



Nigrostriatal dopamine system dysfunction and subtle motor deficits in manganese-exposed non-human primates

Tomás R. Guilarte^{a,*}, Ming-Kai Chen^a, Jennifer L. McGlothan^a, Tatyana Verina^a, Dean F. Wong^b, Yun Zhou^b, Mohab Alexander^b, Charles A. Rohde^c, Tore Syversen^d, Emmanuel Decamp^e, Amy Jo Koser^e, Stephanie Fritz^e, Heather Gonczi^e, David W. Anderson^e, Jay S. Schneider^e

^a Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

^b Department of Radiology, Johns Hopkins Hospital, Baltimore, MD, USA

^c Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

^d Department of Neuroscience, Norwegian University of Science and Technology, Trondheim, Norway

^e Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA

Received 14 February 2006; revised 23 June 2006; accepted 30 June 2006

Abstract

We tested the hypothesis that movement abnormalities induced by chronic manganese (Mn) exposure are mediated by dysfunction of the nigrostriatal dopamine system in the non-human primate striatum. Motor function and general activity of animals was monitored in parallel with chronic exposure to Mn and Positron Emission Tomography (PET) studies of *in vivo* dopamine release, dopamine transporters and dopamine receptors in the striatum. Analysis of metal concentrations in whole blood and brain was obtained and post-mortem analysis of brain tissue was used to confirm the *in vivo* PET findings. Chronic Mn exposure resulted in subtle motor function deficits that were associated with a marked decrease of *in vivo* dopamine release in the absence of a change in markers of dopamine (DA) terminal integrity or dopamine receptors in the striatum. These alterations in nigrostriatal DA system function were observed at blood Mn concentrations within the upper range of environmental, medical and occupational exposures in humans. These findings show that Mn-exposed non-human primates that exhibit subtle motor function deficits have an apparently intact but dysfunctional nigrostriatal DA system and provide a novel mechanism of Mn effects on the dopaminergic system.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Parkinson's disease; Motor function; Dopamine release; Manganese; Positron emission tomography

Introduction

Emerging evidence suggests that environmental factors play an important role in the etiology of Parkinson's disease (PD) (DiMonte, 2003; Gorell et al., 1999). Exposure to high levels of manganese (Mn) is known to produce a behavioral

syndrome with parkinsonian features (Aschner, 2000; Mena et al., 1967; Pal et al., 1999). However, there is a paucity of knowledge on the neurological consequences of chronic, low-level exposures. The classical description of "manganism" includes the early expression of behavioral, psychiatric and memory disturbances that are typically followed by parkinsonian symptoms including action tremor, ataxia, dystonia, bradykinesia, gait abnormalities and postural instability (Aschner, 2000; Mena et al., 1967; Pal et al., 1999). However, differences in the clinical presentation of PD and Mn-induced parkinsonism have been noted (Calne et al., 1994; Jankovic, 2005; Olanow, 2004). For example, in Mn-

* Corresponding author. Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe St., Room E6622, Baltimore, MD 21205, USA. Fax: +1 410 502 2470.

E-mail address: tguilart@jhsph.edu (T.R. Guilarte).

induced parkinsonism, there is action tremor rather than resting tremor as in PD, more dystonia and severe gait disturbance with difficulty in backward walking (Kim et al., 2002; Pal et al., 1999). Further, while L-dopa therapy is an effective means to treat the early clinical symptoms of PD, this therapy does not appear to be effective in Mn-induced parkinsonism (Lu et al., 1994). Therefore, there is controversy in the scientific literature on the neurobiology of Mn-induced parkinsonism.

Recently, there has been a renewed interest in understanding the neurological consequences of chronic exposure to low levels of Mn. This interest stems from the concern by regulatory agencies and the scientific community on the potential for adverse neurological health effects to the general population from chronic exposure to increases in ambient levels of Mn (Davis, 1999; Kaiser, 2003). An anthropogenic source that has the potential to markedly increase Mn exposure to the general population is from the automobile combustion of gasoline containing methylcyclopentadienyl manganese tricarbonyl (MMT) (Davis, 1999; Kaiser, 2003). Moreover, there are reports that workers in occupations in which chronic exposure to Mn occurs may have a higher prevalence of developing parkinsonism at an earlier age (Racette et al., 2001, 2005a; Sadek et al., 2003).

