Short-term manganese inhalation decreases brain dopamine transporter levels without disrupting motor skills in rats

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ABSTRACT — Manganese (Mn) is used in industrial metal alloys and can be released into the atmosphere during methycyclopentadienyl manganese tricarbonyl combustion. Increased Mn deposition in the brain after long-term exposure to the metal by inhalation is associated with altered dopamine metabolism and neurobehavioral problems, including impaired motor skills. However, neurotoxic effects of short-term exposure to inhaled Mn are not completely characterized. The purpose of this study is to define the neurobehavioral and neurochemical effects of short-term inhalation exposure to Mn at a high concentration using rats. Male Sprague-Dawley rats were exposed to MnCl2 aerosol in a nose-only inhalation chamber for 3 weeks (1.2 μm, 39 mg/m3). Motor coordination was tested on the day after the last exposure using a rotarod device at a fixed speed of 10 rpm for 2 min. Also, dopamine transporter and dopamine receptor protein expression levels in the striatum region of the brain were determined by Western blot analysis. At a rotarod speed of 10 rpm, there were no significant differences in the time on the bar before the first fall or the number of falls during the two-minute test observed in the exposed rats, as compared with controls. The Mn-exposed group had significantly higher Mn levels in the lung, blood, olfactory bulb, prefrontal cortex, striatum, and cerebellum compared with the control group. A Mn concentration gradient was observed from the olfactory bulb to the striatum, supporting the idea that Mn is transported via the olfactory pathway. Our results demonstrated that inhalation exposure to 39 mg/m3 Mn for 3 weeks induced mild lung injury and modulation of dopamine transporter expression in the brain, without altering motor activity.

Key words: Manganese, Dopamine transporter, Motor skill, Inhalation exposure, Rats, Lung injury

INTRODUCTION

Manganese (Mn) exposure is closely associated with significant neurotoxic effects, including motor and physiological disturbances in humans (Dobson et al., 2004; Mergler, 1999). Many studies using animals have also demonstrated a positive relationship between Mn deposition in the brain and subsequent neurochemical and behavioral changes. For example, Normandin et al. demonstrated that Mn was deposited in the brain of the rats following exposure to 3 mg Mn/m3 via inhalation (Normandin et al., 2004; Tapin et al., 2006) and exposure through drinking water (Nachtman et al., 1986) and both intra-striatal (Inoue et al., 1975) and intraperitoneal (Nam and Kim, 2008) injections of Mn all resulted in behavioral changes in rats. Intranasal instillation of Mn in mice and rats also alters behavioral organization and impairs neurochemical homeostasis (Kim et al., 2012; Ye and Kim, 2015a, 2015b). Several studies have attempted to define the role of routes of Mn deposition in the neurotoxic effects of the metal. The globus pallidus and the nigrostriatal pathway have been identified as important target sites of Mn neurotoxicity in humans (Pal et al., 1999; Zheng et al., 1998); Mn preferentially accumulates in the stria-
physiological and neurochemical changes that resulted from exposure.

MATERIALS AND METHODS

Chemicals
Mn (II) chloride (MnCl₂) was obtained from Sigma Aldrich (St. Luis, MO, USA). MnCl₂ is in a reddish, water-soluble powder form. All other chemicals were of the highest possible quality.

Animals
All animals were treated in accordance with Institutional Animal Care and Use Committee protocols established by the Korea Institute of Toxicology. A total of 24 8-week-old male Sprague-Dawley rats weighing 250-290 g (Charles River Orient Bio Inc., Seoul, Korea) were given rodent chow (PMI Nutrition, Richmond, VA, USA) and tap water ad libitum, except during Mn exposure. Animals were acclimated starting from approximately 2 weeks before the exposure started in a HEPA-filtered room maintained at 19-25°C and 40-60% relative humidity in an animal facility accredited by Association for Assessment and Accreditation of Laboratory Animal Care. Two rats were housed in each cage with a 12:12-hr day/night light cycle.

