

**Roles of monoamine oxidase-B (MAO-B) in the neurotoxicity of  
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice**

**(マウスにおける 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)の  
神経毒性発現に関する monoamine oxidase-B (MAO-B)の役割)**

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## **General Introduction**

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by resting tremor, muscular rigidity and akinesia, and is the second most frequent neurodegenerative disorders after Alzheimer's disease. The incidence of PD rises steeply with age, from 17.4 in 100,000 between 50 and 59 years of age to 93.1 between 70 and 79 years, with a lifetime risk of developing the disease of 1.5% [6, 15]. A selective degeneration of dopaminergic (DA-ergic) neurons in the substantia nigra pars compacta (SNpc) accompanied by three distinctive intraneuronal inclusions: Lewy body, pale body, and Lewy neurite are the pathological hallmarks of PD [49]. Although PD is regarded as a sporadic disorder, remarkably few environmental causes or triggers have been so far identified [7, 74, 76].

In 1982, drug addicts in the San Francisco Bay area, while attempting to make an illegal heroin analog, overcooked the concoction, which resulted in the formation of a compound known as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Injection of MPTP resulted in acute "end stage" parkinsonism in these individuals, due to the rapid loss of the same set of neurons that destroyed in PD [45] and administration of MPTP to experimental animals resulted in the similar effects [46, 47]. Thus, MPTP is widely used to induce an animal model of parkinsonism. MPTP is metabolized by an enzyme, monoamine oxidase B (MAO-B) to 1-methyl-4-phenylpyridinium ( $MPP^+$ ) in liver cells and endothelial cells of microvasculature responsible for the blood-brain barrier (BBB) [64, 81]. Since  $MPP^+$  cannot pass through the BBB, MPTP that had escaped from the metabolism can enter into the brain parenchyma [65]. There MPTP is converted into  $MPP^+$  by astrocytic MAO-B, and  $MPP^+$  enters into DA-ergic neurons with a dopamine transporter (DAT) on the plasma membrane. Inside the DA-ergic neurons,  $MPP^+$  injures the

mitochondrial respiratory complex I, causes energy depletion, and finally leads to neuronal death (Fig. 1) [3, 71]. Like MPP<sup>+</sup>, rotenone and annonacin lead to selective degeneration of DA-ergic neurons by inhibition of mitochondrial complex I in rats [11, 32]. Thus, some forms of parkinsonism might be induced by environmental toxins that affect the activity of complex I, and the finding of a complex I deficiency in the SNpc of PD patients provides a direct link between PD and experimental models of parkinsonism [68]. In addition, MPTP and rotenone are capable of inducing the formation of intracellular fibrillar protein inclusions immunoreactive to  $\alpha$ -synuclein and reminiscent of Lewy bodies [4, 23]. From these reports, MPTP is helpful in the studies of parkinsonism including PD.

There are species differences in the susceptibility to MPTP injection; for example, human and monkeys are susceptible, whereas rats and hamsters are relatively resistant [9, 12, 44, 55]. Mice are middle susceptible, but show marked strain differences [73, 63, 26]; C57BL/6 mice are susceptible, while BALB/c mice are resistant [69, 79, 22]. However, the reason for such strain differences remains still unclear. One possible factor responsible for this strain difference that has been the subject of investigation is MAO-B. Most previous studies suggested that MAO-B activity is less responsible for the strain difference [37, 72, 25], on the other hand, another previous study reported that MAO-B activity is responsible for the strain difference [82]. However, the expression level of MAO-B between mouse strains has never been examined. Besides the direct toxic action of MPP<sup>+</sup> on the mitochondrial respiration, it is proposed that excessive production of nitric oxide (NO) also contributes to MPTP neurotoxicity (Fig. 1). NO is synthesized from L-arginine by nitric oxide synthase (NOS) [13], and an excessive amount of NO produced in the

brain by the enzyme reacts with superoxide species produced by MPP<sup>+</sup>, and forms peroxynitrite, which is implicated in the neuronal damage [58]. There is a possibility that the different NOS expression level between mouse strains is responsible for the strain differences of susceptibility to MPTP toxicity.

In addition, it was shown that ip administration of MPTP also induces apoptosis of the subventricular zone (SVZ) cells [27, 29]. In the brains of adult animals, including rodents, the SVZ lining the lateral ventricles is one of the primary regions of adult neurogenesis [1, 20]. The SVZ is composed of four types of cells: doublecortin (Dcx)-positive neural progenitor cells (migrating neuroblasts, A cells), glial fibrillary acidic protein (GFAP)-positive neural stem cells (astrocytes, B cells), epidermal growth factor receptor (EGFR)-positive neural precursor cells (rapidly dividing transit amplifying cells, C cells) and ependymal cells [20, 21, 60]. Of these SVZ cells, ip administration of MPTP induces apoptosis of A cells [27, 29]. In addition, such apoptosis was inhibited by the pretreatment with selective MAO-B inhibitors, R(-)-deprenyl (deprenyl, selegiline) or N-(2-Aminoethy)-4-chlorobenzamide (Ro 16-6491) [28]. However, whether such apoptosis is induced by direct toxicity of MPP<sup>+</sup> or not remains unclear.

The purpose of Chapter 1 and 2 is to clarify factor(s) influencing the mouse strain differences between susceptible C57BL/6 and resistant BALB/c. Clarifying this factor(s) might provide insights into the different genetic background between susceptible and resistant humans for PD. In addition, the purpose of Chapter 3 is to examine whether the MPTP-induced neuroblast (A cell) apoptosis is caused by MPP<sup>+</sup> toxicity. It is reported that SVZ cells of PD patients decrease, thus clarifying the mechanisms of MPTP-induced SVZ apoptosis would have an important role. This

thesis consists of the following three chapters: Chapter 1) Different susceptibility to MPP<sup>+</sup>-induced nigro-striatal dopaminergic cell loss between C57BL/6 and BALB/c mice is not related to the difference of MAO-B; Chapter 2) Neuronal or inducible nitric oxide synthase (NOS) expression level is not involved in the different susceptibility to nigro-striatal dopaminergic neurotoxicity induced by MPTP between C57BL/6 and BALB/c mice; and Chapter 3) MPTP-induced neuroblastic apoptosis in the subventricular zone is caused by MPP<sup>+</sup> converted from MPTP through MAO-B.

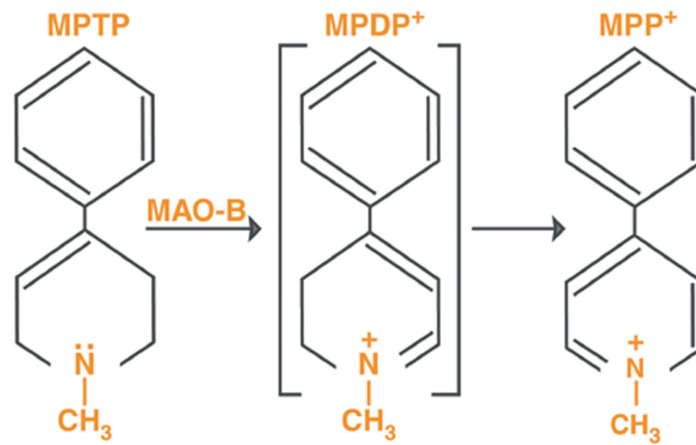
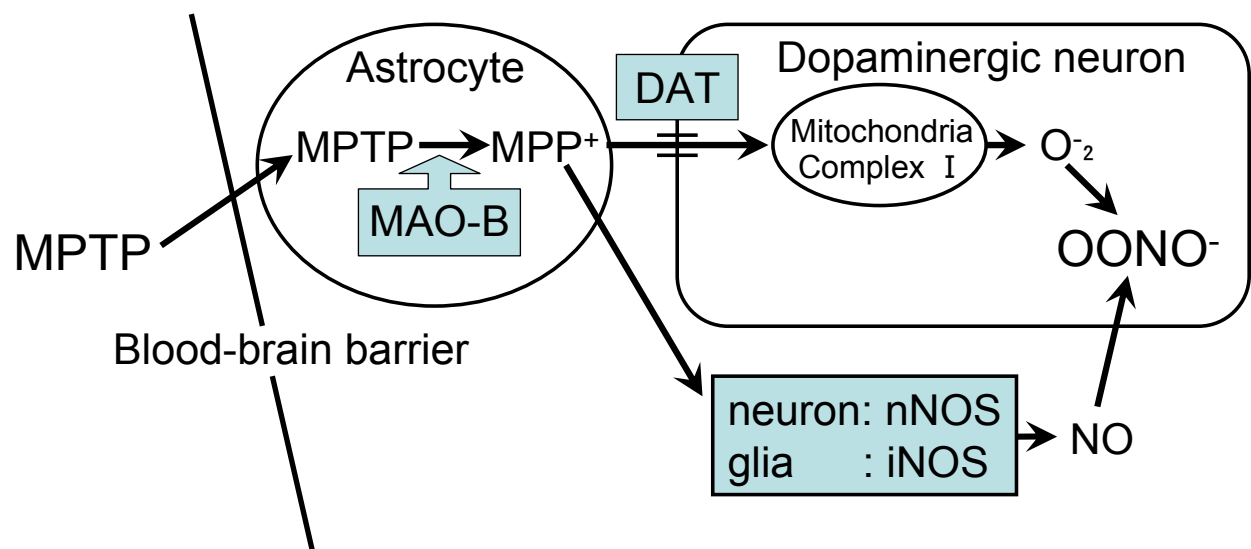
**A****B**

Fig. 1. Chemical structures of MPTP and metabolites (A) and schematic drawing indicating the mechanisms of MPTP toxicity (B).



## **Abbreviations**

**BBB:** blood-brain barrier

**DA:** dopamine

**DAT:** dopamine transporter

**Dcx:** doublecortin

**EGFR:** epidermal growth factor receptor

**GFAP:** glial fibrillary acidic protein

**icv:** intracerebroventricular(ly)

**ip:** intraperitoneal

**MAO-B:** monoamine oxidase-B

**MPDP<sup>+</sup>:** 1-Methyl-4-phenyl-2,3-dihydropyridinium

**MPP<sup>+</sup>:** 1-methy-4-phenylpyridinium

**MPTP:** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

**NO:** nitric oxide

**NOS:** nitric oxide synthase

**PD:** Parkinson's disease

**sc:** subcutaneous

**SNpc:** substantia nigra pars compacta

**SVZ:** subventricular zone

**TH:** tyrosine hydroxylase

**TUNEL:** terminal deoxynucleotidyl transferase-mediated dUTP endlabeling

## **Chapter 1**

**Different susceptibility to MPP<sup>+</sup>-induced nigro-striatal dopaminergic cell loss between C57BL/6 and BALB/c mice is not related to the difference of MAO-B**

## Abstract

In mice, marked strain differences in the susceptibility to MPTP-injection have been reported. To clarify which factor(s) cause the strain differences, MPTP or MPP<sup>+</sup> was icv injected into adult C57BL/6 (highly susceptible to MPTP) and BALB/c (resistant to MPTP) mice. The brain tissues including the striatum and SNpc were examined immunohistochemically using an antibody to TH. MPP<sup>+</sup>-injected C57BL/6 mice showed a significant decrease in TH-immunopositive areas in the striatum at 3 and 7 days (d) post injection ( $p < 0.01$ ), and TH-positive cells in the SNpc at 1, 3 and 7 d ( $p < 0.01$ ), respectively, compared to saline-injected control mice. In addition, MPP<sup>+</sup>-injected BALB/c mice showed a significant decrease in TH-positive areas in the striatum at 1 and 3 d, and SNpc TH-positive cells in the SNpc at 3 d, respectively ( $p < 0.05$ ). However, the decrease rates in the BALB/c mice were lower than that in C57BL/6 mice. MPTP-injected C57BL/6 mice, however, showed no lesions in the striatum and SNpc at 1 and 7 d after icv injection. All the present findings indicate that factors other than MAO-B can influence the strain susceptibility between C57BL/6 and BALB/c mice after the conversion from MPTP to MPP<sup>+</sup>.