Human and non-human primate studies examining the consequences of toxic Mn exposures focus on understanding the role of the basal ganglia and in particular the dopaminergic system. Magnetic resonance imaging (MRI) in humans and non-human primates demonstrates that Mn accumulates in basal ganglia structures (Dietz et al., 2001; Eriksson et al., 1992; Josephs et al., 2005; Kim et al., 1999; Newland et al., 1989; Racette et al., 2005b). Functional imaging using [^{18}F]-fluorodopa Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT) with dopamine transporter (DAT) radioligands has assessed dopamine terminal integrity in the striatum of Mn-exposed subjects (Eriksson et al., 1992; Huang et al., 2003; Kim et al., 2002; Racette et al., 2005b; Shinotoh et al., 1995, 1997; Wolters et al., 1989). While these markers are decreased in the striatum of PD patients (Brooks et al., 2003; Pirker et al., 2002; Thobois et al., 2004), similar studies in Mn-exposed humans or non-human primates have produced equivocal results. For the most part, [^{18}F]-fluorodopa PET has been normal (Shinotoh et al., 1995, 1997; Wolters et al., 1989; but see Racette et al., 2005b), while decreased DAT levels have been described (Eriksson et al., 1992; Huang et al., 2003; Kim et al., 2002). Thus, the extent to which Mn exposure may produce dopaminergic neuronal degeneration or dysfunction is not resolved. It has been suggested that Mn-induced parkinsonism may not be associated with deficits in nigrostriatal dopaminergic function, but that it may be related to Mn effects on downstream neuronal pathways (Calne et al., 1994; Huang et al., 1998; Olanow, 2004).

In the present report, we provide evidence of a novel mechanism by which chronic, low-level Mn exposure results in nigrostriatal dopamine (DA) system dysfunction in non-human primates expressing subtle deficits in motor function.

Materials and methods

Mn administration and general procedures

Five young adult male research naive Cynomolgus macaques (5 to 6 years of age at the start of the study and housed at Thomas Jefferson University) were used. All animal studies were reviewed and approved by the Johns Hopkins and the Thomas Jefferson University Animal Care and Use Committee. One of the animals could not be used for imaging studies due to technical problems secondary to venous complications from the chronic Mn infusions. Three additional young adult animals of similar age that did not receive Mn exposure, behavioral evaluations or imaging studies served as a comparison group to the Mn-exposed animals for post-mortem analyses of the brain.

Behavioral ratings and motor function were monitored on a regular schedule and animals were trained to perform a variety of cognitive tasks. The effects of Mn exposure on cognition will be described separately. Animals were transferred to Johns Hopkins for baseline imaging studies once cognitive training was completed. Imaging studies comprised magnetic resonance imaging (T1-weighted MRI), magnetic resonance spectroscopy (MRS) and PET studies. The MRI and MRS results will be reported separately. Upon their return and once a stable level of behavioral performance was confirmed, Mn exposure was initiated. Animals received i.v. injections of manganese sulfate (10–15 mg MnSO_4/kg or 3.26–4.89 mg Mn/kg) into the saphenous vein under light isoflurane (1–3% isoflurane) anesthesia approximately once per week. A needle and catheter were inserted into the vein and flushed with sterile saline. A sterile and warm MnSO_4 solution (50 mg/ml in isotonic physiological saline) was administered at a rate of 0.5 ml/min over a 4 to 6 min period, based on body weight. Vital signs were monitored during the Mn administration. At the end of the Mn infusion, 1.0 ml of sterile saline was pushed through the catheter. Animals were returned to their home cage and observed for any adverse events. At the first imaging time point after initiation of Mn exposure (Mn-1) and the second imaging time point (Mn-2), animals received the same PET and MRI/MRS studies as in baseline. On average, animals received a mean \pm SEM of 34.2 ± 1.2 Mn injections given once per week over a period of 39.6 ± 1.0 weeks. Animals did not receive any Mn administration during the time that they were at Johns Hopkins for imaging studies or after the Mn-2 imaging set. Animals were euthanized by ketamine injection (20–30 mg/kg) followed by an overdose of pentobarbital (100 mg/kg) and the brain harvested for confirmation of PET studies, neuropathological assessment and metals analysis.

