Manganese does not alter the severe neurotoxicity of MPTP

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We utilized a mice model of Parkinsonism: (1) to evaluate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity; and (2) to evaluate whether manganese (Mn) exposure can affect MPTP-induced neurotoxicity. A $2 \times 3$ experimental design (MPTP $\times \pm$ Mn) was as follows: SS, MPTP($-$) $\times$ low Mn($+$); SLMn, MPTP($-$) $\times$ high Mn($+$); SHMn, MPTP($+$) $\times$ low Mn($+$); MpS, MPTP($+$) $\times$ Mn($-$); MpLMn, MPTP($+$) $\times$ high Mn($+$). We administered MPTP (30 mg/kg per day) to male C57BL/6 mice intraperitoneally, once a day for 5 days. Subsequently, mice were treated with either 2 or 8 mg/kg of MnCl$_2$·4H$_2$O intraperitoneally, once a day for 3 weeks.

Blood and striatal Mn levels were elevated in the Mn-exposed groups. The number of tyrosine hydroxylase (TH)-immunoreactive (ir) neurons in the substantia nigra pars compacta were decreased significantly in the MPTP-exposed groups. The densities of TH-ir axon terminals in caudate-putamen (CPU) were significantly decreased in the MPTP-treated groups. However, Mn treatment did not affect MPTP neurotoxicity. The densities of glial fibrillary acidic protein (GFAP)-ir astrocytes in the CPU or globus pallidus were significantly increased in the MPTP-treated groups. Concentrations of dopamine in the striatum were decreased significantly in the MPTP-exposed groups only, but Mn had no effect. Human & Experimental Toxicology (2007) 26, 203–211

Key words: manganese; MPTP; Parkinson disease; neurotoxicity; dopamine

Introduction

The importance of environmental agents, such as manganese (Mn) and pesticides, in the etiology of Parkinson’s disease (PD) is gaining recognition. However, epidemiologic data on the role of Mn as a risk factor for PD is contradictory. Animal model data on the contribution of Mn to the development of Parkinsonism is also contradictory. It is well known that Mn causes a certain type of Parkinsonism, PD-like basal ganglia dysfunction (manganism), which is different from PD. However, recently, several reports suggest that Mn might cause PD as well. Hence, mechanistic animal model data, which determine the contribution of Mn to the development of Parkinsonism, are needed. We utilized a mice model of Parkinsonism: (1) to evaluate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity; and (2) to evaluate whether Mn exposure can affect MPTP-induced neurotoxicity.

Materials and methods

The present study utilized a MPTP mice model of Parkinsonism and a $2 \times 3$ experimental design ($\pm$ MPTP $\times \pm$ Mn) as follows: SS, MPTP($-$) $\times$ Mn($-$); SLMn, MPTP($-$) $\times$ low Mn($+$); SHMn, MPTP($-$) $\times$ high Mn($+$); MpS, MPTP($+$) $\times$ Mn($-$); MpLMn, MPTP($+$) $\times$ low Mn($+$); MpHMn, MPTP($+$) $\times$ high Mn($+$). Each group contained seven to ten mice.
We simultaneously treated three sets of animals with the same dosing regimen and assessed several neurochemical outcomes: (1) Mn levels in blood and brain (striatum); (2) immunohistochemistry; and (3) concentrations of dopamine and its metabolites, in each set of animals.

Animals

Male C57BL/6J mice (8-weeks-old, body weight 25-30 g) were caged in an air-conditioned room maintained at 22 ± 2°C, relative humidity 50 ± 10%, with a 12:12 hour light:dark cycle. The animals had free access to tap water and were fed a conventional rat chow diet *ad libitum*. The animals were acclimated for 2 week prior to the start of the study. All procedures related to animal care were in accordance with the guidelines for the Care and Use of Laboratory Animals of College of Medicine, Pusan National University. We administered MPTP (30 mg/kg per day) to the MpS, MpLMn and MpHMn group, into the peritoneum, once a day for 5 days. The SS, SLmN, and SHMn group received the same volume of saline intraperitoneally. Subsequently, the MpLMn and SLMn group were treated intraperitoneally with 0.2 mL of MnCl$_2$·4H$_2$O (2 mg/kg body weight), and the MpHMn and SHMn group were treated with 0.8 mL of MnCl$_2$·4H$_2$O (8 mg/kg body weight) once a day for 3 weeks. The MpS and SS group received the same volume of saline intraperitoneally.