MnCl₂ exposure
Rats were exposed to 4-hr MnCl₂ inhalation periods, 5 days/week, for 3 weeks. The rats were randomly divided into 2 groups (N = 12 rats/group) and exposed to either HEPA-filtered air (control group) or 39 mg MnCl₂/m³ (exposed group). The chamber temperature (22-25°C) and relative humidity (25-60%) were held constant during the study. The MnCl₂ aerosol was generated using a mist generator (NB-2N, Sibata, Saitama, Japan) with a generation air-flow of 11 L per minute. The average aerosol mass concentration was determined using mass weight filter samples. The mass median aerodynamic diameter (MMAD) was measured using a cascade impactor (MiniMOUDI, MSP Corp., Shoreview, MN, USA).

Rotarod test
Rats were tested for motor coordination on a rotarod device (B.S Technolab). For two days before the test, the rats were acclimated to the rotarod at speeds 4 rpm and 7 rpm on the first day, 7 rpm and 10 rpm on the second day, for two trials/day at 2 min/trial. After the last exposure, rats were tested on the rotarod with a fixed speed of 10 rpm for 2 min. The time on the bar before the first fall and the number of falls were recorded.
Tissue collection
Following rotarod testing, the rats were euthanized by isoflurane overdose, followed by exsanguination and collection of blood, liver, brain, and lung tissues. Brains were removed and hemisected in the sagittal plane. The right hemisphere was collected for metal analysis. The left hemisphere was immediately frozen in liquid nitrogen and stored at -70°C for protein analysis. Each hemisphere was dissected into four regions: the olfactory bulb, prefrontal cortex, striatum, and cerebellum. All left lungs were preserved for histopathology. Right lungs were used for Western blot analysis, metal analysis, and non-heme iron analysis. Blood and liver were collected for metal analysis. Wet tissues and blood for metal analysis were weighed and then stored at -70°C until analysis. Serum was obtained from centrifugation of blood at 4°C for 15 min at 3,500 × g and used for non-heme iron analysis.

Metal level analysis using inductively coupled plasma mass spectrometry (ICP MS)
Metal levels in blood, brain regions (olfactory bulb, prefrontal cortex, striatum, and cerebellum), lung, and liver tissues were determined using a NexION™ 300D ICP MS (Perkin-Elmer, Waltham, MA, USA). The instrument was optimized according to the recommendations of the manufacturer, and its performance was evaluated daily prior to sample analysis by aspiration of a 1 μg/L multi-element solution containing Be, Ce, Fe, In, Li, Mg, Pb, and U in 1% HNO₃. Method parameters and instrument conditions are summarized in Table 1.

For sample preparation for the analysis, tissues were freeze-dried in a freeze dryer (Ilshin, Yangju-si, Korea) at -70°C and 5 mTorr for 24 hr and then digested in 5 mL concentrated HNO₃ and 5 mL ultrapure water (Direct-Q 3 UV, Millipore, Darmstadt, Germany), while blood samples were digested in 3 mL concentrated HNO₃ and 6 mL ultrapure water (Direct-Q 3 UV, Millipore), using a microwave digestion system (Multiwave 3000). A 2-ng/mL solution of rhodium in 1% HNO₃ was used as the internal standard. This solution was mixed with the calibration standards and samples online. Samples were analyzed using the external calibration method with 5 standard concentrations ranging from 0.1-50 ng/mL.

Non-heme iron analysis
The tissue samples were combined with protein precipitation solution (10% trichloroacetic acid in 3 M HCl) in 1.5-mL microcentrifuge tubes (Axxygen Scientific, Union City, CA, USA), incubated in a water bath at 65°C for 20 hr, and then cooled to room temperature. The supernatants were mixed with chromogen solution (10% thioglycolic acid, 1% bathophenanthroline disulfonic acid (BPS) in a half-saturated sodium acetate solution) at a supernatant:chromogen ratio of 1:10, v/v and vortex-mixed. After 30 min at room temperature, absorbance was measured at 535 nm using a spectrophotometer (BioTek Instruments, Winoosk, VT, USA). Sample values were calculated in relation to serially diluted iron standard solutions prepared at concentrations of 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39 μg/mL, and 0 μg/mL (acid only), in protein precipitation solution.

