in distilled water at 4 °C. Non-specific binding was determined using 10 µM haloperidol (Sigma).

Autoradiographic localization of dopamine uptake sites was detected using [<sup>3</sup>H]mazindol according to the method of Przedborski et al<sup>15)</sup> with minor modifications<sup>16)</sup>. The sections were pre-incubated for 15 min at 4 °C in 50 mM Tris-HCl buffer (pH 7.9) containing 120 mM NaCl and 5 mM KCl. The sections were then incubated with 15 nM [<sup>3</sup>H]mazindol (New England Nuclear, specific activity 24 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.9) containing 300 nM NaCl, 5 mM KCl and 0.3 µM desmethylimipramine (Sigma). Desmethylimipramine was added to block the binding of [<sup>3</sup>H]mazindol to norepinephrine uptake sites, as described previously<sup>17)</sup>. After incubation, the sections were washed twice in fresh buffer for 3 min at 4 °C and dipped in ice-cold distilled water. Non-specific binding was determined using 30 µM benztropine (Sigma).

### *Data analysis*

The sections were quickly dried under a cold air stream and then exposed for 1-3 weeks with [<sup>3</sup>H]-labeled graded standards (Amersham) to tritium-sensitive imaging plates (Fuji Photo Film, Japan) coated with minutes crystals of photostimulable phosphor. A computer-assisted image-processing system, BAS5000 (Fuji Photo Film, Japan), was used for the quantitative analysis of radioactivity. Regions of interest (ROIs) on the autoradiograms were placed at the dorsolateral and ventromedial parts of the striatum separately and in the whole substantia nigra according to an atlas of mouse brain. The radioactivity of each ROI was quantified using the calibration lines obtained from [<sup>3</sup>H]-labeled graded standards, and the values for radioactivities were converted to fmol/mg tissue. Specific binding activities of each ligand in the striatum and substantia nigra were calculated by subtracting the non-specific binding from the total binding. All values were expressed as means±S.E. and statistical significance was evaluated using an analysis of variance (ANOVA) followed by Dunnett's multiple range test (two-side).

### *Measurement of dopamine and its metabolite*

The mice were killed by cervical dislocation at 3 and 7 days after MPTP treatment. After decapitation, brains were quickly removed and the two striata were rapidly dissected out freehand on an ice-cold glass Petri dish. Samples were immediately weighted, then frozen and stored at -80 °C until assay. The dissection procedure was performed in less than 2 min. Striata were sonicated ice-cold 0.2 M per chloric acid containing 100 ng/ml isoproterenol as internal standard. Homogenates were centrifuged at 2,500 rpm for 15 min at 4 °C. The supernatant was filtered (pore size 0.45 µm, Millipore filter) and a 30 µl aliquot of the supernatant was used for determination the dopamine, 3,4-dihydroxyphenyl acetic acid (DOPAC) and isoproterenol by high-performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (Eicom, Japan). The mobile phase consisted of 0.1 M sodium citrate-0.1 M sodium acetate solution (pH 3.5) including 1.064 M octane sulfonic acid 0.013 mM EDTA 2Na and 15% (v/v) methanol. The recoveries of dopamine, DOPAC and isoproterenol through the present procedures were > 93%. Levels of dopamine and its metabolite were calculated from the comparison of simple peak area with internal standard peak region and were expressed as µg/g tissue weight. Each group contained 5-9 mice. All values were expressed as means±S.E. and statistical significance was evaluated using an analysis of variance (ANOVA) followed by Dunnett's multiple range test (two-side).

## **Results**

### *Receptor autoradiography*

[<sup>3</sup>H]SCH23390 binding showed no significant change in either the dorsolateral and ventromedial striatum or the substantia nigra of MPTP-treated mice compared with saline-

treated group for the 7 days after MPTP treatment (Table 1).

[<sup>3</sup>H]Raclopride binding showed no significant change in both the striatum and substantia nigra 3 days after MPTP treatment, as compared with saline-treated group. Seven days after MPTP administration, a significant decrease in [<sup>3</sup>H]raclopride binding was observed in the substantia nigra. However, the striatum showed no significant change in [<sup>3</sup>H]raclopride binding (Table 1).

[<sup>3</sup>H]Mazindol binding showed a marked reduction in the striatum 3 and 7 days after MPTP treatment, as compared with saline-treated group. In the substantia nigra, a significant decrease in [<sup>3</sup>H]mazindol binding was also observed 3 and 7 days after MPTP administration (Table 1).