Behavioral ratings, general activity and fine motor skills

Animals were rated for a variety of behaviors using a modified rating scale based on a parkinsonian symptoms rating scale for non-human primates (Schneider and Kovelowski, 1990). Each item was rated as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe). A score of 33 represented the highest

possible disability score. The presence and severity of dystonia and dyskinesias were also rated. A score of 21 represented maximum severity.

Level of activity during 2–3 consecutive 24 h periods was recorded prior to Mn exposure (1–3 separate sessions) and post-Mn exposure (1 session every 2–4 weeks) using an activity monitor (Actitrack; IM Systems, Inc.) placed into a pocket on the back of a jacket worn by the animals during the evaluation period.

Fine motor skills were assessed using 2 boards made of clear plexiglass and containing 16 wells of small (14 mm) or large diameter (22 mm) and 17 mm deep. The “easy” board consisted of 12 large and 4 small wells while the “difficult” board consisted of 12 small and 4 large wells. Animals were required to retrieve a standard reward pellet from each well as quickly as possible. The primary measure obtained was the number of errors made per well (i.e., the number of attempts made to remove a pellet from a well and/or the number of times a pellet was dropped).

Assessment of D2-dopamine receptor (D2-DAR) binding potential (BP) and DA release using [¹¹C]-raclopride with amphetamine PET and dopamine transporter (DAT) levels using [¹¹C]-methylphenidate PET

General PET imaging protocols have been described previously (Chen et al., 2006). [¹¹C]-methylphenidate and [¹¹C]-raclopride with amphetamine (AMPH) challenge PET scans were performed on the same day for each animal. For *in vivo* DA release and D2-DAR BP, animals were studied with a bolus (*B*) plus continuous infusion (*I*) method (*B/I*=75) using the D2-DAR ligand [¹¹C]-raclopride (Carson et al., 1997; Endres et al., 1997; Watabe et al., 1998). All monkeys received a continuous infusion of [¹¹C]-raclopride over a 90-min scanning period with AMPH (2.0 mg/kg IV) delivered at 40 min from the onset of [¹¹C]-raclopride infusion. The change in [¹¹C]-raclopride binding following AMPH was measured and used to infer the magnitude of DA release. For each PET study, regions-of interest (ROIs) were drawn on multiple slices encompassing the left and right striata and the cerebellum. Time–activity curves (TACs) were generated for each region. A simplified reference tissue model (SRTM) with cerebellum as reference tissue was used to estimate BP (Lammertsma and Hume, 1996). BP is estimated by fitting SRTM model to the measured striatum TACs with cerebellum TACs as input function. To estimate AMPH-induced DA release, a hybrid modeling technique (Lammertsma and Hume, 1996) was used as below:

DA release = $\frac{[C_{\text{SRTM}}([60-90])/C_{\text{REF}}([60-90]) - 1] - [C([60-90])/C_{\text{REF}}([60-90]) - 1]}{[C_{\text{SRTM}}([60-90])/C_{\text{REF}}([60-90]) - 1]}$ * 100 / $\frac{[C_{\text{SRTM}}([60-90])/C_{\text{REF}}([60-90]) - 1]}{[C_{\text{SRTM}}([60-90])/C_{\text{REF}}([60-90]) - 1]}$. Where C_{SRTM} is extrapolated TACs for post-AMPH phase from the SRTM model with the parameters identified from pre-AMPH phase [¹¹C]-raclopride kinetics measured by PET. C ([60–90]), C_{REF} ([60–90]) and C_{SRTM} ([60–90]) are the average of TACs of striatum and cerebellum, and extrapolated TACs of striatum tracer radioactivity within 60 to 90 min post-tracer bolus injection.

[¹¹C]-methylphenidate BP was used as a measure of striatal DAT levels. SRTM with cerebellum as the reference region was used to estimate BP from the [¹¹C]-methylphenidate dynamic PET study (Watabe et al., 1998). BP was estimated by fitting SRTM model to the measured striatum TACs with cerebellum TACs as input function.