Serum samples were mixed with chromogen solution (10% thioglycolic acid in 1 M sodium acetate). The background absorbance was read at 535 nm using a spectrophotometer. Then, the sample mixture was mixed with 1% BPS for colorimetric reaction. The absorbance was read at 535 nm, and the background values were subtracted from the readings. Sample values were calculated in relation to serially diluted iron standard solutions prepared at concentrations of 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39 μg/mL, and 0 μg/mL (acid only), in 1 M sodium acetate (Kim et al., 2011).

Western blot analysis
Snap-frozen lung and brain tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, pH 7.5) containing protease inhibitors (Complete Mini, Roche, Mannheim, Germany) with 0.5 mM phenylmethylsulfonylfluoride (PMSF). Tissue homogenates were centrifuged at 16,000 x g for 5 min at 4°C. Protein concentrations in the homogenates were determined using the Bradford assay. The tissue extracts (40-50 μg protein) were electrophoresed on 10% gels and transferred to pol-

| Table 1. Method parameters and instrument conditions. |
|------------------------------------------------------|
| **ICP MS** | **Settings** |
| RF power | 1600 W |
| Nebulizer gas flow | 1.0 L/min |
| Auxiliary gas flow | 1.2 L/min |
| Nebulizer type | Meinhard TR3-A30 glass nebulizer |
| Scanning mode | Peak hopping |
| Sample uptake rate | 250 μL/min |
| Number of sweeps | 30 |
| Number of replicates | 5 |
| Blank subtraction | After internal standard |
| Calibration | Linear through 0 |
| Auto Lens | On |
| Dwell time | 50 msec |
| Sample flush | 35 sec |
| Read delay | 15 sec |
| Wash | 45 sec |

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yvinylidene difluoride (PVDF) membranes at 210 mA for 2 hr. The membranes were incubated with blocking solution (5% non-fat milk in Tris-buffered saline for 1 hr at room temperature, followed by incubation with primary antibodies in 2% non-fat milk overnight at 4°C. The primary antibodies employed were rabbit anti-dopamine receptor (D1R; 1:500, Santa Cruz, Santa Cruz, CA, USA) and goat anti-dopamine transporter (DAT; 1:100, Santa Cruz). The blots were also probed with mouse anti-ac tin (1:5000, MP Biomedicals, Santa Ana, CA, USA) as a loading control. The secondary antibodies employed were peroxidase-labeled goat anti-mouse (1:1000, Santa Cruz), goat anti-rabbit (1:1000, Santa Cruz), and donkey anti-goat (1:1000, Santa Cruz). Immunoreactivity was detected using ECL Amersham (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The resulting protein bands were visualized by ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) and their intensities were quantitated using Quantity One software (version 4.6.6; Bio-Rad) (Chang et al., 2014).

Histological examination

Brain, lung, and nasal cavity specimens were fixed in 10% neutral buffered formalin for 48 hr. After routine processing, the tissues were embedded in paraffin. Tissue sections, 2-3 μm in thickness, were stained with H&E for histological examination. The stained sections were evaluated by light microscopy (Olympus BX51, Olympus Co., Tokyo, Japan).

Fluorojade staining procedure

Brain sections were mounted on silane-coated slides and then air dried on a slide warmer at 50°C for at least 30 min. The slides were then immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min. This was followed by 2 min in 70% alcohol and 2 min in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 10 min, with gentle shaking on a rotating platform. The slides were rinsed for 2 min in distilled water and were then transferred to 0.01% fluorojade staining solution and gently agitated for 30 min. After staining, the sections were rinsed with three × 1 min changes of distilled water. Excess water was drained out, and the slides were dried on a slide warmer. The dry slides were cleared by immersion in xylene for at least 1 min and then cover-slipped with DPX (Sigma Chem. Co., St. Louis, MO, USA) mounting media. Sections were examined with an epifluorescence microscope (Olympus BX53, Olympus Co.) with blue (450-490 nm) excitation light.

Statistical analysis

Values reported were expressed as mean ± S.E.M. Differences between the control and Mn-exposed groups were analyzed using an unpaired, two-tailed t-test. A significant difference was considered at p < 0.05.

RESULTS

Test atmospheres

The overall average mass concentration and mass median aerodynamic diameter (MMAD) of the MnCl₂ aerosol were 39.2 ± 2.8 mg/m³ and 1.2 μm, respectively. The mean daily chamber temperatures ranged from 22-25°C, and the relative humidity ranged from 26-55%.