## **Introduction**

There are species differences in the susceptibility to MPTP injection; for example, human and monkeys are susceptible, whereas rats and hamsters are relatively resistant [9, 12, 44, 55]. Mice are middle susceptible, but show marked strain differences [73, 63, 26]; C57BL/6 mice are susceptible, while BALB/c mice are resistant, to MPTP toxicity [69, 79, 22]. However, the reason for such strain differences remains unclear. To clarify factor(s) influencing the mouse strain differences, MPTP and MPP<sup>+</sup> were icv injected into susceptible C57BL/6 and resistant BALB/c mice, and the lesions were histologically examined.

## Materials and Methods

### *Animals*

Eight-week-old male C57BL/6J and BALB/cByJ mice, weighting 21 to 24 g and 26 to 29 g, respectively, were purchased from Japan CLEA (Tokyo, Japan). The mice were housed in a room maintained under constant temperature ( $23 \pm 2$  °C) and humidity ( $55 \pm 5$  %) conditions with a 12-h light/dark cycle using an isolator caging system (Niki Shoji, Tokyo). Water and food were accessible *ad libitum*. All the experimental procedures used in the present study were approved by the Committee of Animal Experiments of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

### *Drug injection*

MPP<sup>+</sup> iodide (Sigma, St. Luis, MO) was dissolved in saline. After being anesthetized with ethyl ether, C57BL/6 and BALB/c mice (n = 3 to 5) were injected with 10 µl of solution containing 18 µg or 22 µg of MPP<sup>+</sup> iodide (0.8 mg/kg), respectively, into the unilateral ventricle. Control mice were injected with 10 µl of saline. The stereotaxic coordinates of injection site were as follows: bregma -0.5 mm, lateral 1.0 mm, depth 2.0 mm (C57BL/6) and bregma -1.0 mm, lateral 1.0 mm, depth 2.0 mm (BALB/c), respectively. The mice were sacrificed by cervical dislocation under ethyl ether anesthesia at 1, 3, and 7 days after injection (1, 3 and 7 d). In addition, 10 µl of a solution containing 36 µg or 162 µg of MPTP-HCl (Sigma, St. Luis, MO, 1.54 mg/kg and 7.14 mg/kg respectively) or 10 µl of saline (control) were icv injected into C57BL/6 mice (n = 3). The mice were killed by cervical dislocation at 1 and 7 d after injection.

### *TH immunohistochemistry*

The brain samples were fixed in a 10% neutral buffered formalin solution for 4 days, processed routinely, and embedded in paraffin. Four  $\mu\text{m}$ -thick transverse sections encompassing the striatum and the SNpc were used for immunohistochemical examination. The primary antibody used was rabbit anti-TH (1:500, Millipore, Temecula, CA). Following the treatment with 8% skim milk at 37 °C for 40 min, sections were incubated with the primary antibody at 4 °C overnight, followed by secondary antibody reactions at 37 °C for 40 min using an EnVision Anti-rabbit conjugation system (Dako, Carpinteria, CA). Finally, the positive reaction was visualized with 0.05% 3,3'-diaminobenzidine (DAB) and 0.03% hydrogen peroxide in a Tris-HCl buffer, and the sections were counterstained with methyl green. TH-positive areas in the striatum were measured with an image analyzing program, NIH Image-J. In addition, TH-positive neurons in the SNpc were counted as previously described [57]. The boundary between the SNpc and ventral tegmental area was defined with the aid of the mouse brain atlas [24]. The number of TH-positive neurons on each representative mesencephalic section was counted under a magnification of  $\times 200$ . The mean number of total TH-positive neurons was calculated for each mouse. Statistical significance was evaluated using the Student's *t*-test.

## Results

### *Different susceptibility to MPP<sup>+</sup> in C57BL/6 and BALB/c mice*

After MPP<sup>+</sup> icv injection, the lesions were found mainly adjacent to the lateral ventricles in the striatum and in the ventral cells of the SNpc in both C57BL/6 and BALB/c mice (Figs. 1-1 and 1-2). In C57BL/6 mice, TH-positive areas in the striatum were significantly decreased at 3 and 7 d after MPP<sup>+</sup> injection, whereas TH-positive cells in the SNpc were significantly decreased at 1, 3 and 7 d (Fig. 1-3). The results suggest that the MPP<sup>+</sup> first affects specifically DA-ergic cells in the SNpc and that the lesion then spreads to the striatum. In BALB/c mice, both TH-positive areas in the striatum and the number of TH-positive cells in the SNpc also decreased significantly at 1 and 3 d, and at 3 d, respectively (Fig. 1-3). Using one-way analysis of variance (ANOVA) between MPP<sup>+</sup>-injected C57BL/6 and BALB/c mice, strain differences of the percentage of the decrease compared to control was shown in Table 1-1. The percentage was higher in C57BL/6 mice compared to that in BALB/c mice at each time point.

### *Susceptibility to MPTP in C57BL/6 mice*

In both low (36µg) and high (162µg) doses icv MPTP-treated C57BL/6 mice, no decrease in TH-positive areas nor decrease in TH-positive cells was found either in the striatum or SNpc at 1 and 7 d (Figs. 1-4 and 1-5).

## Discussion

Previous reports indicated a more obvious loss of DA-ergic neurons in the SNpc and their nerve endings in the striatum in C57BL/6 mice than that in BALB/c mice after ip injection of MPTP [69, 79, 22], though the mechanism of the different strain susceptibility to MPTP neurotoxicity remains unclear. Candidates responsible for such susceptibility include different expression and/or activity of MAO-B in brain astrocytes [82] or endothelial cells constituting the BBB [63]. Our present observation revealed that icv injection of MPP<sup>+</sup> induced different neurotoxicity between C57BL/6 and BALB/c mice, being similar to those of previous reports [69, 79, 22] using ip injection of MPTP. The results of the present and previous studies suggest that MAO-B in astrocytes and BBB endothelial cells is less responsible for the strain difference. Other previous reports [37, 72, 25] showed the similar strain difference in mice. In addition, transgenic mice expressing high neuronal MAO-B levels did not result in increased susceptibility to MPTP [2]. Thus, the main factor(s) influencing the strain difference in MPTP-susceptibility are present after the conversion of MPTP to MPP<sup>+</sup>.

After metabolization by MAO-B, MPP<sup>+</sup> is transported into DA neurons through DAT, injures mitochondrial complex I, and finally induces neuronal death [71]. In addition, MPP<sup>+</sup> can be taken into cytoplasmic vesicles by the action of the vesicular monoamine transporter (VMAT) [52, 17]. Thus, DAT and VMAT might be responsible for the strain difference, but strain differences in their activities have not been found until the present.

On the other hand, MPP<sup>+</sup> is thought to induce microglial and astroglial activation, resulting in a release of inflammatory cytokines and chemokines from the



cells [80]. There are previous reports indicating that MPTP neurotoxicity was prevented by NSAIDs [43, 66, 54]. In addition, microglia inactivation was shown to be neuroprotective in the MPTP model [78]. Thus, neuroinflammation can be responsible for the strain difference. After ip administration of MPTP, the activation of microglia and astrocytes in the striatum and the increased levels of the inflammatory cytokines and chemokines in the cerebrospinal fluid (CSF) were observed in C57BL/6 mice, but not in BALB/c mice [79]. The two strains also have different immunological backgrounds; C57BL/6 mice are Th1 dominant while BALB/c mice are Th2 dominant [31]. Th1 cells are believed to induce brain inflammation and Th2 cells anti-inflammatory responses [41]. Inflammatory reactions in the brain induced by MPTP and/or MPP<sup>+</sup> in C57BL/6 mice may be responsible for the lesions in the SNpc and striatum, and the less inflammation in BALB/c mice may be involved in their lower susceptibility.

In the present study, icv injection of MPP<sup>+</sup> into C57BL/6 mice induced a significant reduction in the TH-positive area in the striatum from 3 d, whereas TH-positive cells in the SNpc significantly decreased from 1 d, suggesting that MPP<sup>+</sup> initially affects the DA-ergic cell body in the SNpc from the third ventricle and then the striatum to which DA-ergic cells project. On the other hand, icv-injected MPTP induced no lesions in the striatum or SNpc of C57BL/6 mice at 1 and 7 d. It is possible that icv-injected MPTP rapidly disappears from CSF before the conversion to MPP<sup>+</sup> and/or that MPTP is detoxified by drug-metabolizing enzymes such as glutathione S-transferase and cytochrome P450. Diffusion to the brain parenchyma from the CSF is more favorable for lipophilic than for hydrophilic drugs [5]. While MPTP is a highly lipophilic molecule, MPP<sup>+</sup> is far less lipophilic than MPTP. Thus,

most icv-injected MPTP might diffuse to the SVZ, where MPTP induce apoptosis of neuroblasts [35, 27], before it reaches the third ventricle. The previous report [27] revealed the phagocytosis of apoptotic bodies by microglia. As a result, the microglia in the SVZ might become a barrier preventing the striatum from MPTP toxicity by phagocytosis of apoptotic bodies containing MPP<sup>+</sup>.

Region	Strain	TH loss at 1 d	TH loss at 3 d	TH loss at 7 d
		after injection <sup>a</sup>	after injection <sup>a</sup>	after injection <sup>a</sup>
Striatum	C57BL/6	-2.77 <sup>b</sup>	36.7 <sup>c</sup>	46.2 <sup>d</sup>
	BALB/c	11.3	14.5	3.28
SNpc	C57BL/6	52.3 <sup>c</sup>	49.7 <sup>c</sup>	48.1
	BALB/c	17.7	20.2	16.9

<sup>a</sup>% of control

<sup>b</sup> $p < 0.01$  versus BALB/c striatum

<sup>c</sup> $p < 0.05$  versus BALB/c striatum

<sup>d</sup> $p < 0.01$  versus BALB/c striatum

<sup>e</sup> $p < 0.05$  versus BALB/c SNpc

Table. 1-1 Decrease % in TH-positive area in the striatum and TH-positive cells in the SNpc after icv injection of MPP<sup>+</sup>.

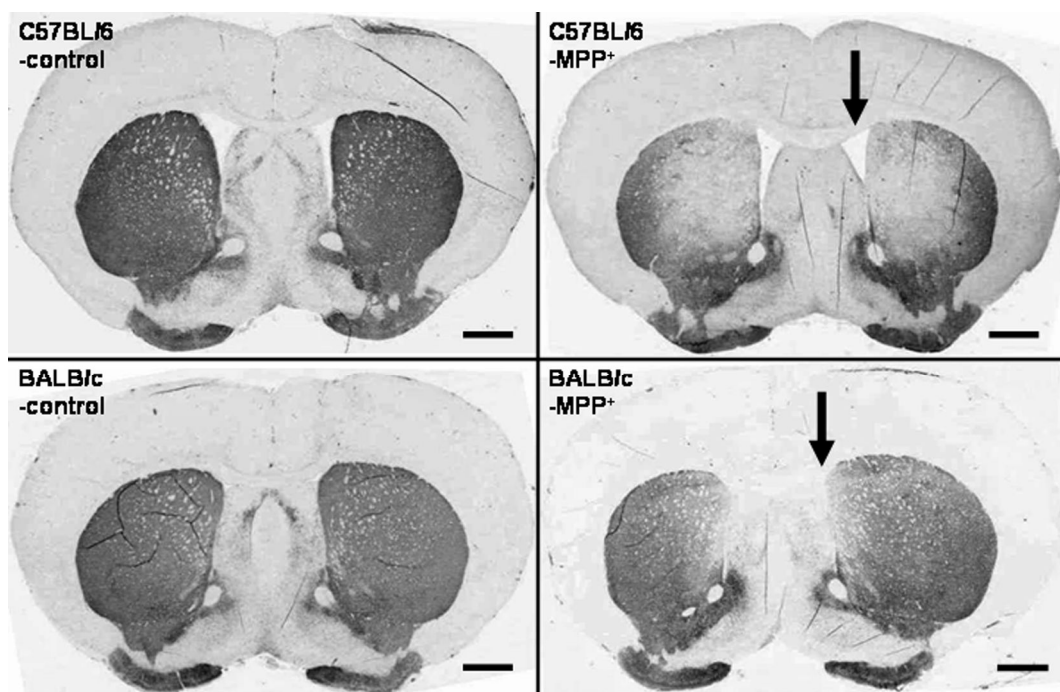


Fig. 1-1. TH-immunoreactivity in the striatum at 7 d after injection. In MPP<sup>+</sup>-treated mice, a reduction in TH-positive areas was observed at the injection (arrows) and contralateral sides of the striatum, especially adjacent to the lateral ventricles in C57BL/6 and BALB/c mice. However, no lesions were found in control C57BL/6 and BALB/c mice. EnVision method. Counterstained with methyl green. Bar = 1mm.