After the last Mn injection, the mice were sacrificed. The whole brain was removed, sliced in a contour-fit brain matrix, and divided into the cerebral cortex, the striatum, thalamus, ventral midbrain – including the substantia nigra (SN), the cerebellum, pons, and medulla. We determined Mn concentrations in the whole blood and the striatum using a flameless graphite furnace atomic absorption spectrophotometry (Spectra AA880-GTA 100; Varian, Australia).

Immunohistochemical analysis

Animals were anesthetized with pentobarbital sodium (50 mg/kg) and sacrificed by intracardiac perfusion with 4% paraformaldehyde in a 0.1-M phosphate buffer, pH 7.4. Brains were post-fixed for 4 hours at 4°C and cryoprotected with 30% sucrose. Tissue was frozen with a compound embedding medium in dry ice powder. Coronal sections were cut 30-µm thick using cryostat and processed for immunohistochemistry. Every sixth section of the corpus striatum and midbrain were reacted with immunohistochemistry, as previously described. Sections were incubated in a blocking buffer (0.3% Triton X-100 and 10% goat serum in phosphate buffered saline; PBS) for 1 hour, followed by overnight incubation with primary antibodies to tyrosine hydroxylase (TH) (rabbit monoclonal anti-TH, 1:500, Chemicon), glial fibrillary acidic protein (GFAP) (rabbit polyclonal anti-GFAP, 1:200; Dako) in an incubation buffer (0.1% Triton X-100, 1% goat serum and 1% bovine serum albumin in PBS) at 4°C. Sections were then washed three times for 10 min each with PBS and incubated for 2 hours at room temperature in biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) to detect TH and GFAP, respectively. Sections were washed and processed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) with 0.01% H$_2$O$_2$. For all incubation and rinse steps, the sections were agitsted on a shaker table.

After processing, the sections were washed, mounted on coated slides, dried, dehydrated through graded alcohols, cleared in xylene, and coverslipped with an Entellan mountant medium (Merck). In order to control staining conditions, we ran the sections from the normal and MPTP-treated mice through the histochemical staining steps at the same time.

Morphological analysis

Three rostrocaudal levels (2.92 mm posterior to bregma, 3.28 mm posterior to bregma, 3.40 mm posterior to bregma) of the SN were chosen for image analysis to assess the regional difference of Mn and/or MPTP effects. The SN region containing TH-immunoreactive (ir) neurons was photographed with a digital camera at 200 × magnification and combined into a single section with Photoshop 6.0 software (Adobe, USA). The borders of the SN at all

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Mn level (µg/dL)</th>
<th>Striatal Mn level (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS (n = 9)</td>
<td>3.4 ± 0.5*</td>
<td>0.46 ± 0.05**</td>
</tr>
<tr>
<td>SLmN (n = 7)</td>
<td>8.1 ± 1.6**</td>
<td>0.77 ± 0.39</td>
</tr>
<tr>
<td>SHMn (n = 8)</td>
<td>17.7 ± 2.3</td>
<td>1.48 ± 0.26</td>
</tr>
<tr>
<td>MpLS (n = 7)</td>
<td>3.7 ± 0.3*</td>
<td>0.34 ± 0.07**</td>
</tr>
<tr>
<td>MpLMn (n = 7)</td>
<td>8.2 ± 0.7**</td>
<td>0.66 ± 0.26</td>
</tr>
<tr>
<td>MpHMn (n = 7)</td>
<td>16.1 ± 2.8</td>
<td>0.94 ± 0.18</td>
</tr>
</tbody>
</table>

Concentrations of Mn in blood (µg/dL) and the corpus striatum (µg/g tissue) were measured.

*Significant difference versus SLMn, MpLMn, SHMn, MpHMn (P < 0.05) based on Scheffe’s post-hoc analyses.

**Significant difference versus SHMn, MpHMn (P < 0.05) based on Scheffe’s post-hoc analyses.

Mean ± SD;

n, number of mice.
Figure 1. Photomicrographs showing tyrosine hydroxylase (TH)-immunoreactive (ir) neurons in the rostral level (A, D, G, J, M, P), middle level (B, E, H, K, N, Q) and caudal level (C, F, I, L, O, R) of substantia nigra in the SS group (A–C), MpS (D–F), SLMn (G–I), MpLMn (J–L), SHMn (M–O) and MpHMn (P–R). The numbers of TH-ir neurons are significantly decreased in the MPTP-treated groups (MpS, MpLMn, MpHMn), especially in the rostral level. Scale bar in R is 75 μm.
The number of tyrosine hydroxylase (TH)-immunoreactive (ir) neurons in the substantia nigra pars compacta (SNpc) and TH-ir axon terminals in the caudate-putamen (CPU) were measured. Means of number of TH-ir neurons are statistically different among treatment groups ($P < 0.001$). The optical densities of TH-ir axon terminals in the CPU, which were corrected for non-specific background density, are presented as a percentage area of the measured total area ($\%$ area). Means of optical density of TH-ir axon terminals are statistically different among treatment groups ($P < 0.001$).