### *Dopamine and its metabolite*

Striatal levels of dopamine and DOPAC are illustrated in Fig. 1. In mice subjected to MPTP, the dopamine concentrations were markedly decreased (82.2%) in the striatum 3 days after MPTP treatment as compared with saline-treated group. Also, the DOPAC concentrations were significantly reduced (59.7%) in the striatum 3 days after MPTP administration. Seven days after MPTP treatment, furthermore, the dopamine and DOPAC concentrations were significantly reduced (74.2 and 57.1%) in the striatum, respectively. In addition, the dopamine and DOPAC concentrations in the striatum of saline-treated mice were similar to the values of a previous report<sup>3</sup>.

### **Discussion**

The purpose of the present study was to determine the changes in dopamine D<sub>1</sub> and D<sub>2</sub> receptors and dopamine uptake sites in the striatum and substantia nigra of mouse brain at early stages after acute administration of MPTP. The results of the present study show that MPTP does not cause the changes in dopamine D<sub>1</sub> and D<sub>2</sub> receptors in the striatum up to 7 days after MPTP treatment. In contrast, dopamine uptake sites showed a marked reduction in the striatum 3 and 7 days after acute treatment with MPTP. In the substantia nigra, on the other hand, dopamine D<sub>1</sub> receptors exhibited no significant change up to 7 days of postlesion, whereas dopamine D<sub>2</sub> receptors showed a significant reduction 7 days after MPTP treatment. Dopamine uptake sites showed a marked decrease in the substantia nigra 3 and 7 days after MPTP treatment. These findings suggest that the change in dopamine uptake sites precede any change in dopamine D<sub>1</sub> and D<sub>2</sub> receptors in the striatum and substantia nigra after acute treatment with MPTP. Interestingly, many studies utilizing rat brains have reported that the localization of dopamine receptors in the substantia nigra is heterogeneous for the two subtypes. Dopamine D<sub>1</sub> receptors are distributed on the terminals of striatonigral projections, which are well-recognized to be far denser in the pars reticulata than in pars compacta of the substantia nigra<sup>18,19</sup>. In contrast, dopamine D<sub>2</sub> receptors in the substantia nigra are located

on the dopaminergic cell bodies or their dendrites in the pars compacta, and the density of dopamine D<sub>2</sub> receptors is shown to be minimal in the pars reticulata<sup>20-22</sup>). Therefore, it is conceivable that the difference between the effects of MPTP on dopamine D<sub>1</sub> and D<sub>2</sub> receptors observed in the present study may be due to their heterogeneous localization in the substantia nigra of mice.

In clinical studies, there have been several reports on striatal dopamine D<sub>1</sub> receptors in patients with Parkinson's disease utilizing PET (Positron Emission Tomography) or autopsied brains. Some reports have demonstrated no significant change in dopamine D<sub>1</sub> receptors<sup>23,24</sup>), but others have described an up-regulation of dopamine D<sub>1</sub> receptors<sup>25,26</sup>) at post-synaptic sites. Also, there have been a few reports on dopamine D<sub>1</sub> receptors in the substantia nigra of patients with Parkinson's disease. Cortés et al<sup>24</sup>) demonstrated no significant alteration in nigral dopamine D<sub>1</sub> receptors, whereas Rinne et al<sup>26</sup>) described a significant decline in the binding using autopsied brains. Thus, several evidence for changes in dopamine D<sub>1</sub> receptors is contradictory. On the other hand, there have also been many studies on dopamine D<sub>2</sub> receptors in the striatum of patients with Parkinson's disease using PET or autopsied brains. Most studies have suggested an up-regulation in untreated patients with early Parkinson's disease<sup>27-29</sup>), but the down-regulation of dopamine D<sub>2</sub> receptors might underlie the fluctuating response to L-DOPA observed in chronically treated Parkinson's patients<sup>27,28</sup>). Interestingly, Cortés et al<sup>24</sup>) demonstrated no significant change in dopamine D<sub>2</sub> receptors in the substantia nigra between Parkinson's disease and control using autopsied brains. In contrast, Murray et al<sup>30</sup>) demonstrated marked reduction of dopamine D<sub>2</sub> receptors in the substantia nigra of Parkinson's disease using autopsied brains. From these observations, it is conceivable that receptor autoradiographic approach under the same experimental conditions may help to explain the dopamine receptor changes in Parkinson's disease.