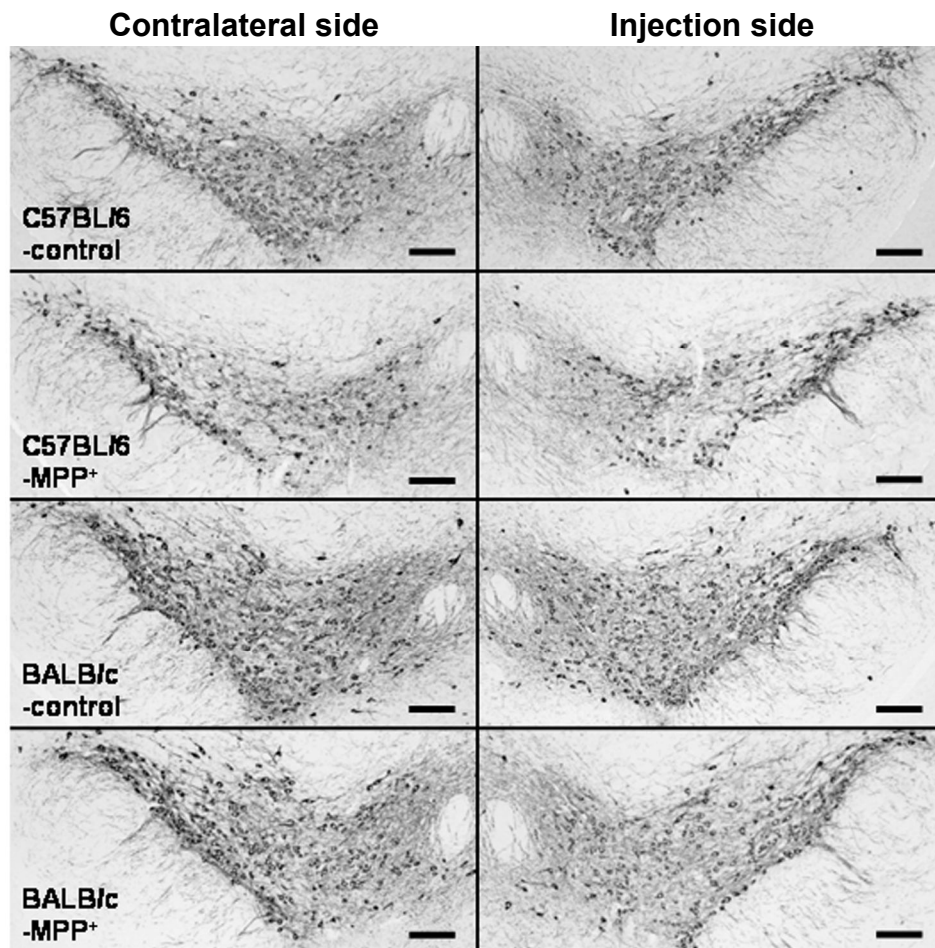


Fig. 1-2. TH-immunoreactivity in the SNpc at 7 d after injection. In MPP<sup>+</sup>-treated mice, a reduction in TH-positive cells was observed, especially in the ventral area of both the injection and contralateral sides of the SNpc. However, no lesions were found in control mice. EnVison method. Counterstained with methyl green. Bar = 200µm.

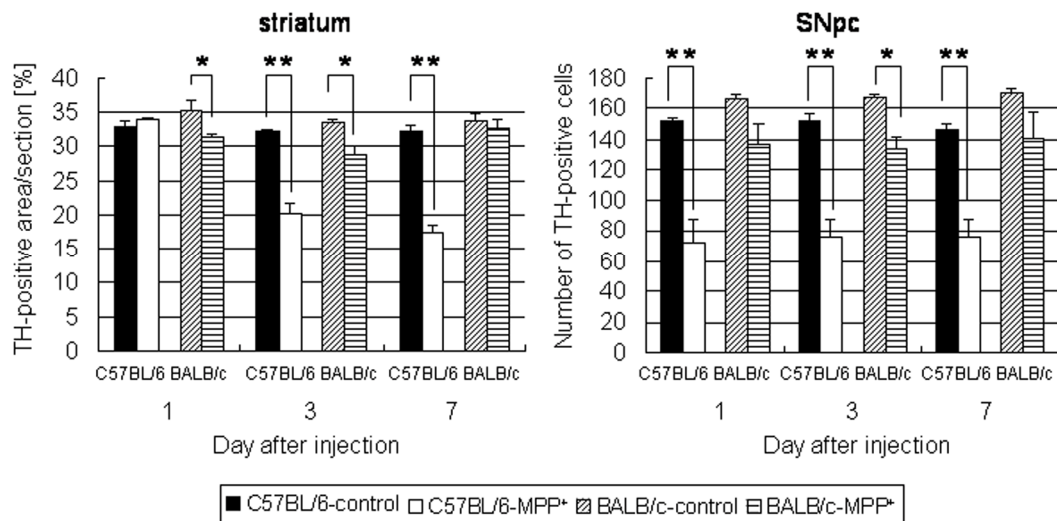


Fig. 1-3. Different strain susceptibility to MPP<sup>+</sup> of C57BL/6 and BALB/c mice. Significant decreases in TH-positive areas in the striatum and TH-positive cells in the SNpc were documented in C57BL/6 mice at 3 and 7 d, and at 1, 3 and 7 d, respectively. In BALB/c mice, significant decreases in TH-positive areas and TH-positive cells were observed at 1 and 3 d, and 3 d after injection, respectively. The decrease rates of BALB/c mice were smaller compared to those in C57BL/6 mice. Data are expressed as the mean  $\pm$  standard error (n=3 to 5). \* $p < 0.05$  and \*\* $p < 0.01$ .

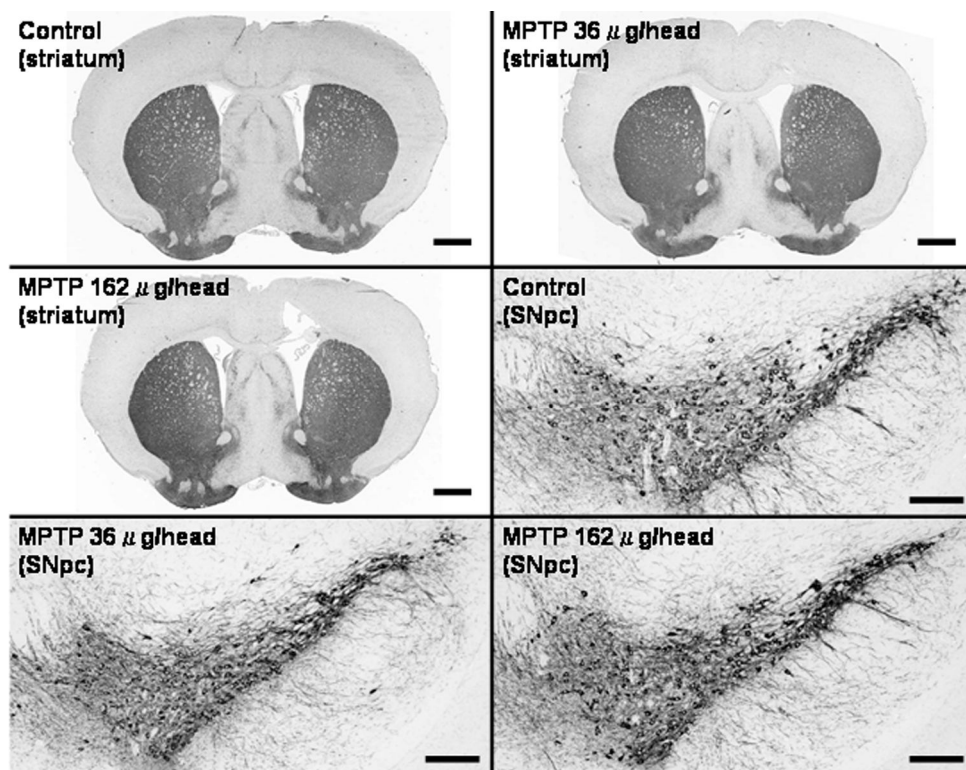


Fig. 1-4. TH-immunoreactivity in the striatum and at the injection side of the SNpc of MPTP-injected C57BL/6 mice at 7 d after injection. No lesions were found in MPTP-treated (36 $\mu$ g and 162 $\mu$ g) or control C57BL/6 mice. EnVison method. Counterstained with methyl green. Bar = (striatum) 1mm; (SNpc) 200 $\mu$ m.

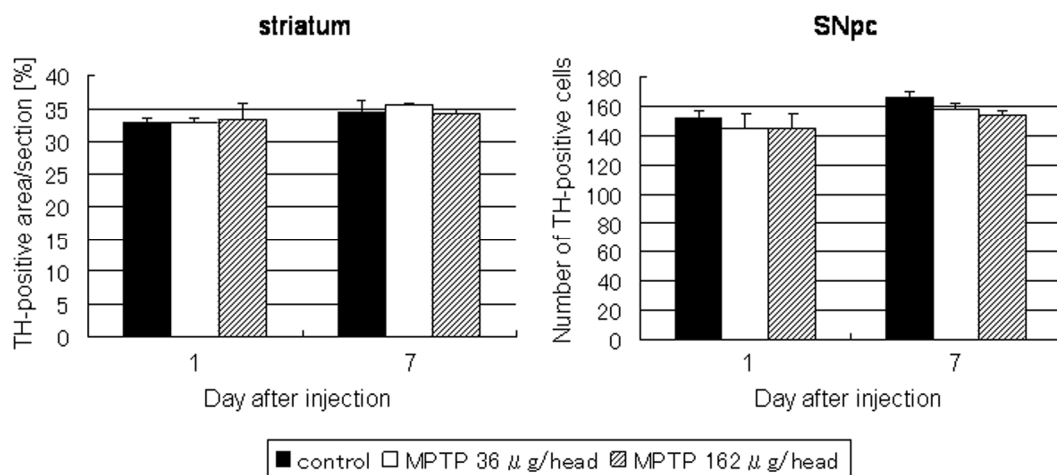


Fig. 1-5. TH-immunoreactivity in the striatum and SNpc of MPTP-injected C57BL/6 mice at 1 and 7 d. In MPTP-treated and control mice, no significant decrease in TH-positive areas or TH-positive cells was observed. Data are expressed as the mean  $\pm$  standard error (n=3).