Fig. 1. The effect of MnCl₂ on body weight. Body weights of the rats exposed to 39 mg manganese chloride/m³ or filtered air for 3 weeks during exposure and right before necropsy were recorded. Data are presented as mean ± S.E.M. (N = 12 per group) and were analyzed by an unpaired two-tailed t-test, with the level of significance set at p < 0.05.
Food and water metal content

The average Mn, iron, copper, and zinc concentrations in rodent pellet chow were 78.9 μg/g, 249.7 μg/g, 9.5 μg/g, and 38.4 μg/g and 0.0006 mg/L, 0.002 mg/L, 0.4 mg/L, and 0.3 mg/L in water, respectively.

Physiological and hematological characteristics

Animal body weight was recorded. There was no significant decrease in body weight during or after 3-week exposure to MnCl₂ aerosol, as compared to the control group (Fig. 1). Hematocrit, brain weights, and lung weights were not significantly different in the rats in the exposure group compared to those in the control group (Table 2). While the liver weight decreased, the relative liver to body weight did not differ significantly from that in the control group. These results indicate that there are no physiological alterations as a result of a short-term Mn exposure.

Motor skill assessment

Motor skill was assessed by the rotarod test, where the time on the bar before falling and the number of falls during the two-minute test were examined. At a speed of 10 rpm, there were no significant differences in the group exposed to MnCl₂ for 3 weeks, as compared to the controls (86 vs 83 sec; p = 0.86 and 0.8 vs 0.8; p = 0.87) (Fig. 2). These results demonstrate that short-term exposure to Mn aerosol does not cause motor impairment in the rats.

Blood and tissue metal levels

To examine if Mn inhalation alters the homeostasis of Mn and other essential metals, Mn, iron, zinc, and copper levels were determined in the olfactory bulb, prefrontal cortex, striatum, and cerebellum. Significant increases in Mn levels in the olfactory bulb, prefrontal cortex, striatum, and cerebellum were observed in Mn-exposed animals (Fig. 3). Specifically, olfactory bulb, prefrontal cortex and striatum demonstrated a decreasing pattern of Mn concentration, indicating anterograde axonal transport of Mn (Henriksson et al., 1999) through the olfactory bulb to distant regions of the brain. In addition, no other essential metals in the brain were altered as a result of Mn exposure.

In addition, since high levels of Mn can affect the homeostasis of iron, we determined iron levels in these tissues. There was no significant change in iron levels observed in the brain, lung, liver, and blood of the Mn-exposed group when measured by ICP MS.

Mn and iron levels in other tissues are presented in Table 3. The blood Mn levels were increased by two-fold in the Mn-exposed group, as compared with the control group, and the lung Mn levels were also increased by

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**Table 2. Physiological and hematological characteristics.**

| Characteristics | Control | Mn-exposed |
|-----------------|---------|------------|
| Terminal body weight (TBW) (g) | 334.8 ± 7.6 | 316.1 ± 8.8 |
| Hematocrit (%) | 49.5 ± 0.7 | 48.8 ± 0.6 |
| Brain weight (g) | 2.1 ± 0.03 | 2.0 ± 0.02 |
| Brain weight/TBW | 0.6 ± 0.01 | 0.6 ± 0.01 |
| Lung weight (g) | 1.2 ± 0.04 | 1.2 ± 0.03 |
| Lung weight/TBW | 0.4 ± 0.01 | 0.4 ± 0.01 |
| Liver weight (g) | 12.2 ± 0.4 | 11.1 ± 0.4* |
| Liver weight/TBW | 3.6 ± 0.04 | 3.5 ± 0.05 |

Data are expressed as mean ± S.E.M. Differences between control and Mn exposed groups were analyzed using an unpaired two-tailed t-test. *p < 0.05.
three folds. However, Mn levels in the liver were not significantly affected by Mn inhalation. These results suggest that Mn inhalation increases Mn levels in the brain and lung, the two primary sites of Mn deposition after inhalation exposure, but not in the liver (the site of Mn storage).