*Significant difference versus MpS, MpLMn and MpHMn ($P < 0.05$), based on Scheffe’s post-hoc analyses.

Mean $\pm$ SD; $n$, number of mice.

**Table 2** The number of TH-ir neurons in the SNpc and density of TH-ir axon terminals in the CPU

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of TH-ir neurons in SNpc</th>
<th>Densities of TH-ir axon terminals in CPU ($%$ area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>62.1 $\pm$ 11.3 ($n = 9$)*</td>
<td>36.2 $\pm$ 3.8 ($n = 9$)*</td>
</tr>
<tr>
<td>SLMn</td>
<td>47.8 $\pm$ 12.4 ($n = 9$)*</td>
<td>31.6 $\pm$ 4.6 ($n = 10$)*</td>
</tr>
<tr>
<td>SHMn</td>
<td>46.7 $\pm$ 12.8 ($n = 8$)*</td>
<td>31.4 $\pm$ 4.1 ($n = 10$)*</td>
</tr>
<tr>
<td>MpS</td>
<td>32.6 $\pm$ 11.2 ($n = 8$)</td>
<td>10.7 $\pm$ 3.3 ($n = 9$)</td>
</tr>
<tr>
<td>MpLMn</td>
<td>38.2 $\pm$ 9.2 ($n = 8$)</td>
<td>8.4 $\pm$ 3.0 ($n = 10$)</td>
</tr>
<tr>
<td>MpHMn</td>
<td>38.8 $\pm$ 8.9 ($n = 8$)</td>
<td>9.2 $\pm$ 2.8 ($n = 9$)</td>
</tr>
</tbody>
</table>

The number of tyrosine hydroxylase (TH)-immunoreactive (ir) neurons in the substantia nigra pars compacta (SNpc) and TH-ir axon terminals in the caudate-putamen (CPU) were measured. Means of number of TH-ir neurons are statistically different among treatment groups ($P < 0.001$). The optical densities of TH-ir axon terminals in the CPU, which were corrected for non-specific background density, are presented as a percentage area of the measured total area ($\%$ area). Means of optical density of TH-ir axon terminals are statistically different among treatment groups ($P < 0.001$).

*Significant difference versus MpS, MpLMn and MpHMn ($P < 0.05$), based on Scheffe’s post-hoc analyses.

Mean $\pm$ SD; $n$, number of mice.

levels in the rostrocaudal axis were defined. The medial border was defined by a vertical line passing through the medial tip of the cerebral peduncle, thereby excluding the TH-positive cells in the ventral tegmental area. The ventral border followed the dorsal border of the cerebral peduncle, thereby including the TH-positive cells in the substantia nigra pars reticulata (SNpr), and the area extended laterally to include the pars lateralis in addition to the substantia nigra pars compacta (SNpc). A labeled cell was defined as a TH-ir soma with or without a visible nucleus. Pieces of cells were counted if they were larger than half the size of nearby cells with clearly visible nuclei. We excluded from the count faintly-stained cells encountered at the rostral-medial portion of SNpc.

The optical densities of TH-ir axon terminals and GFAP-ir astrocytes in the striatum were measured using the Metamorpho 4.7 Image program (Universal Image Corporation, USA) on a personal computer connected to a digital camera with constant illumination. The relative optical density for each animal at the three rostrocaudal levels of striatum (1.1 mm anterior to bregma, 0.14 mm anterior to bregma and 0.82 mm posterior to bregma) were measured according to the atlas of Paxinos and Watson,\(^{27}\) to assess the regional difference due to Mn and/or MPTP effects. The data were presented as a percentage area of the total measured area ($\%$ area).

**Figure 2** Photomicrographs showing tyrosine hydroxylase (TH)-immunoreactive (ir) structures in the rostral level (A, D, G, J, M, P), middle level (B, E, H, K, N, Q) and caudal level (C, F, I, L, O, R) of striatum in the SS group (A–C). MpS (D–F), SLmM (G–I), MpLMn (J–L), SHMn (M–O) and MpHMn (P–R). The density of TH-ir axon terminals is significantly decreased in the MPTP-treated groups (MpS, MpLMn, MpHMn), especially in the rostral level. Scale bar in R is 140 $\mu$m.