## **Chapter 2**

**MAO-B, DAT and NOS expression levels are not involved in the different  
susceptibility to nigro-striatal dopaminergic neurotoxicity induced by MPTP  
between C57BL/6 and BALB/c mice**

## **Abstract**

MPTP induces severe degeneration of DA-ergic neurons when administrated to C57BL/6 mice, but such lesions are not observed in BALB/c mice. To clarify the factors which influence such marked strain differences in the susceptibility to MPTP, the involvement of neuronal NOS (nNOS) and inducible NOS (iNOS) was investigated. MPTP was ip administrated to adult C57BL/6 (highly sensitive) and BALB/c (resistant) mice. Immunohistochemical analysis using an antibody to TH showed a significant decrease in TH-immunopositive areas in the striatum and TH-positive cells in the SNpc of MPTP-treated C57BL/6 mice at 1 and 7 days (d) after administration, compared to control C57BL/6 mice. On the other hand, MPTP-treated BALB/c mice showed no significant changes. By Western blot analysis, TH, MAO-B, DAT, nNOS and iNOS protein expression levels were examined in intact and MPTP-treated mice. Intact BALB/c mice showed higher DAT protein expression in the striatum and TH protein expression in the midbrain than intact C57BL/6 mice. In addition, MPTP-treated BALB/c mice showed a more significant increase of MAO-B expression than MPTP-treated C57BL/6 mice at 12 hours (h). The increase of nNOS and iNOS protein expressions in MPTP-treated BALB/c mice was more pronounced in the striatum and midbrain than in MPTP-treated C57BL/6 mice at 12 h and 2 d. These results indicate that MAO-B, DAT, nNOS or iNOS expression levels do not influence the different strain susceptibility to MPTP.

## Introduction

It is proposed that excessive production of nitric oxide (NO) contributes to MPTP neurotoxicity. NO is synthesized from L-arginine by NOS [13]. NOS is present in the central nervous system (CNS) in three different isoforms; two constitutive enzymes (neuronal-nNOS and endothelial-eNOS) and an inducible enzyme (iNOS). nNOS and eNOS are distributed in neurons and the vasculature, respectively. iNOS is located predominantly in activated macrophages, microglia and astrocytes. It has been reported that, although the eNOS expression level does not alter following MPTP administration [56], nNOS and iNOS expression levels in the striatum or midbrain increase after MPTP administration [10, 36, 51]. In addition, nNOS and/or iNOS inhibitors are protective against MPTP-induced pathology and mutant mice that lack the nNOS or iNOS gene are more resistant to MPTP treatment than wild-type mice [16, 40, 50, 61].

There are species and strain differences in the susceptibility to MPTP administration. Mice are moderately susceptible, but show marked strain differences [26, 73]; C57BL/6 mice are susceptible, while BALB/c mice are resistant [22, 69, 79]; however, the reason for such strain difference remains unclear. To investigate whether nNOS and/or iNOS influences the mouse strain difference, nNOS and iNOS expression levels were examined in C57BL/6 and BALB/c mice.

## Materials and Methods

### *Animals*

Eight-week-old male C57BL/6J and BALB/cByJ mice, weighing 21 to 25 g and 25 to 28 g, respectively, were purchased from Japan CLEA. The mice were housed in a room maintained under constant temperature ( $23 \pm 2$  °C) and humidity ( $55 \pm 5$  %) conditions with a 12-h light/dark cycle using an isolator caging system (Niki Shoji, Tokyo). Water and food were accessible *ad libitum*. All the experimental procedures used in the present study were approved by the Committee of Animal Experiments of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

### *Drug administration*

C57BL/6 and BALB/c mice ( $n = 3$  each) received four ip injections of either 20 mg/kg MPTP-HCl (Sigma) or saline at 2-hour (h) intervals within a single day. The mice were sacrificed by cervical dislocation under ethyl ether anesthesia at 1 and 7 days (d) for immunohistochemical analysis and at 6 h, 12 h, 1 d and 2 d for Western blot analysis after the last MPTP administration.

### *Immunohistochemistry*

The brain samples were fixed in a 10% neutral-buffered formalin solution for 4 d, processed routinely, and embedded in paraffin. Four  $\mu$ m-thick transverse sections encompassing the striatum and the substantia nigra pars compacta (SNpc) were used for immunohistochemical analysis. The primary antibody was rabbit anti-tyrosine hydroxylase (TH) (1:500; Millipore). Following treatment with 8% skim milk at 37°C

for 40 min, sections were incubated with the primary antibody at 4°C overnight, followed by secondary antibody reactions at 37°C for 40 min using the EnVision anti-rabbit conjugation system (Dako). Finally, the positive reaction was visualized with 0.05% 3,3'-diaminobenzidine (DAB) and 0.03% hydrogen peroxide in Tris-HCl buffer, and the sections were counterstained with methyl green.

### *Western blotting*

Mouse striatum or midbrain was homogenized in a solution of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 2mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 % NP-40 and 0.1 % SDS, and centrifuged at 12,000×g at 4 °C for 20 min. The supernatant was then loaded onto a 10 % SDS-PAGE gel, electrophoresed, and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The blots were first probed with antibodies to TH (1:6,000; Millipore), MAO-B (1:200; Santa Cruz Biotechnology), nNOS (1:1,000; Cell Signaling Technology, Beverly, MA), iNOS (1:200; Santa Cruz Biotechnology) and β-actin (1:2,000; Cell Signaling Technology). After incubation with an appropriate secondary antibody conjugated to horseradish peroxidase (anti-rabbit; Amersham, Buckinghamshire, UK or anti-goat; Santa Cruz Biotechnology), detection was performed with the ECL Plus kit (Amersham).

### *Measurement and statistics*

TH-positive areas in the striatum were measured with an image analyzing program, NIH Image-J. In addition, TH-positive neurons in the SNpc were counted as previously described [57]. The boundary between the SNpc and ventral tegmental area was defined with the aid of the mouse brain atlas [24]. The number

of TH-positive neurons in each representative mesencephalic section was counted at ×200 magnification. The mean number of total TH-positive neurons was calculated for each mouse. The bands in Western blotting were also quantified using NIH Image-J. Statistical significance was evaluated using Student's *t*-test.

## Results

### *Different susceptibility to MPTP between C57BL/6 and BALB/c mice*

After MPTP ip administration, lesions were found in both the striatum and SNpc of C57BL/6 mice (Figs. 2-1 and 2-2). TH-positive areas in the striatum were significantly decreased at 1 and 7 d after MPTP injections. TH-positive cells in the SNpc were also significantly decreased at 1 and 7 d. The number of cells was decreased more significantly at 7 d than 1 d. On the other hand, BALB/c mice showed no change in the striatum and SNpc (Figs. 2-1 and 2-2).

### *Protein expressions in intact C57BL/6 and BALB/c mice*

The expressions of DAT protein in the striatum and of TH protein in the midbrain of C57BL/6 mice were significantly higher than those of BALB/c mice (Fig. 2-3). In contrast, the expressions of MAO-B, nNOS and iNOS in the striatum and midbrain showed no significant differences between the strains.

### *Protein expressions in MPTP-treated C57BL/6 and BALB/c mice*

In the striatum (Fig. 2-4), MPTP-treated C57BL/6 mice showed a more significant decrease of TH protein expression than MPTP-treated BALB/c mice from 6 h to 2 d after administration. MPTP-treated BALB/c mice showed a significant increase of MAO-B, nNOS and iNOS expressions compared to MPTP-treated C57BL/6 mice at 12 h. In the midbrain (Fig. 2-5), TH protein expression was significantly decreased in MPTP-treated C57BL/6 mice compared to BALB/c mice at 2 d. nNOS and iNOS protein expressions of MPTP-treated BALB/c mice showed a significant increase compared to MPTP-treated C57BL/6 mice at 2 d.

## Discussion

ip administration of MPTP induces more obvious loss of DA-ergic neurons in the striatum and SNpc in C57BL/6 mice than in BALB/c mice [22, 69, 79]. The present immunohistochemical and Western blot analyses for TH showed similar results to previous reports. In addition, the present study indicates that MAO-B, DAT, nNOS or iNOS expression levels do not influence the different susceptibility of the two strains to MPTP.

Different expression and activity of MAO-B in brain astrocytes [82] and those of DAT in DA-ergic neurons [39, 48] are candidate factors responsible for the susceptibility to MPTP toxicity. MPTP is metabolized by MAO-B to MPP<sup>+</sup> [8, 62]. MPP<sup>+</sup> further enters DA-ergic neurons with a DAT on the plasma membrane and induces neuronal death. In the present study, the MAO-B expression level in the striatum of MPTP-treated BALB/c mice showed a more significant increase than that of MPTP-treated C57BL/6 mice at 12 h, and the DAT expression level in the striatum of intact BALB/c mice was higher than that in the striatum of intact C57BL/6 mice. These results suggest that neither the MAO-B nor DAT expression level is involved in the strain susceptibility to MPTP.

The involvement of nNOS and iNOS enzymes in the strain differences of susceptibility to MPTP toxicity has never been examined. An excessive amount of NO produced in the brain by NOS reacts with superoxide species produced by MPP<sup>+</sup>, and forms peroxynitrite, which is implicated in neuronal damage [58]. Among three NOS isoforms, nNOS and/or iNOS expression increased after MPTP administration in the striatum [10, 36, 51]. In addition, nNOS or iNOS inhibitors are protective against MPTP toxicity, and mutant mice that lack the nNOS or iNOS gene are more resistant



to MPTP than wild-type mice [16, 40, 50, 61]. A previous report [36] suggested that increased expressions of nNOS and/or iNOS might contribute to the higher susceptibility to MPTP of old mice than young mice. In the present study, we compared expression levels of nNOS and iNOS between susceptible C57BL/6 mice and resistant BALB/c mice. By Western blot analysis, nNOS and iNOS expression levels in the striatum and midbrain showed no significant difference between the two intact mouse strains. In addition, after MPTP administration, BALB/c mice showed a more significant increase of nNOS and iNOS expressions than those of C57BL/6 mice at 12 h and 2 d in the striatum and midbrain. These results indicate that the nNOS or iNOS expression level is not involved in the different susceptibility to MPTP between the two strains; however, there is a report describing the significant increase of nNOS and iNOS expressions in the striatum and SNpc in C57BL/6 mice after MPTP administration [10, 36]. The significant increase of iNOS and/or nNOS expressions might occur later than 2 d in MPTP-treated C57BL/6 mice. The tendency that higher nNOS and iNOS expression levels of MPTP-treated BALB/c mice than those of MPTP-treated C57BL/6 mice might indicate that the protective response is more significant than the toxic response of nNOS and iNOS after MPTP administration in BALB/c mice. Though factor(s) influencing the strain difference in the susceptibility to MPTP toxicity is still unknown at present, the protective factor(s) might play important role. Using two-way ANOVA between MPTP-treated C57BL/6 and BALB/c mice, significant lower nNOS expression level of MPTP-treated BALB/c mice was shown at 6 h in the striatum. This result might indicate that lower nNOS expression level contributes to the MPTP resistance in BALB/c mice at very early time point.