### Non-heme iron levels

While ICP-MS measurements provide the information about “total” iron levels in both non-heme and heme iron forms, non-heme iron serves as a surrogate for body iron status. Therefore, we further determined non-heme iron concentrations in the liver and serum to examine any possible changes in body iron stores. There was no significant difference in non-heme iron levels either in serum or in the liver between the Mn-exposed and control groups (2.1 vs 2.3 ppm in serum; p = 0.36 and 50.5 vs 49.8 ppm in liver; p = 0.86) (Fig. 4). These results combined with ICP MS determination indicate that the short-term exposure to Mn by inhalation does not cause any apparent disruption of body iron homeostasis.

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**Table 3.** Mn and iron levels in lung, liver, and blood.

|                | Mn (μg/g wet tissue) | Iron (μg/g wet tissue) |
|----------------|----------------------|------------------------|
|                | Control | Mn-exposed | Control | Mn-exposed |
| Lung           | 0.20 ± 0.01 | 0.61 ± 0.02*** | 76.12 ± 1.76 | 80.88 ± 2.33 |
| Liver          | 2.76 ± 0.07 | 2.95 ± 0.09   | 101.43 ± 2.22 | 104.97 ± 4.35 |
| Blood          | 10.20 ± 0.42 | 19.90 ± 0.42*** | 470.02 ± 12.48 | 497.21 ± 11.31 |

Data are expressed as mean ± S.E.M. Differences between control and Mn-exposed groups were analyzed using an unpaired two-tailed t-test. ***p < 0.001.
Striatal dopamine transporter and dopamine receptor levels

Since Mn exposure is known to be related to disruption of dopamine pathway, we determined expressions of dopamine-related proteins by Western blot analysis as shown in Fig. 5. Mn exposure reduced dopamine transporter (DAT) levels, while there were no significant differences in the levels of striatal dopamine receptor (D1R).

Brain and lung pathology following MnCl₂ exposure

Since Mn inhalation can influence the respiratory system as well as the brain, we conducted histological tests using lungs and brains. Lung analysis by hematoxylin and eosin (H&E) staining showed minimal inflammatory cell infiltration at respiratory bronchiles in the 3-week Mn-exposed group (Fig. 6). Brain histopathology was examined using H&E and fluorojade staining (Fig. 7). No brain histopathological damages or neuronal degeneration was observed in the Mn-exposed group. These results demonstrate minor lung injury with no other clinical signs of injury in other tissues.

DISCUSSION

The micron-sized Mn aerosol employed in this study mimicked real-life exposure to Mn in humans. In particular matter, 80% of Mn is associated with particles with < 5 μm MMAD (Environmental Health Criteria 17). In this study, rats were exposed to a total dose of 86.6 mg MnCl₂/kg, based on the calculation described by Alexander et al. (2008). This can be converted to an equivalent human dose of 15 mg/m³ over the same duration (Reagan-Shaw et al., 2008). Mn exposure in humans can originate from MMT emitted to the atmosphere during the combustion of gasoline, while high concentrations of Mn can also be detected in some working environments, where Mn concentrations of 0.093 mg/m³ (Lucchini et al., 1997), 0.21 mg/m³ (Myers et al., 2003), 0.948 mg/m³ (Roels et al., 1992), 1.26 mg/m³ (Jiang et al., 2007), 4.8 mg/m³, and even 35.7 mg/m³ (Nakata et al., 2006) have been recorded.

Therefore the dose of 15 mg/m³ used in this study represents the higher end of the dose to which humans are exposed in the working environments. Because the rats were exposed to the dose in the span of three weeks, this study captures the scheme of short-term exposure to a high dose of Mn.

There was a significant difference in Mn levels observed in all of the brain regions examined in the Mn-exposed group, as compared with the control group (Fig. 3A). Mn deposition was observed in the olfactory bulb, which has been demonstrated to provide a direct pathway whereby Mn can enter the brain via the nose (Brenneman et al., 2000). As stated in the Results section, deposition of Mn in the olfactory bulb, prefrontal cortex, and striatum in a decreasing pattern demonstrates anterograde axonal transport of Mn (Henriksson et al., 1999) from the olfactory bulb to distant regions of the brain. This result is consistent with those reported by Tapin et al. (2006), where significant nasal absorption into the subfrontal cortex occurred via the olfactory bulb (Tjälv et al., 1999). Evidence for Mn uptake via the olfactory pathway was also provided by Tjälv et al. (1996), who
demonstrated that direct movement of $^{54}\text{Mn}$ to the olfactory bulb and telencephalon via transport through secondary olfactory neurons occurs after intranasal instillation of $^{54}\text{MnCl}_2$ in rats; this route circumvents the blood-brain barrier.