**Neurochemical measurements**

Monoamine enzymes in brain tissue were inactivated in microwave ovens as soon as the brain was extracted. Brain tissue was then weighed and homogenized on ice with 20:1 (v/w) of a degassed buffer (0.4 M sodium perchlorate, 0.5 M acetic acid, 0.5 M sodium acetate, 2 mM Na$_2$ EDTA) containing the internal standard 3,4-dihydroxybenzylamine. The homogenate was centrifuged (30 min at 48 000 $\times$ g at 4°C), and the supernatant was stored at $-$80°C until analysis. The supernatant fraction (20 $\mu$L aliquots) was used for the assay. Dopamine (DA), 3,4 dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) in the corpus striatum were measured using HPLC (model 307; Gilson) with an electrochemical detector (ICA-3063; Toa).

$C_{18}$ 3-$\mu$m reversed-phase 150 $\times$ 4.6 mm column (Nakalai) was used. The mobile phase was 0.1 M KH$_2$PO$_4$ (pH 3.2)/methanol containing 2.5 mM 1-octane sulfate and 10 $\mu$M EDTA (85/15), and set at a flow rate of 0.9 mL/min. The working electrode potential for the detector was set at 0.5 V and the range selector switch at 5 nA full scale. Concentrations of DA and its metabolites were expressed as $\mu$g/gram striatal tissue weight. DA turnover was evaluated as (DOPAC+HVA)/DA ratio.

**Data analysis**

Three or five morphologic sections were obtained from each mouse, and mean difference of overall morphologic sections in each group was compared by one-way ANOVA. If the one-way ANOVA showed statistical significance among the six groups at $P < 0.05$, the Scheffe’s multiple comparison test was used to identify which subgroup was significantly different from the other subgroups. All analyses were performed using SPSS, tenth edition.

**Results**

**Mn concentrations**

The Mn concentration in the blood was significantly increased in the high Mn-exposed groups (SHMn, MpHMn) compared to low Mn-exposed groups (SLMn, MpLMn), as well as unexposed groups (SS, MpS) (Table 1). The Mn concentration in the striatum was significantly increased in the high Mn-exposed groups (SHMn, MpHMn) compared to the non-exposed groups (SS, MpS).
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Figure 2
**Immunohistochemical analysis**

Dopaminergic neurons in the SNpc have TH-immunoreactivity in the soma and dendritic processes (Figure 1). The number of TH-ir neurons in the SNpc was significantly decreased in the MPTP-exposed groups (MpS, MpLMn and MpHMn) in comparison with the unexposed groups (SS or SLMn and SHMn groups) especially in the rostral level (Figure 1). The numbers of TH-ir neurons were counted in the total rostro-caudal levels of SN (Table 2).

TH-ir axon terminals were distributed evenly throughout the CPU in the control SS group. The densities of TH-ir axon terminals in CPU were significantly decreased in the MPTP-treated groups (MpS, MpLMn and MpHMn) in comparison with the unexposed groups (SS or SLMn and SHMn groups) especially in the rostral level (Figure 1). The numbers of TH-ir neurons were counted in the total rostro-caudal levels of SN (Table 2).

GFAP-ir astrocytes in the globus pallidus (GP) had small cell bodies with relatively thick processes and were more densely distributed in comparison with those of the CPU, including the SS group (Figures 3 and 4). The relative densities of GFAP-ir astrocytes in the CPU and GP were significantly increased in the MPTP-treated groups in comparison with the MPTP untreated groups. The relative densities of GFAP-ir astrocytes in the GP were slightly increased in the SHMn group in comparison with the SS group, although the increase was statistically insignificant. However, Mn treatment did not affect MPTP neurotoxicity (Table 3).

**Concentrations of DA and its metabolites**

Concentrations of DA in the corpus striatum were decreased significantly in the MPTP-exposed groups only, but Mn had no effect (Table 4). DA was reduced

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**Figure 3** Photomicrographs showing glial fibrillary acidic protein (GFAP)-immunoreactive (ir) astrocytes in the caudate-putamen (CPU) of SS, SLMn, SHMn, MpS, MpLMn and MpHMn groups. The GFAP-ir astrocytes are significantly increased by MPTP treatment, but GFAP immunoreactivity is not effected by Mn treatment. Scale bar in MpHMn is 100 μm.