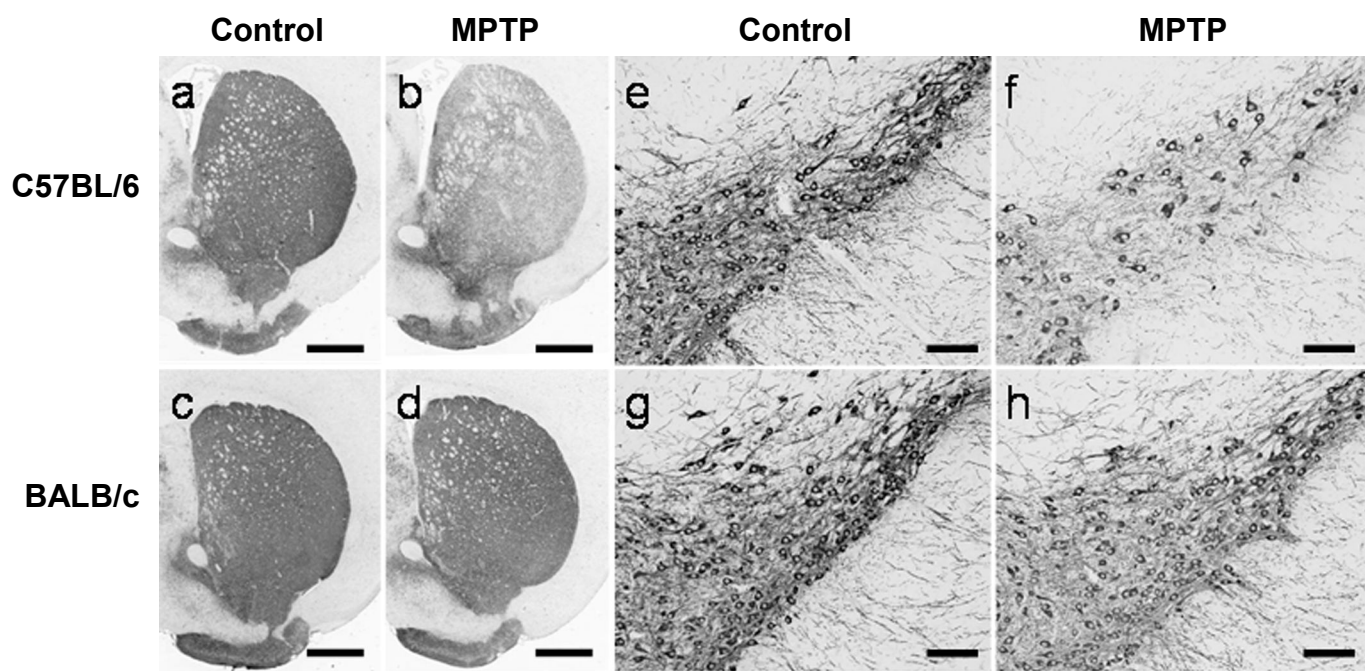


Fig. 2-1. TH immunoreactivity in the striatum and SNpc at 7 d after the last MPTP ip administration. Representative photographs of the striatum of control C57BL/6 (a), MPTP-treated C57BL/6 (b), control BALB/c (c), and MPTP-treated BALB/c (d) mice, respectively. The SNpc of control C57BL/6 (e), MPTP-treated C57BL/6 (f), control BALB/c (g), and MPTP-treated BALB/c (h) mice, respectively. A reduction in TH-positive areas in the striatum (b) and TH-positive cells in the SNpc (f) was observed in MPTP-treated C57BL/6 mice; however, no obvious lesions were found in MPTP-treated BALB/c mice (d and h). EnVision method. Counterstained with methyl green. Scale bars = 1 mm (a to d) and 100  $\mu$ m (e to h).

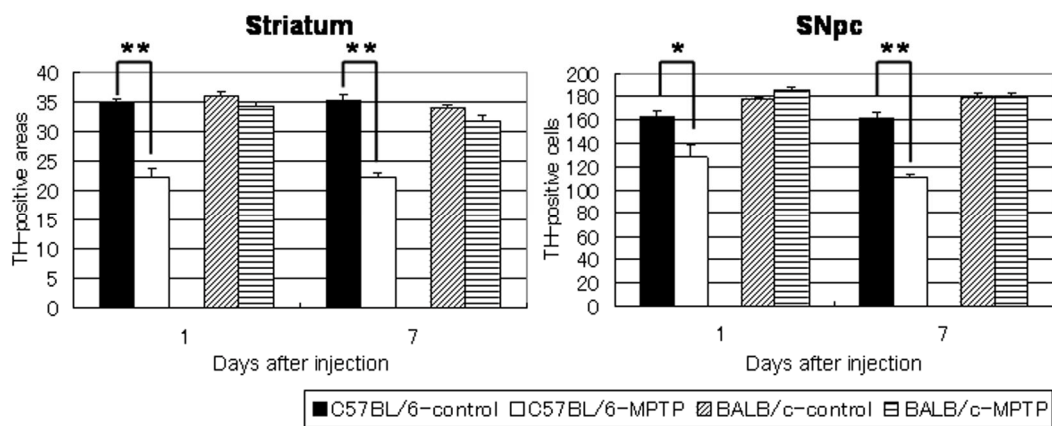


Fig. 2-2. Different strain susceptibility to MPTP between C57BL/6 and BALB/c mice. Significant decreases in TH-positive areas in the striatum and TH-positive cells in the SNpc were documented in C57BL/6 mice at 1 and 7 d. In BALB/c mice, however, no significant decreases in TH-positive areas and TH-positive cells were observed. Data are expressed as the mean  $\pm$  standard error (n=3). \* $p < 0.05$  and \*\* $p < 0.01$ .

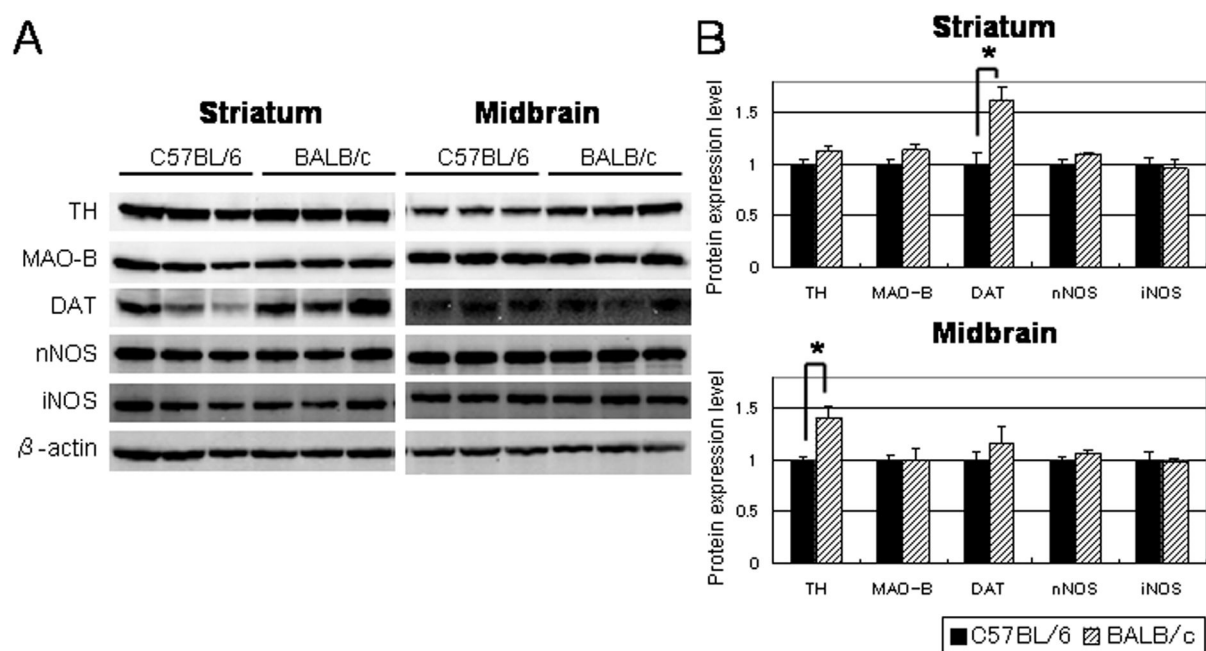


Fig. 2-3. (A) Representative photographs of Western blot analysis. The striatum and midbrain of intact C57BL/6 and BALB/c mice. (B) Results of quantitative Western blot analysis. Protein expression levels are represented as the ratio to the level of  $\beta$ -actin  $\pm$  standard error ( $n=3$ ). \* $P < 0.05$  when compared between C57BL/6 and BALB/c mice.

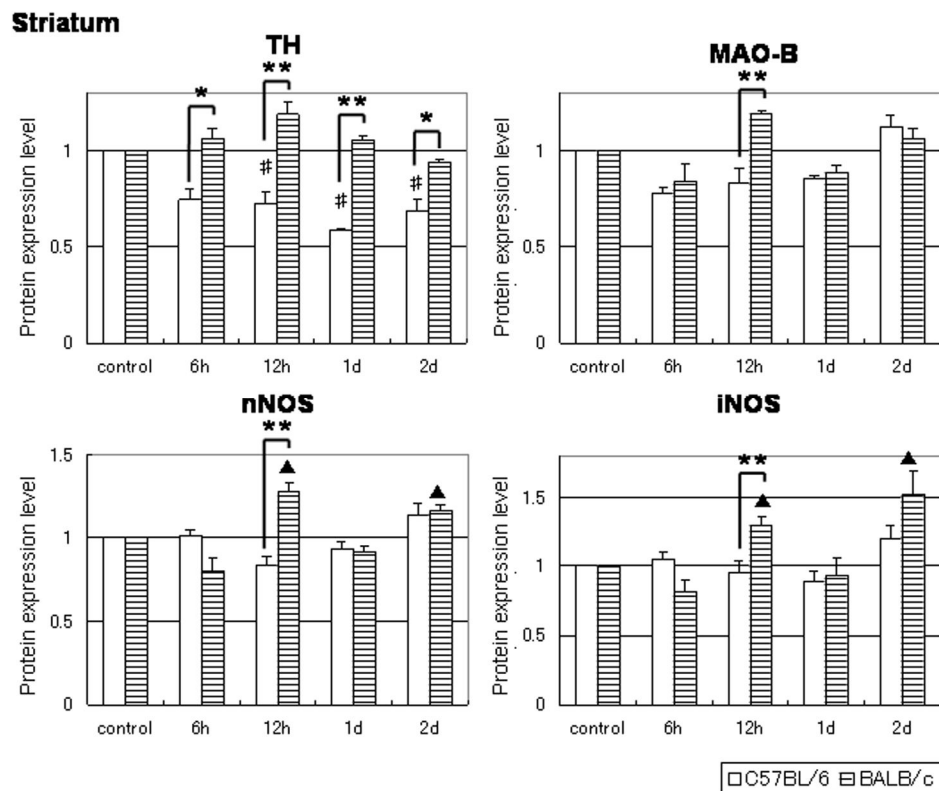


Fig. 2-4. Results of quantitative Western blot analysis in the striatum of MPTP-treated C57BL/6 and BALB/c mice. Protein expression levels are calculated as the ratio to the level of  $\beta$ -actin, and represented as the ratio to control mice  $\pm$  standard error ( $n=3$ ). \* $P < 0.05$  and \*\* $P < 0.01$  when compared between C57BL/6 and BALB/c mice. # $P < 0.05$  when compared with control C57BL/6 mice. ▲ $P < 0.05$  when compared with control BALB/c mice.

# Midbrain

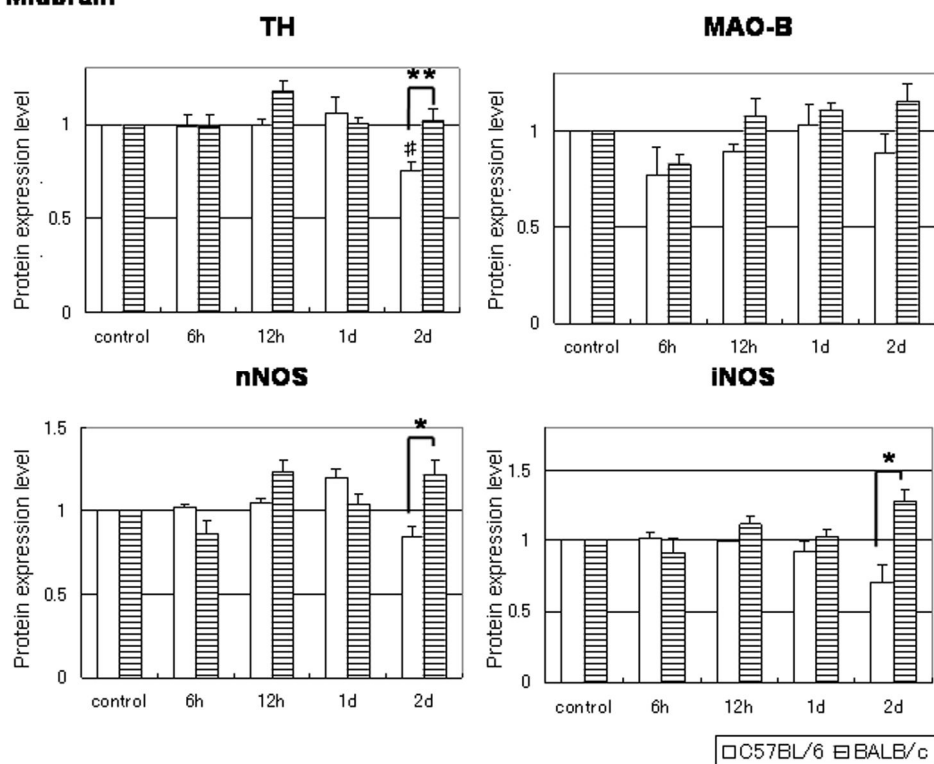


Fig. 2-5. The results of quantitative Western blot analysis in the midbrain of MPTP-treated C57BL/6 and BALB/c mice. Protein expression levels are calculated as the ratio to the level of  $\beta$ -actin, and represented as the ratio to control mice  $\pm$  standard error (n=3). \* $P < 0.05$  and \*\* $P < 0.01$  when compared between C57BL/6 and BALB/c mice. # $P < 0.05$  when compared with control C57BL/6 mice.