When administered by intratracheal instillation and intraperitoneal injection, Roels et al. (1997) detected the highest Mn deposition in the striatum, as compared with the cortex and cerebellum. This probably reflects Mn transport through the blood-brain barrier. Our results are consistent with those of Vitarella et al. (2000), Dorman et al. (2001), and Tapin et al. (2006), where the highest Mn deposition was detected in the olfactory bulb after inhalation exposure. Although the cerebellum is not considered a primary site for Mn neurotoxicity (Pal et al., 1999), there was a significant increase in Mn level in this region in our study. This is the contribution of systemic delivery of blood Mn absorbed by inhalation through lungs. A further study is warranted to investigate the mechanism by which inhaled Mn distributes to the cerebellum.

Metal uptake into the brain can influence the uptake and levels of other metals (Smith et al., 1997). The present study, however, did not detect any changes in the levels of iron, zinc, or copper in the brain (Fig. 3B-D). Iron, zinc, and copper are essential for normal brain function, playing numerous biological roles in the brain. Zinc

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**Fig. 5.** Protein expression levels in the brain and lung of the rats exposed to MnCl$_2$ aerosol. Lung transferrin receptor-1 (A), striatal dopamine transporter (B), and striatal dopamine receptor (C) expression levels were determined by Western blot analysis. Relative intensities of protein bands were determined using Quantity One software (version 4.6.6; Bio-Rad) and normalized to actin. Data were presented as mean ± S.E.M. (N = 4 per group) and were analyzed using an unpaired two-tailed t-test, with the level of significance set at $p < 0.05$. 
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modulates brain oxidative metabolism, DAT function, and glutamate receptors, while copper is a cofactor for dopamine β-hydroxylase, which catalyzes the conversion of dopamine to norepinephrine (Garcia et al., 2006; Erikson et al., 2004). Chen et al. (2006) also found no change in the levels of zinc and copper in the brain after a single intraperitoneal injection of MnCl₂. In the present study, rat brain metal homeostasis was not altered by inhalation exposure to 39 mg MnCl₂/m³ for 3 weeks, suggesting potential adaptive mechanisms in response to

Fig. 6. Mild lung injury observed in MnCl₂-exposed rats. Histopathologic changes in hematoxylin and eosin-stained lung sections from the (A) control group and (B) Mn-exposed group were examined. The black arrow indicates inflammatory cell infiltration.

Fig. 7. Hematoxylin and eosin, fluorojade stained section of globus pallidus of the rats exposed to MnCl₂ aerosol. Hematoxylin and eosin (A: control group; B: Mn-exposed group) and fluorojade staining (C: control group; D: Mn-exposed group) were performed to observe Mn-related histopathologic changes.
short-term Mn exposure to maintain the homeostasis of essential metals in the brain.

It has been shown that iron and Mn share the same transporters (Garrick et al., 2003). Iron overload has also been shown to reduce Mn accumulation in the brain and in other organs (Thompson et al., 2006). Conversely, Mn exposure by drinking water alters iron pharmacokinetics (Molina et al., 2011). However, it is largely unknown whether Mn inhalation could modify body iron status. We therefore determined the levels of non-heme iron, an established marker for iron status, with a particular focus on liver and serum levels. There was no significant change in the liver and serum non-heme iron levels (Fig. 4) in the Mn-exposed group, as compared with controls. In addition, the hematocrit and total iron levels in lungs, liver, and blood also showed no significant change (Tables 2, 3). Similar results were observed in mice after intranasal instillation of Mn for 3 weeks (Ye et al., 2015a); iron status in the liver and brain were unaltered upon Mn instillation. However, Mn exposure by drinking water for 5 weeks significantly decreased iron levels in the liver (Alsulimani et al., 2015). These differences could result from different exposure route (inhalation vs. ingestion) rather than exposure period.