[<sup>3</sup>H]Mazindol is a specific ligand for dopamine uptake sites<sup>15,17</sup>) and its binding is located on pre-synaptic terminals of dopaminergic axons originating in the substantia nigra, especially in the pars compacta<sup>17</sup>). Therefore, measurement of dopamine uptake sites is useful for detecting functional changes of dopaminergic neurons in Parkinson's disease or in animal models of Parkinson's disease. Many studies have reported marked reduction of dopamine uptake sites in the striatum and substantia nigra using [<sup>3</sup>H]mazindol in MPTP-treated animals, such as monkeys<sup>31</sup>), cats<sup>32</sup>) and marmosets<sup>33</sup>). Furthermore, Alexander et al<sup>31</sup>) reported that there was a good correlation between the reductions in [<sup>3</sup>H]mazindol binding sites and in tissue dopamine levels in the striatum. The present study showed that marked reduction in [<sup>3</sup>H]mazindol binding was evident in the striatum where severe reductions of dopamine and DOPAC contents were found 3 and 7 days after acute treatment with MPTP. Therefore, our findings also suggest that there is a good correlation between the decreases in [<sup>3</sup>H]mazindol binding sites and in dopamine levels in the striatum.

In conclusion, the present study demonstrates that severe functional damage in dopamine uptake sites occurs in the striatum and substantia nigra 3 and 7 days after acute treatment with MPTP. In contrast, dopamine D<sub>1</sub> receptors are unaltered in the striatum and substantia nigra after the treatment. Dopamine D<sub>2</sub> receptors are significantly decreased in the substantia nigra 7 days after the MPTP treatment, whereas no significant change in the receptors is observed in the striatum. Our results also suggest that marked reduction in dopamine and its metabolite (DOPAC) contents is found in the striatum 3 and 7 days after acute treatment with MPTP. Our studies may provide valuable information for the pathogenesis of acute stage of Parkinson's disease.

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**Table 1. Time course of the changes in [<sup>3</sup>H]SCH23390, [<sup>3</sup>H]raclopride and [<sup>3</sup>H]mazindol binding in the striatum and substantia nigra of mice after MPTP treatment**

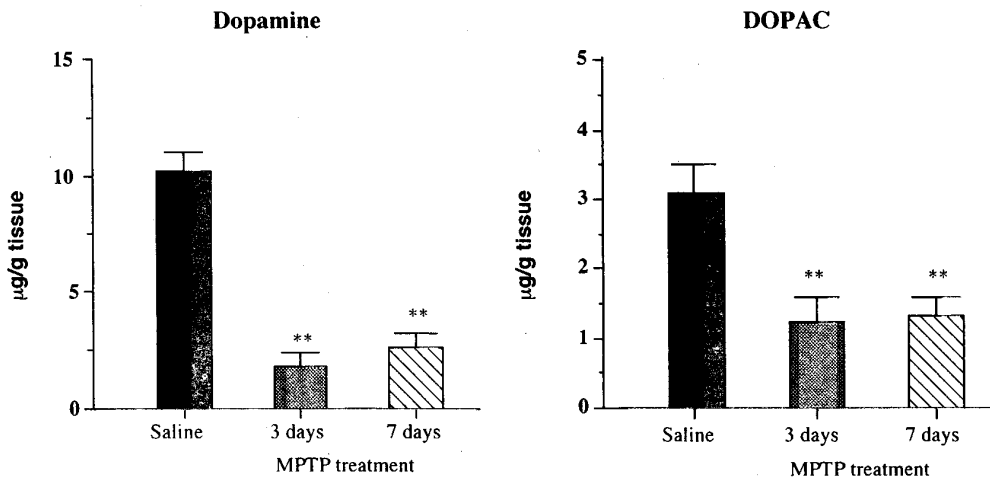
	After MPTP treatment		
	Saline (n=12)	3 days (n=6)	7 days (n=6)
<b>[<sup>3</sup>H]SCH23390 binding</b>			
Striatum lateral	734 ± 26	694 ± 45	727 ± 46
Striatum medial	686 ± 30	655 ± 39	686 ± 48
Substantia nigra	310 ± 8	291 ± 8	327 ± 7
<b>[<sup>3</sup>H]Raclopride binding</b>			
Striatum lateral	68 ± 1.8	66 ± 2.6	65 ± 2.8
Striatum medial	55 ± 1.9	56 ± 0.8	55 ± 1.9
Substantia nigra	12 ± 0.3	12 ± 0.3	9 ± 0.4**
<b>[<sup>3</sup>H]Mazindol binding</b>			
Striatum lateral	141 ± 4	42 ± 6**	44 ± 5**
Striatum medial	120 ± 4	21 ± 4**	22 ± 4**
Substantia nigra	37 ± 3	18 ± 3**	13 ± 3**

Values are mean ± S.E. (fmol/mg tissue).

\* p<0.05, \*\* p<0.01 vs. saline-treated group (Dunnett's multiple range test). n=6-12 mice.

Striatum (lateral): dorsolateral part.

Striatum (medial): ventromedial part.



**Fig. 1. Striatal concentration of dopamine and its metabolite (DOPAC) 3 and 7 days after acute treatment with MPTP in mice. n=6 mice. \* p<0.05, \*\* p<0.01 vs. saline-treated group (Dunnett's multiple range test).**