Metal analysis in blood and brain tissue using high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS)

Concentrated nitric acid (HNO₃) (Suprapur, Merck) was added to the dried whole blood and brain samples. Blood samples were placed at room temperature for 24 h and digested on a heat block for 1 h at 70°C, 1 h at 100°C and 1 h at 110°C. Brain samples were placed at room temperature for 24 h and digested either on a heat block (QBT4, Grant) for 3 h at 70°C or using a microwave oven (Multiwave 3000, Anton Paar) using ramp 200W for 10 min and then held for 10 min. Samples were then diluted with 0.6 M HNO₃ with 18.2 MΩ water. Blood and brain samples were analyzed for metal content by HR-ICP-MS using a Thermo (Finnigan) model Element 2 instrument (Bremen, Germany), as published (Erikson et al., 2004) except that radio frequency power was set at 1250W. ⁵⁵Mn, ⁵⁷Fe, ⁶³Cu and ⁶⁷Zn were measured at medium resolution. Elements were analyzed at medium resolution in order to maintain a low detection limit, high analytical accuracy and the absence of chemical interferences.

Quantitative receptor autoradiography

Brain sections (20 μm) were thaw-mounted onto poly-L-lysine-coated slides (Sigma) and stored at –20°C until used. DAT autoradiography using [¹²⁵I]-RTI-121 was performed as described (Strazielle et al., 1998) except that 10 μM GBR-12909 was used to assess non-specific binding. D2-DAR autoradiography using [³H]-raclopride was performed as described (Nader et al., 2002). Brain sections were apposed to Kodak Bio-Max MR film with [¹²⁵I]-Microscales (Amersham) for 18–24 h (for DAT) or with calibrated [³H]-standards for 8–9 weeks (for D2-DAR). Images were acquired using the Inquiry system (Loats Associate, Westminster, MD, USA) and quantified using NIH Image v1.62.

Tyrosine hydroxylase (TH)-immunohistochemistry

We used standard immunohistochemistry with rabbit anti-TH antibodies (Chemicon, 1:2000, 48 h at 4°C), the avidin–biotin–peroxydase reaction and visualized with 3,3′-diaminobenzidine. Images were acquired using the Inquiry system (Loats Associate, Westminster, MD) and optical densities measured using NIH Image v1.62.

HPLC analysis of DA and homovallinic acid (HVA) levels in brain tissue

Brain samples were assayed for DA and its metabolite HVA using a high-pressure liquid chromatography (HPLC) with electrochemical detection (ESA Inc., Chelmsford, MA)

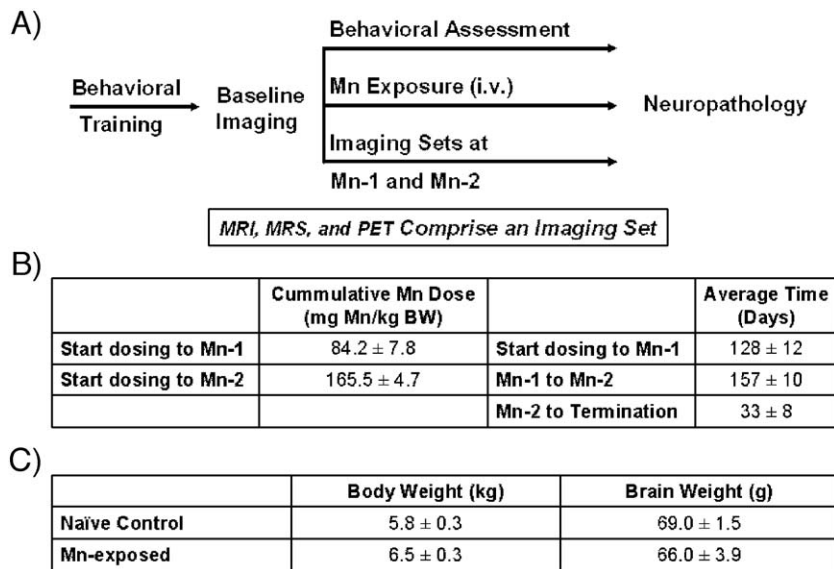


Fig. 1. Experimental design scheme. (A) Time sequence of experimental design from behavioral training to neuropathology. (B) Cumulative Mn doses and time periods from the first administration of Mn to the first post-Mn imaging set (Mn-1) to the second post-Mn imaging set (Mn-2) and to termination of study. (C) Physical characteristics of animals used in the study. Each value is the mean ± SEM of $n=3$ (control) and $n=4$ (Mn-exposed) animals.

according to a previously described protocol (Schneider and Yuwiler, 1989).