Our study identified no significant change in liver Mn levels after Mn exposure (Table 3). This lack of exposure-dependence in liver Mn levels has been observed in several studies of Mn inhalation in rats (Tapin et al., 2006; Yu et al., 2003; St-Pierre et al., 2001); this may be related to the short half-life of Mn in blood following short-term inhalation exposure (Smargiassi and Mutti, 1999).

The Mn concentrations in the lungs after exposure to 39 mg MnCl₂/m³ for 3 weeks were 0.61 μg/g (Table 3). In a previous study reported by Dorman et al. (2001), the lung Mn levels were 14.7 μg/g after exposure to 3 mg Mn tetroxide/m³ for 14 days. Even though we used a higher Mn concentration, our study found lower Mn levels in the lung. Mn solubility has been shown to affect its clearance from the lung. Since MnCl₂ has a higher solubility than Mn tetroxide, the clearance of MnCl₂/m³ for 14 days. Even though we used a high-

been shown to be more dependent on DAT than on D1R (Erixon-Lindroth et al., 2005; Anderson et al., 2007). DAT expression was decreased in the 3-week exposure group, with D1R levels showing a trend towards down-regulation (Fig. 5B-C). This result is consistent with the findings of Kim et al., 2012) who showed a decrease in DAT expression after intranasal administration of MnCl₂. Mn has been suggested to induce internalization of DAT and suppress dopamine uptake in vitro (Roth et al., 2013), likely resulting in elevated levels of extracellular dopamine. Indeed, Kim et al. (2012) demonstrated a slight up-regulation of extracellular dopamine in the striatum after intranasal instillation of MnCl₂. We have, however, noted a difference between two studies: while MnCl₂-instilled rats showed decreased rotorod task performance along with a significant down-regulation of striatal D1R (Kim et al., 2012), our results showed no significant differences in motor function and no change in D1R expression in the striatum of the Mn-exposed group (Fig. 5C), suggesting that the effects of Mn on motor performance may be correlated more closely with striatal D1R expression than with DAT (Willuhn and Steiner, 2008). However, we cannot exclude the possibility that both central (e.g., cerebellum, cerebral cortex) and peripheral (muscle) mechanisms influence motor activity, and that these could be modified differentially by different forms of Mn, the exposure level, and the duration of airborne Mn exposure. It is also possible that other dopamine receptors (D2-5R) are affected by inhaled Mn. Future studies are warranted to characterize these relationships.

Mn deposition in the lung altered lung histopathology with some induction of inflammatory cell infiltration (Fig. 6). Brain morphology analysis revealed no histopathological changes, and no neuronal degeneration. Brain Mn deposition in this study did not result in structural damage to rat brain (Fig. 7). The same observation was made after Mn inhalation exposure for 13 weeks and after Mn exposure in drinking water for 10 weeks (Normandin et al., 2004; Calabresi et al., 2001). In addition to the effect of manganese exposure on structural damage in tissues, we also assessed if our Mn exposure condition could modify neurobehavioral function. In this study, the rotorod test used to quantify motor skill following Mn exposure. This test has been used to detect motor impairment after brain injury and was shown to be more effective than other motor function assessment methods, such as beam-balance and beam-walking tasks (Hamm et al., 1994). After exposure to Mn for 3 weeks, the exposed group showed a similar performance as the control group (Fig. 2). Our results are different from other studies that reported Mn neurotoxicity. For example, welders exposed
to Mn have demonstrated decreased motor function (Bowler et al., 2006; Ellingsen et al., 2008), and a previous Mn exposure study in rats also showed altered locomotor activity (Normandi et al., 2004; Tapin et al., 2006; St-Pierre et al., 2001). It is possible that in our study, the exposure duration may not have been long enough to induce structural brain damage and neurobehavioral changes, but sufficient to elicit altered expression of dopamine transporter. Conversely, a temporal, adaptive change in dopamine transporter expression may be able to maintain sufficient concentrations of extracellular dopamine and to support proper dopaminergic neurotransmission, which therefore provides a protective mechanism against Mn-induced neurotoxicity due to an acute or sub-acute exposure by inhalation. A future study will address this question by the characterization of the kinetics of dopamine transporters and its associated receptors.

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**Conflict of interest**--- The authors declare that there is no conflict of interest.

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