### **Chapter 3**

**MPTP-induced neuroblastic apoptosis in the subventricular zone is caused by**

**MPP<sup>+</sup> converted from MPTP through MAO-B**

## Abstract

ip administration of MPTP induces apoptosis of SVZ Dcx-positive neural progenitor cells (migrating neuroblasts, A cells). Actually, a metabolite of MPTP, 1-methy-4-phenylpyridinium (MPP<sup>+</sup>), is responsible for such neural progenitor cell toxicity. In the present study, to examine whether the MPTP-induced SVZ cell apoptosis is caused directly by MPP<sup>+</sup> metabolized through MAO-B, MPTP or MPP<sup>+</sup> was icv injected into C57BL/6 mice. At 1 day (d) post-injection, many TUNEL-positive cells were observed in the SVZ of both low (36µg) and high (162µg) dose MPTP- and MPP<sup>+</sup>-injected mice. The number of Dcx-positive A cells significantly decreased following high dose of MPTP- or MPP<sup>+</sup>-injection on 1 and 3 d, respectively, whereas that of EGFR-positive C cells showed no change in mice with any treatment. In addition, prior icv injection of a MAO-B inhibitor, R(-)-deprenyl (deprenyl), inhibited MPTP-induced apoptosis, but not MPP<sup>+</sup>-induced apoptosis. MAO-B- and GFAP- double positive cells were detected in the ependyma and SVZ in all mice. It is revealed from these results that icv injection of MPTP induces apoptosis of neural progenitor cells (A cells) in the SVZ via MPP<sup>+</sup> toxicity. In addition, it is suggested that the conversion from MPTP to MPP<sup>+</sup> is caused mainly by MAO-B located in ependymal cells and GFAP- positive cells in the SVZ.



## Introduction

In the brains of adult animals, including rodents, the SVZ lining the lateral ventricles is one of the primary regions of neurogenesis [1, 20]. The SVZ is composed of four types of cells: Dcx-positive neural progenitor cells (migrating neuroblasts, A cells), GFAP-positive neural stem cells (astrocytes, B cells), EGFR-positive neural precursor cells (rapidly dividing transit amplifying cells, C cells) and ependymal cells [20, 21, 60]. Neural stem B cells divide into clusters of precursor C cells, which in turn generate neuroblastic A cells [21]. Newly generated A cells in the SVZ migrate through a network of tangential pathways in the lateral wall of the lateral ventricle and then converge onto the rostral migratory stream (RMS) to enter the olfactory bulb, where they differentiate into interneurons [19].

Recently, it was shown that ip administration of MPTP induces apoptosis of SVZ A cells [27, 29]. In addition, such apoptosis was inhibited by the pretreatment with selective MAO-B inhibitors, R(-)-deprenyl (deprenyl, selegiline) or N-(2-Aminoethy)-4-chlorobenzamide (Ro 16-6491) [28], suggesting the inhibition was mediated by blockage of the conversion from MPTP to MPP<sup>+</sup>. In the present study, to examine whether the MPTP-induced neuroblast apoptosis is actually caused by MPP<sup>+</sup> toxicity, icv injections of MPTP or MPP<sup>+</sup> were given to adult C57BL/6 mice.

## Materials and Methods

### *Animals*

Eight-week-old male C57BL/6J mice, weighing 22 to 26 g, were purchased from Japan CLEA. The mice were housed in a room maintained under a constant temperature ( $23 \pm 2$  °C) and humidity ( $55 \pm 5$  %) condition with a 12-h light/dark cycle using an isolator caging system (Niki Shoji). Water and food were accessible *ad libitum*. All the experimental procedures used in the present study were approved by the Committee of Animal Experiments of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

### *Drug injection*

MPTP-HCl (Sigma) and MPP<sup>+</sup> iodide (Sigma) were dissolved in saline. After being anesthetized with ethyl ether, C57BL/6 mice ( $n = 3$  to  $4$ ) were injected with 10  $\mu$ l of a solution containing 36  $\mu$ g (low dose) or 162  $\mu$ g (high dose) of MPTP-HCl, or 18  $\mu$ g of MPP<sup>+</sup> iodide, respectively, into the unilateral ventricle. Control mice were injected with 10  $\mu$ l of saline. The stereotaxic coordinates of the injection site were as follows: bregma -0.5 mm, lateral 1.0 mm and depth 2.0 mm. The mice were sacrificed by cervical dislocation under ethyl ether anesthesia at 1 and 3 days after injection (1 and 3 d). For MAO-B inhibition, the animals ( $n = 3$  to  $4$ ) were icv injected with 5  $\mu$ l of a solution containing 36  $\mu$ g of R(-)-deprenyl (Biomol, Plymouth, PA) or saline. One hour after the injection, the animals were icv injected with 5  $\mu$ l of a solution containing 36  $\mu$ g or 162  $\mu$ g of MPTP-HCl, 18  $\mu$ g of MPP<sup>+</sup> iodide or saline. The mice were sacrificed as mentioned above at 1 day after injection (1 d).

### *Tissue preparation*

Brain samples were fixed in a 10% neutral buffered formalin solution for 4 days, processed routinely, and embedded in paraffin. Four  $\mu$ m-thick transverse sections encompassing the entire SVZ were used.

### *Detection of apoptotic cells*

Apoptotic cells containing fragmented DNA were detected by the terminal deoxynucleotidyl transferase-mediated dUTP endlabeling (TUNEL)-diaminobenzidine (DAB) method with an ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions.

### *Immunohistochemistry*

The primary antibodies used were goat anti-Dcx (1: 200, Santa Cruz Biotechnology) and rabbit anti-EGFR (1: 200, Bethyl Laboratories). Biotinylated anti-goat Ig-G (1: 400, Kirkegaard and Perry, Gaithersburg, MD) or the EnVision Anti-rabbit conjugation system (Dako) were used as secondary antibodies. Finally, the positive reaction was visualized with 0.05% 3,3'-diaminobenzidine (DAB) and 0.03% hydrogen peroxide in a Tris-HCl buffer, and the sections were counterstained with methyl green.

### *Double-labeling immunofluorescence*

After visualization of the binding of the goat anti-MAO-B (1: 10, Santa Cruz Biotechnology) with donkey anti-goat IgG- Alexa 488 (1: 100, Molecular Probes, Eugene, OR), the tissue sections were incubated with rabbit anti-GFAP (1: 50, Dako).

Visualization of the second primary antibody was accomplished using donkey anti-rabbit IgG- Alexa 594 (1: 100, Molecular Probes). The sections were then mounted with Vectashield (Vector Laboratories, Burlingame, CA) and observed using a Zeiss LSM510 confocal laser scanning microscope.

#### *Measurement and statistics*

Serial sections of the brain, including the SVZ corresponding to the bregma 0.50 to 0.98 mm (anterior-posterior coordinate), were collected with the aid of the mouse brain atlas [24]. At least one of every 15 sections of the contralateral side (total 4 to 6 sections per animal) was blindly counted for positive cells for TUNEL, EGFR or Dcx. Statistical significance was evaluated using the Student's *t*-test.

## Results

*TUNEL-positive cells in the SVZ are increased following icv injection of MPTP or MPP<sup>+</sup>*

A representative photograph of a hematoxylin and eosin (HE)-stained coronal section including the SVZ is shown in Figure 3-1A. At 1 d after MPTP- or MPP<sup>+</sup>-injection, TUNEL-positive cells were significantly increased in both the injection and contralateral sides (Figs. 3-1B and 3-1C). By 3 d, the number of TUNEL-positive cells declined markedly (Fig. 3-1C). Especially, high dose (162μg) MPTP-treated mice showed a prominent decrease in the number of TUNEL-positive cells.

*Dcx-positive cells in the SVZ decreased after icv injection of MPTP or MPP<sup>+</sup>*

A previous report [27] indicated that the cells undergoing apoptosis in the SVZ after ip-administration of MPTP were Dcx-positive migrating neuroblasts (A cells). To confirm that icv-injected MPTP or MPP<sup>+</sup> induces apoptosis of A cells, Dcx- and EGFR-positive cells were counted. In high dose icv MPTP- and MPP<sup>+</sup>-injected mice, a significant decrease in the number of SVZ Dcx-positive cells was found at 1 and 3 d, respectively (Fig. 3-2). Low dose icv MPTP-injected mice, however, showed no significant decrease in the number of Dcx-positive cells (Fig. 3-2). EGFR-positive cells showed no significant changes in any mice (Fig. 3-2). These results indicate that the cells undergoing apoptosis are A cells.

*SVZ apoptosis induced by icv injection of MPTP is inhibited by a MAO-B inhibitor (deprenyl)*

In mice following low or high dose MPTP-injection, and MPP<sup>+</sup>-injection,

TUNEL-positive cells were significantly increased at 1 d (Figs. 3-3A and 3-3B), whereas the prior icv injection of deprenyl significantly decreased the number of TUNEL-positive cells induced by low or high dose MPTP-injection, but not that by MPP<sup>+</sup>- injection (Figs. 3-3A and 3-3B). These results indicate that MAO-B inhibitors may protect SVZ neuroblasts from MPTP-induced apoptosis, and such apoptosis is likely to be induced by the direct action of MPP<sup>+</sup>.

*MAO-B is located mainly in GFAP-positive ependymal and SVZ cells*

By double-labeling immunofluorescence analysis, MAO-B was detected in GFAP-positive ependymal cells and GFAP-positive cells in the SVZ in all mice (Fig. 3-4).

## Discussion

Previous reports [27, 29] indicated that ip administration of MPTP induces apoptosis of Dcx-positive A cells in the SVZ. In the present study, icv MPTP-injected mice showed a significant increase in TUNEL-positive cells in the SVZ at 1 d. In addition, following icv injection of high dose MPTP, Dcx-positive A cells significantly decreased at 1 d, whereas EGFR-positive C cells showed no significant change. The results indicate that icv MPTP-injection induces apoptosis of A cells in the SVZ, as reported in the case of ip MPTP-administration. In addition, icv MPP<sup>+</sup>-injected mice also showed a significant increase in TUNEL-positive cells in the SVZ at 1 d and a decrease in Dcx-positive cells at 3 d. MPP<sup>+</sup>-induced A cell apoptosis was observed later than that observed by MPTP-injection. Because MPP<sup>+</sup> is a polar molecule, it may take a lot of time to enter ependymal cells.

MAO-B is associated with not only MPTP-induced DA-ergic neuronal death [30, 14], but also MPTP-induced apoptosis of SVZ neuroblasts because pretreatment with a MAO-B inhibitor, deprenyl or Ro 16-6491, protects SVZ neuroblasts from apoptosis [28]. Also in the present study, deprenyl-pretreatment inhibited MPTP-induced apoptosis, while both chemicals were icv injected. Deprenyl has been suggested to have a unique direct neuroprotective action in addition to the inhibition of MAO-B [75, 53, 42]. However, the antiapoptotic effect of deprenyl is not due to such a neuroprotective action, but to MAO-B inhibition because the pretreatment with deprenyl did not inhibit MPP<sup>+</sup>-induced apoptosis. Accordingly, it was revealed that icv injection of MPTP induces apoptosis of neural progenitor cells (A cells) in the SVZ due to the toxicity of MPP<sup>+</sup> metabolized through MAO-B.