**Figure 4** Photomicrographs showing glial fibrillary acidic protein (GFAP)-immunoreactive (ir) astrocytes in the globus pallidus (GP) of SS, SLMn, SHMn, MpS, MpLMn and MpHMn groups. The GFAP-ir astrocytes are significantly increased by MPTP treatment, and Mn treatment (SHMn group) also induces a slight increase of GFAP-ir astrocytes in comparison to the SS group. Scale bar in MpHMn is 100 μm.
### Table 3  Densities of GFAP-ir structures in the (CPU), and (GP) (% area)

<table>
<thead>
<tr>
<th>Group</th>
<th>GFAP-ir structures in CPU</th>
<th>GFAP-ir structures in GP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>0.11 ± 0.04 (n = 9)*</td>
<td>0.40 ± 0.11 (n = 7)*</td>
</tr>
<tr>
<td>SLMn</td>
<td>0.12 ± 0.02 (n = 9)*</td>
<td>0.50 ± 0.20 (n = 9)*</td>
</tr>
<tr>
<td>SHMn</td>
<td>0.12 ± 0.02 (n = 9)*</td>
<td>0.54 ± 0.14 (n = 9)*</td>
</tr>
<tr>
<td>MpS</td>
<td>0.33 ± 0.70 (n = 8)</td>
<td>0.43 ± 0.44 (n = 8)</td>
</tr>
<tr>
<td>MpLMn</td>
<td>3.22 ± 0.51 (n = 8)</td>
<td>3.42 ± 0.60 (n = 10)</td>
</tr>
<tr>
<td>MpHMn</td>
<td>3.41 ± 1.04 (n = 8)</td>
<td>3.36 ± 0.46 (n = 9)</td>
</tr>
</tbody>
</table>

Densities of glial fibrillary acidic protein (GFAP)-ir structures in the caudate-putamen (CPU), and GFAP-ir structures in the globus pallidus (GP) were measured. The optical densities of GFAP-ir structures, which were corrected for non-specific background density, are presented as a percentage area of the measured total area (% area) in the CPU and GP. Means of optical densities of GFAP-ir structures in the CPU, and GFAP-ir structures in the GP are statistically different among treatment groups (*P < 0.001).

*Significant difference versus MpS, MpLMn and MpHMn (*P < 0.05), based on Scheffe’s post-hoc analyses.

**Significant difference versus MpS and MpHMn (**P < 0.05), based on Scheffe’s post-hoc analyses.

***Significant difference versus MpHMn (**P < 0.05), based on Scheffe’s post-hoc analyses.

### Discussion

There have been many studies examining either the biochemical or morphological aspects of MPTP or Mn-exposed animals, but very few comprehensive studies examining the combined effects of MPTP and Mn.21,28

**Effects of MPTP exposure**

The numbers of TH-ir neurons in the SNpc and the density of TH-ir axon terminals in the CPU in MPTP-treated groups showed a decrease in the amount of TH protein. The TH enzyme is the rate-limiting enzyme of catecholamine synthesis. Down-regulation of TH means reduced dopaminergic activity and susceptibility to insults leading to death. GFAP-ir astrocytes were also significantly increased in the CPU in MPTP-treated groups. However, the increased densities of GFAP-immunoreactivities in the CPU are not a primary pathology, but a secondary reaction of gliosis due to damage to dopaminergic elements by MPTP treatment. Thus, γ-aminobutyric acid (GABA) neurons in the CPU should be evaluated further. Furthermore, DA and its metabolites (DOPAC, HVA) were severely diminished in the corpus striatum (DA% reduction: 85–90%), with the MPTP dose of 150 mg/kg, similar to the 84 ± 3% reduction at the dose of 100 mg/kg.29 Increased DA turnover ratios were seen in MPTP-exposed groups. All of these findings suggest dopaminergic neuronal degeneration. Thus, decreased dopaminergic neurons by MPTP led to decreased DA and its metabolites.30,31

**Effects of Mn exposure**

Blood Mn levels were elevated in Mn-exposed groups compared to unexposed groups. Striatal Mn content was significantly increased by high Mn treatment alone. The reasons for this may be, first, a relatively low Mn dosing regimen should be considered. An intraperitoneal dose of 2 mg/kg per day for 21 days was used in the present study to evaluate low dose effect. The dose of 2 mg/kg per day is rather lower than those of other studies, in which a dose of 4 or 3.5 mg/kg,32,33 for 30 days significantly increased striatal Mn content. Second, rapid brain Mn depression following cessation of exposure or redistribution of Mn should also be considered.34 The alteration in TH level, such as decreased TH immunoreactivities in both the SNpc and CPU in MPTP-treated subjects, was not influenced by Mn exposure. GFAP staining were not influenced by Mn treatment in the CPU. Further-