Generally, MAO-B is localized predominantly in astrocytes and serotonergic

neurons [67], and MPTP, when ip administered, is metabolized through MAO-B in astrocytes in the brain parenchyma [8, 62]. To identify the localization of MAO-B around the lateral ventricle, double-immunofluorescence using antibodies to MAO-B and GFAP was performed in the present study. Localization of MAO-B and GFAP were highly overlapped in the ependyma and SVZ. These results indicate that cells converting injected MPTP to MPP<sup>+</sup> through MAO-B are mainly ependymal cells and GFAP-positive cells in the SVZ. Previous reports indicate that ependymal cells express MAO-B by *in situ* hybridization and histochemistry in adult C3H and MAO-A-deficient mice [34, 77]. On the other hand, in adult mice MAO-B expression in the SVZ has not been reported in adult mice. Thus, whether such MAO-B- and GFAP-double positive cells in the SVZ in the present study were astrocytes or neural stem cells (B cells) remains unclear. A previous report [59] indicated that after ip MPTP-administration, GFAP-positive cells increased in the SVZ, but Ki-67-positive proliferating cells did not change, so the report concluded that the increased GFAP-positive cells in the SVZ were not B cells, but were due to up-regulation of gliogenesis.

Besides the apoptosis of A cells induced by MPTP, a report [33] indicated that the MPTP administration induced reduction of the generation of A cells by C cells by DA depletion. In addition, by high performance liquid chromatography, a significant reduction in striatal dopamine was observed in icv-injected C57BL/6 mice with 18 µg MPP<sup>+</sup> at Day 1 [38]. Thus, in the present study, after icv MPTP and/or MPP<sup>+</sup> injection, the DA depletion might also be involved in the A cell reduction. In addition, icv-injected MPP<sup>+</sup> mice showed C cell reduction. Previous report indicated that apoptotic death occurred in a few B cells [27]. B cell apoptosis might result in C cell



reduction, inducing A cell reduction.

In conclusion, icv-injected-MPTP induces A cell apoptosis in the SVZ at 1 d by MPP<sup>+</sup> toxicity. The conversion from MPTP to MPP<sup>+</sup> is conducted mainly in MAO-B-positive ependymal cells and GFAP-positive cells in the SVZ. Since it is unlikely that either DAT or other monoamine transporters are involved in the MPTP-induced apoptosis [70], the mechanism by which MPP<sup>+</sup> affects SVZ A cells must be clarified.

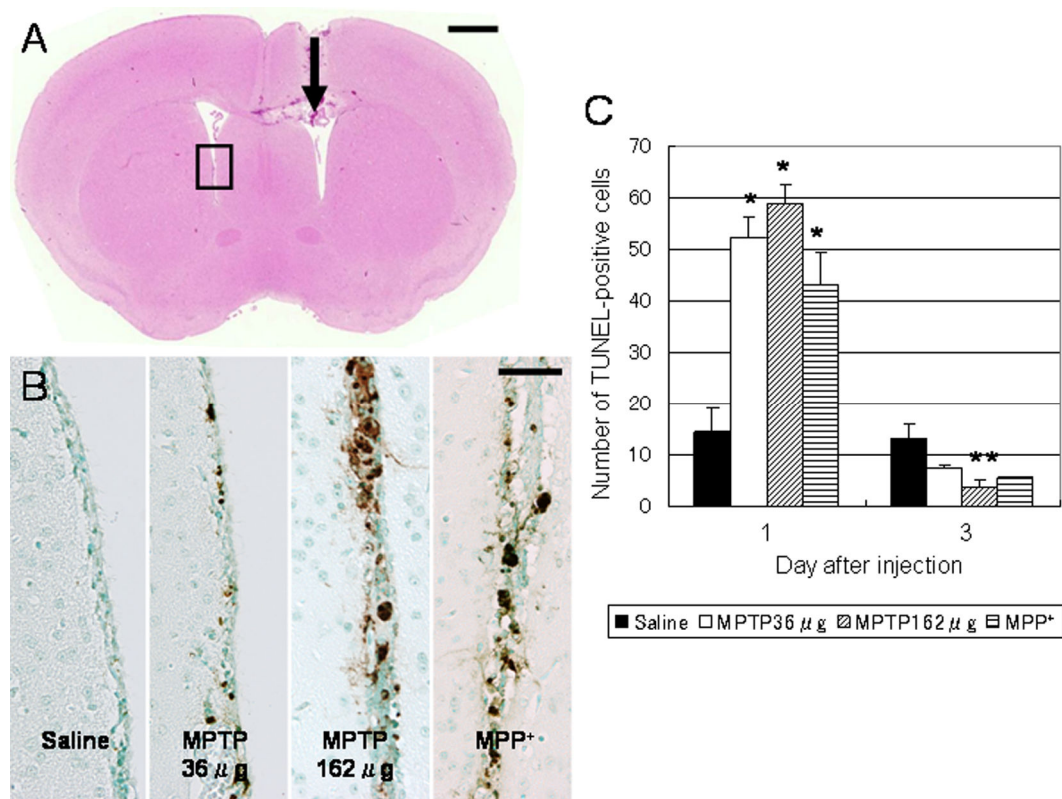


Fig. 3-1. (A) A representative photograph of a hematoxylin and eosin (HE)-stained coronal section, including the SVZ of a saline-injected C57BL/6 mouse, at 1 d. The arrow indicates the injection site and the box indicates the site of observation. (B) Representative photographs of TUNEL-stained coronal sections including the SVZ at 1 d. Many TUNEL-positive cells were detected in both low and high dose MPTP- and MPP<sup>+</sup>-injected mice. (C) The results of quantitative TUNEL analysis. The numbers of TUNEL-positive cells in the SVZ are represented as the mean  $\pm$  standard error ( $n=3$  to 4). \* $P < 0.05$  when compared with saline-injected control mice at 1 d. \*\* $P < 0.05$  when compared with saline-injected mice at 3 d. Scale bars = (A) 1 mm; (B) 50 $\mu$ m.

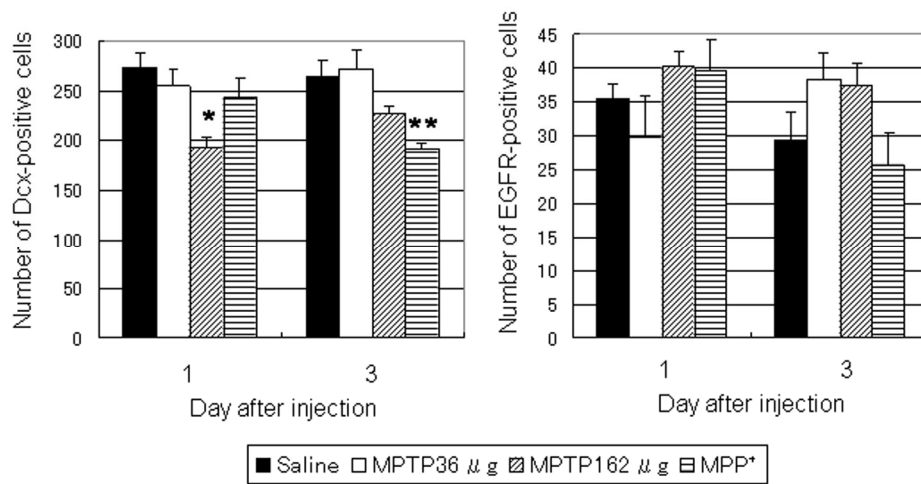


Fig. 3-2. The results of quantitative analysis of Dcx- and EGFR-positive cells in the SVZ of the contralateral injection side. The numbers of Dcx- and EGFR-positive cells are represented as the mean  $\pm$  standard error (n=3 to 4). \* $P < 0.05$  when compared with saline-injected mice at 1 d. \*\* $P < 0.05$  when compared with saline-injected mice at 3 d.

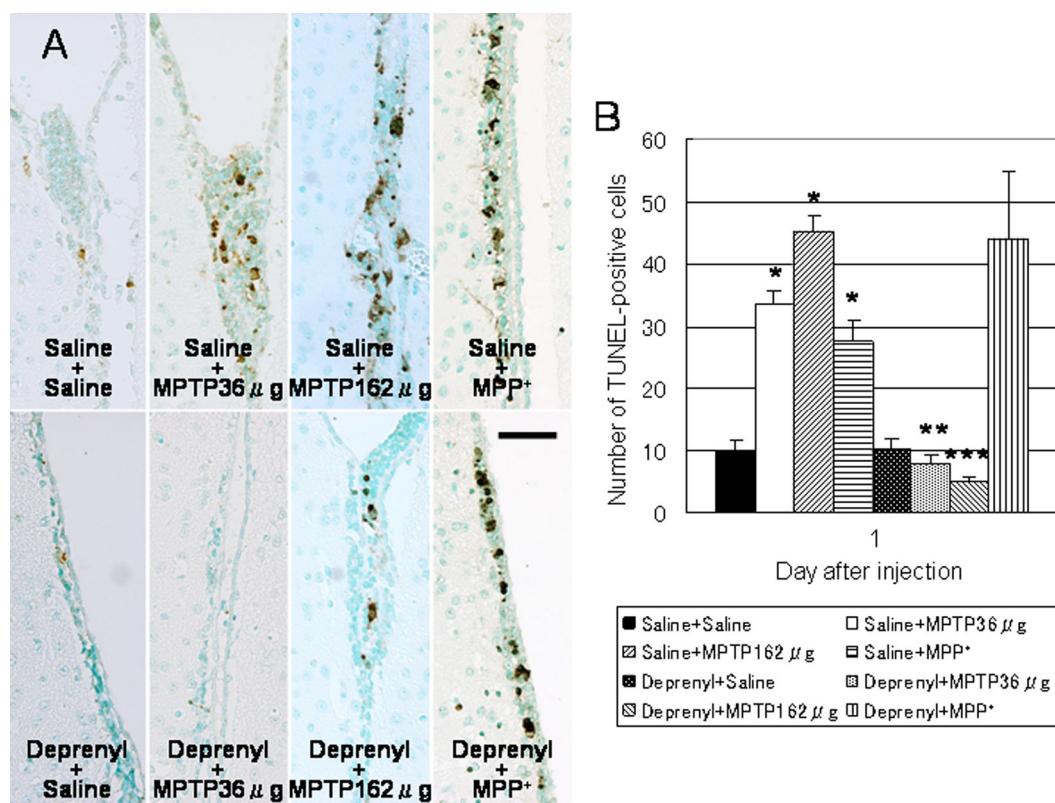


Fig. 3-3. (A) Representative photographs of TUNEL-stained coronal sections including the SVZ at 1 d. Many TUNEL-positive cells were detected in low and high dose MPTP- and MPP<sup>+</sup>-injected mice. In addition, prior icv injection of deprenyl rescued TUNEL-positive cells by MPTP, but not by MPP<sup>+</sup> injection. (B) The results of quantitative TUNEL analysis. The numbers of TUNEL-positive cells in the SVZ are represented as the mean  $\pm$  standard error (n=3 to 4). \* $P$  < 0.05 when compared with the saline + saline injected group at 1 d. \*\* $P$  < 0.05 when compared with the saline + low dose (36 $\mu$ g) MPTP group at 1 d. \*\*\* $P$  < 0.05 when compared with the saline + high dose (162 $\mu$ g) MPTP group at 1 d. Scale bars = 50 $\mu$ m.