### Table 4  Concentrations of DA, DOPAC and HVA in the striatum (µg/g tissue)

<table>
<thead>
<tr>
<th>Group</th>
<th>DA</th>
<th>DOPAC</th>
<th>HVA</th>
<th>DA turnover ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS (n = 6)</td>
<td>33.45 ± 10.57*</td>
<td>3.64 ± 1.10*</td>
<td>3.69 ± 1.19**</td>
<td>0.23 ± 0.06***</td>
</tr>
<tr>
<td>SL Mn (n = 6)</td>
<td>28.05 ± 3.73*</td>
<td>2.93 ± 0.92</td>
<td>3.05 ± 1.27</td>
<td>0.21 ± 0.07***</td>
</tr>
<tr>
<td>SH Mn (n = 7)</td>
<td>29.93 ± 11.55*</td>
<td>4.51 ± 1.07*</td>
<td>4.18 ± 1.89**</td>
<td>0.30 ± 0.09***</td>
</tr>
<tr>
<td>MpS (n = 8)</td>
<td>3.92 ± 1.90</td>
<td>1.44 ± 0.58</td>
<td>1.53 ± 0.41</td>
<td>0.84 ± 0.28</td>
</tr>
<tr>
<td>MpLMn (n = 8)</td>
<td>4.95 ± 2.31</td>
<td>1.65 ± 0.70</td>
<td>2.02 ± 0.75</td>
<td>0.96 ± 0.62</td>
</tr>
<tr>
<td>MpHMn (n = 7)</td>
<td>2.28 ± 2.28</td>
<td>1.40 ± 0.52</td>
<td>1.20 ± 0.44</td>
<td>1.61 ± 0.82</td>
</tr>
</tbody>
</table>

Concentrations of dopamine (DA), 3,4 dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) were measured in the striatum. Means of DA, DOPAC, and HVA in the striatum are statistically different among treatment groups (*P < 0.001).**Significant difference versus MpS, MpLMn and MpHMn (**P < 0.05), based on Scheffe’s post-hoc analyses.

***Significant difference versus MpS, MpLMn and MpHMn (**P < 0.05), based on Scheffe’s post-hoc analyses.

**Significant difference versus MpS and MpHMn (**P < 0.05), based on Scheffe’s post-hoc analyses.

**Significant difference versus MpHMn (**P < 0.05), based on Scheffe’s post-hoc analyses.

Mean ± SD.

n, number of mice.
more, Mn treatment did not affect DA and its metabolites in the striatum. The present findings are compatible with other results in which no measurable changes were shown in striatal dopamine content. The relative densities of GFAP-ir astrocytes in the GP were slightly increased in the SHMn group in comparison with the SS group, although this increase was statistically insignificant. This finding is, however, different from neuropathologic findings on the few Mn-intoxicated human cases, indicating that the GP were preferentially damaged. The caudate nucleus, putamen, and subthalamic nucleus were involved, but to a lesser degree, with the SNpc spared. This difference might be due to masking of Mn neurotoxicity on GP by the severe toxicity of MPTP itself.

Combined effects of Mn and MPTP
The present study showed Mn had no effect on the dopaminergic systems that MPTP targeted. The present findings are supported by other studies in which manganism was caused by damage to the output pathways downstream of the dopaminergic projection, such as the GP and SNpr, with the nigrostriatal pathway remaining intact, whereas damage to the striatal dopaminergic mechanisms is seen in PD. The present study suggested that Mn did not alter the severe neurotoxicity of MPTP, and we cautiously suggest that Mn exposure might have no effect on PD in humans.

The present study had several limitations. First, no neurobehavioral test was performed, which could detect the combined outcome of Mn and MPTP more clearly. Second, the study had poor statistical power due to the small number of mice. The small treatment group size accompanied by limited statistical power might have precluded the detection of a Mn effect. Third, we examined the effect of Mn following severe MPTP toxicity and, therefore, any potentiation of Mn on the MPTP-induced neurotoxic response could be masked by the exacerbated toxicity of MPTP itself. Hence, further study on MPTP dose that produces moderate toxicity (not more than 50% loss of dopamine) is needed.

Acknowledgements
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