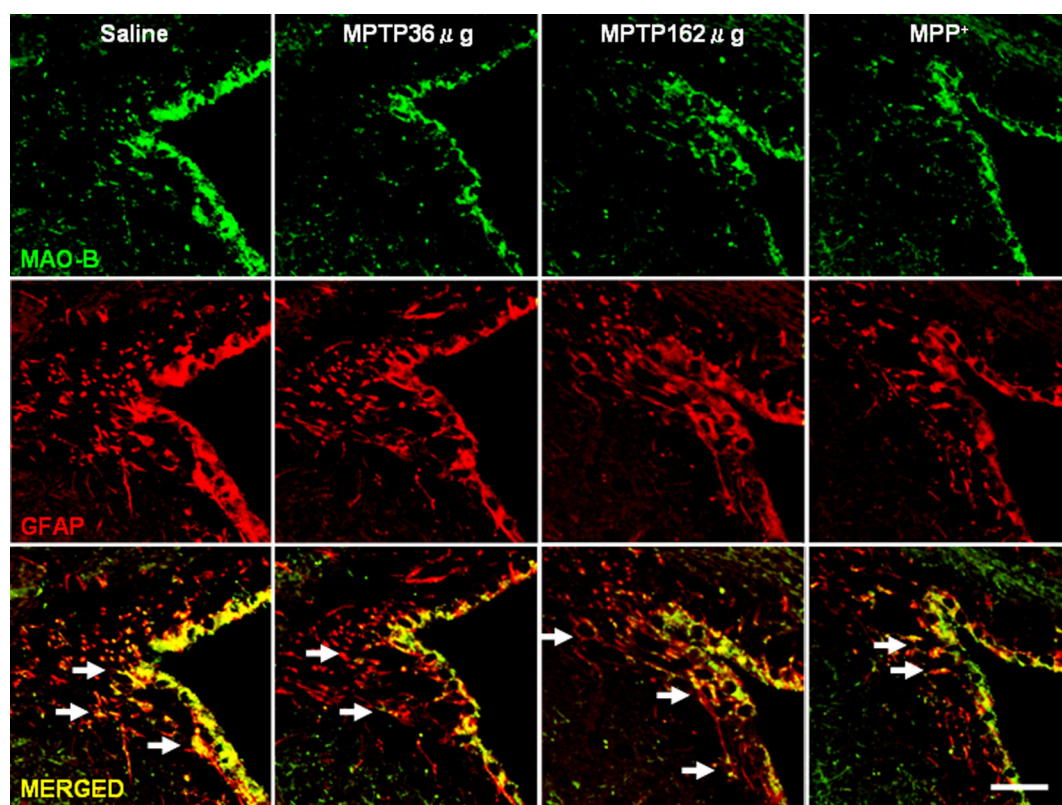


Fig. 3-4. Representative photographs of double-immunolabeling for MAO-B and GFAP. MAO-B-positive cells were merged with GFAP-positive cells in the ependyma and SVZ (arrows). Scale bar = 40 $\mu$ m.

## **Conclusions**

In this thesis, I tried to clarify the roles of MAO-B in the neurotoxicity of MPTP to the nigro-striatal DAergic cells and SVZ neuroblasts.

In Chapter 1, MPP<sup>+</sup> was icv injected into susceptible C57BL/6 and resistant BALB/c mice, and the lesions were histologically examined. My observation revealed that the icv injection induced different neurotoxicity between C57BL/6 and BALB/c mice, being similar to the results using ip injection of MPTP. Namely, C57BL/6 mice were susceptible and BALB/c mice were resistant. In Chapter 2, MAO-B protein expression levels were examined in intact and MPTP-treated mice by Western blot analysis. Intact mice showed no strain differences. MPTP-treated BALB/c mice showed a significant increase of MAO-B expression than MPTP-treated C57BL/6 mice at 12 h after injection. These results indicate that MAO-B expression level does not influence the different strain susceptibility to MPTP. Most previous reports have been indicated that MAO-B activity level is not related to the different strain susceptibility to MPTP. However, the expression level of MAO-B between mouse strains has never been examined. In this thesis, the MAO-B expression level was compared between MPTP susceptible and resistant mice for the first time. From this thesis and previous reports it is revealed that neither MAO-B expression nor activity levels influence the strain differences. In addition, intact BALB/c mice showed a higher DAT protein expression in the striatum than intact C57BL/6 mice. The increase of nNOS and iNOS protein expressions in MPTP-treated BALB/c mice was more pronounced in the striatum and midbrain than in MPTP-treated C57BL/6 mice at 12 h and 2 d. These results indicate that neither DAT, nNOS nor iNOS levels influence the different strain susceptibility to MPTP (Fig. 2A). However, using two-way ANOVA between MPTP-treated C57BL/6 and BALB/c mice, significant lower

nNOS expression level of MPTP-treated BALB/c mice was shown at 6 h in the striatum. This result might indicate that lower nNOS expression level contributes to the MPTP resistance in BALB/c mice at very early time point.

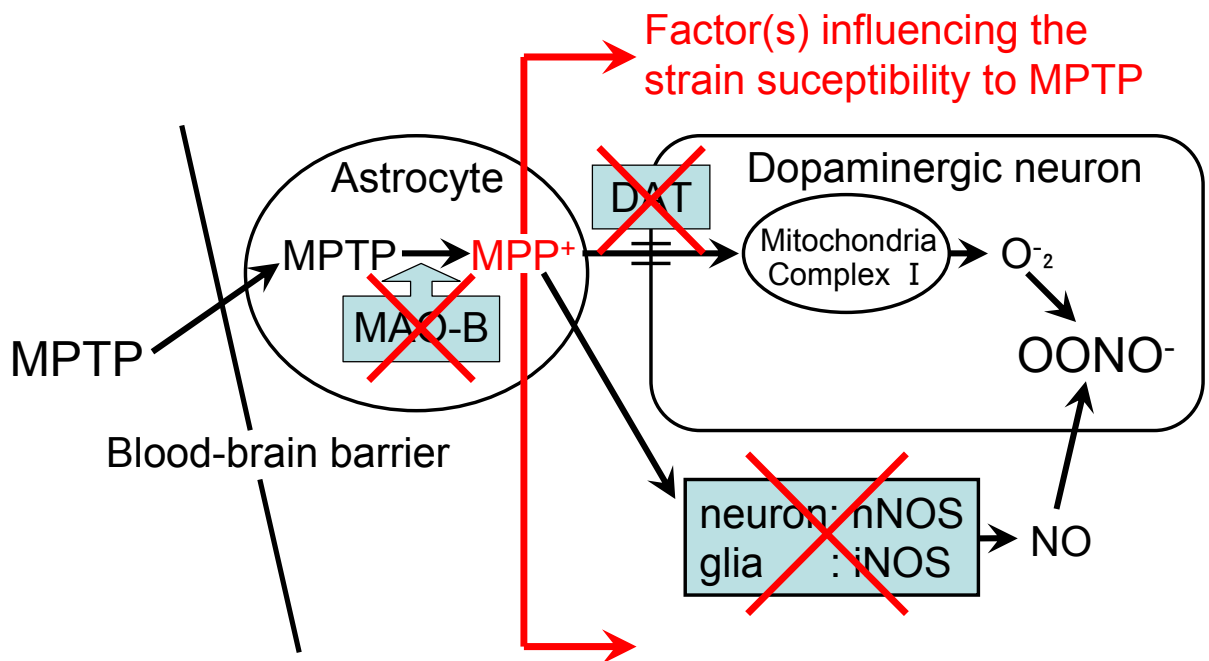
In Chapter 3, I focused on whether the MPTP-induced SVZ cell apoptosis is caused directly by MPP<sup>+</sup> metabolized through MAO-B. MPTP or MPP<sup>+</sup> was icv injected into C57BL/6 mice. It was revealed that icv injection of both MPTP and MPP<sup>+</sup> induced apoptosis of neural progenitor cells (A cells) in the SVZ. In addition, the conversion from MPTP to MPP<sup>+</sup> is caused mainly by MAO-B located in ependymal cells and GFAP- positive cells in the SVZ. Thus, the SVZ A cell apoptosis is induced directly by MPP<sup>+</sup> converted from MPTP by MAO-B (Fig. 2B).

Taken these results together, the expression levels of MAO-B, nNOS, iNOS and DAT are not responsible for the strain different susceptibility to MPTP between susceptible C57BL/6 and resistant BALB/c mice. It is unclear which factor(s) is responsible for the strain differences, though from the result of Chapter 1, the factor(s) might perform after MPTP is converted to MPP<sup>+</sup> in the brain. In addition, from the result using two-way ANOVA between MPTP-treated C57BL/6 and BALB/c mice, lower nNOS expression level might contribute to the MPTP resistance in BALB/c mice at very early time point after MPTP administration. Many similarities between pathogenesis of MPTP induced parkinsonism and that of idiopathic PD are reported. Thus, clarifying the factor(s) responsible for the strain different susceptibility to MPTP would contribute to identify those persons susceptible to parkinsonism including PD. In addition, it is revealed that SVZ cells of PD patients also decrease, thus clarifying the mechanisms of MPTP-induced SVZ apoptosis also would have an important role. The present studies will provide new insights to MPTP neurotoxicity and to the



understanding of PD pathogenesis.

**A**



**B**

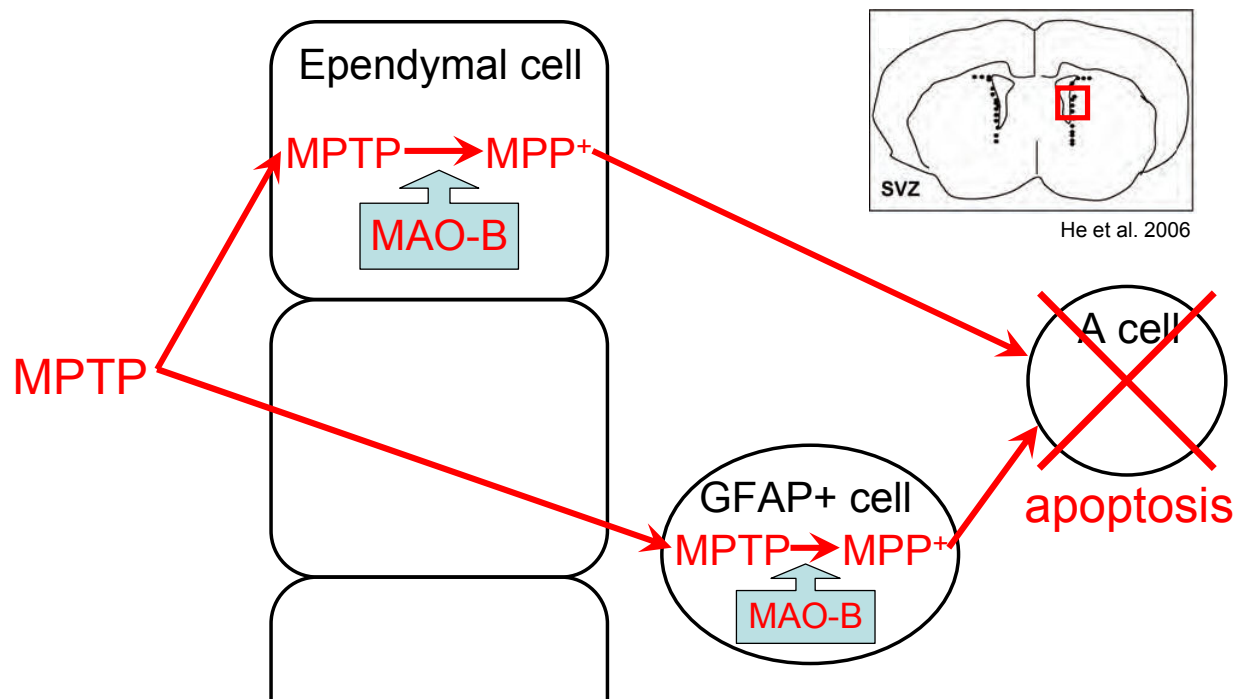


Fig. 2. Schematic drawings indicating the conclusions of Chapter 1 and 2 (A) and Chapter 3 (B).

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