Statistical analysis

Activity and fine motor assessments prior to and following Mn exposure were compared by repeated measures analysis of variance (ANOVA). Pair-wise post hoc comparisons were performed to assess changes between baseline and post-Mn observation periods. Differences between baseline and post-Mn behavioral rating data were compared using a non-parametric Wilcoxon signed rank test. Statistical analysis of PET studies was performed using regression with clustering on animal to account for repeated measures on the same animal over time. Analysis of variance or Student's t test was used where appropriate. Statistical significance was set at $p < 0.05$. A rejection of data test for outliers was used where indicated. Outliers were removed prior to statistical analysis.

Results

Cumulative Mn dose, time of exposure and general characteristics of animals at termination of studies

The experimental design for behavioral training, Mn exposure and imaging studies is depicted in Fig. 1A. The cumulative Mn dose from initiation of Mn administration to the first (Mn-1) and second (Mn-2) post-Mn imaging sets was (mean ± SEM) 84.2 ± 7.8 and 156.7 ± 9.5 mg Mn/kg body weight, respectively. The average time from the initiation of Mn administration to Mn-1 and from Mn-1 to Mn-2 was 128 ± 12.4 and 157 ± 9.7 days, respectively. The time from the last imaging set to euthanasia was 33 ± 8.2 days (Fig. 1B). No significant differences in body weight (in kilograms; control— 5.8 ± 0.3 vs. Mn-exposed— 6.5 ± 0.3 ; $p > 0.05$) or brain weight (in grams; control— 69.0 ± 1.5 vs. Mn—exposed— 66.0 ± 3.9 ; $p > 0.05$) were observed between control naive and Mn-exposed animals (Fig. 1C).

Table 1
Effect of chronic Mn exposure on various measures of motor function

	Baseline	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24	Final
<i>Behavior rating scores</i>								
Rating	1.4 ± 0.4	2.5 ± 0.5	2.3 ± 0.7	2.0 ± 0.9	1.9 ± 0.5	2.8 ± 0.6*	2.8 ± 0.5*	2.9 ± 0.6*
<i>Normalized activity levels</i>								
Activity	100.0 ± 4.4	69.6 ± 7.6	45.6 ± 22.5	109.7 ± 37.6	102.9 ± 11.4	74.5 ± 14.6	65.5 ± 20.4	41.2 ± 3.9*
<i>Fine motor skills task</i>								
“Easy” board	1.0 ± 0.4	1.1 ± 0.7	1.1 ± 0.7	1.7 ± 0.7	0.9 ± 0.9	0.8 ± 0.8	0.6 ± 0.3	0.8 ± 0.5
“Difficult” board	2.5 ± 0.5	6.0 ± 1.9	6.8 ± 4.3	1.8 ± 0.8	2.7 ± 0.4	4.6 ± 2.0	2.3 ± 0.5	6.3 ± 0.2*

Each value is the mean ± SEM of 3–5 animals. * $p < 0.05$ from baseline. Final assessments were performed at 33.8 ± 2.4 weeks for the behavior rating scores and at 28 weeks for the activity levels and fine motor skills task.

Effects of chronic Mn exposure on behavioral ratings, general activity and fine motor skills

Five animals received behavioral ratings, four had fine motor skills assessed and three had general activity data recorded at baseline and throughout the Mn exposure period. Behavioral rating scores increased slightly during the Mn exposure period and became significantly different from baseline from week 20 through the end of the study. However, animals continued to appear to be grossly normal and changes in rating scores, although statistically significant by the end of

the study (Table 1), they were very subtle. None of these animals exhibited any dyskinesias or dystonias at any time during the study. Over the course of Mn exposure, general activity levels varied from one observation period to another but tended to decrease over time and became significantly different from baseline at week 28 (Table 1). On the test of fine motor skills, animals made 0.98 ± 0.4 (mean \pm SEM) errors/well in the “easy” board and 2.45 ± 0.50 errors/well on the “difficult” board at baseline. Over the course of the Mn exposure period, performance on the “easy” board was not affected while animals made significantly more errors on the

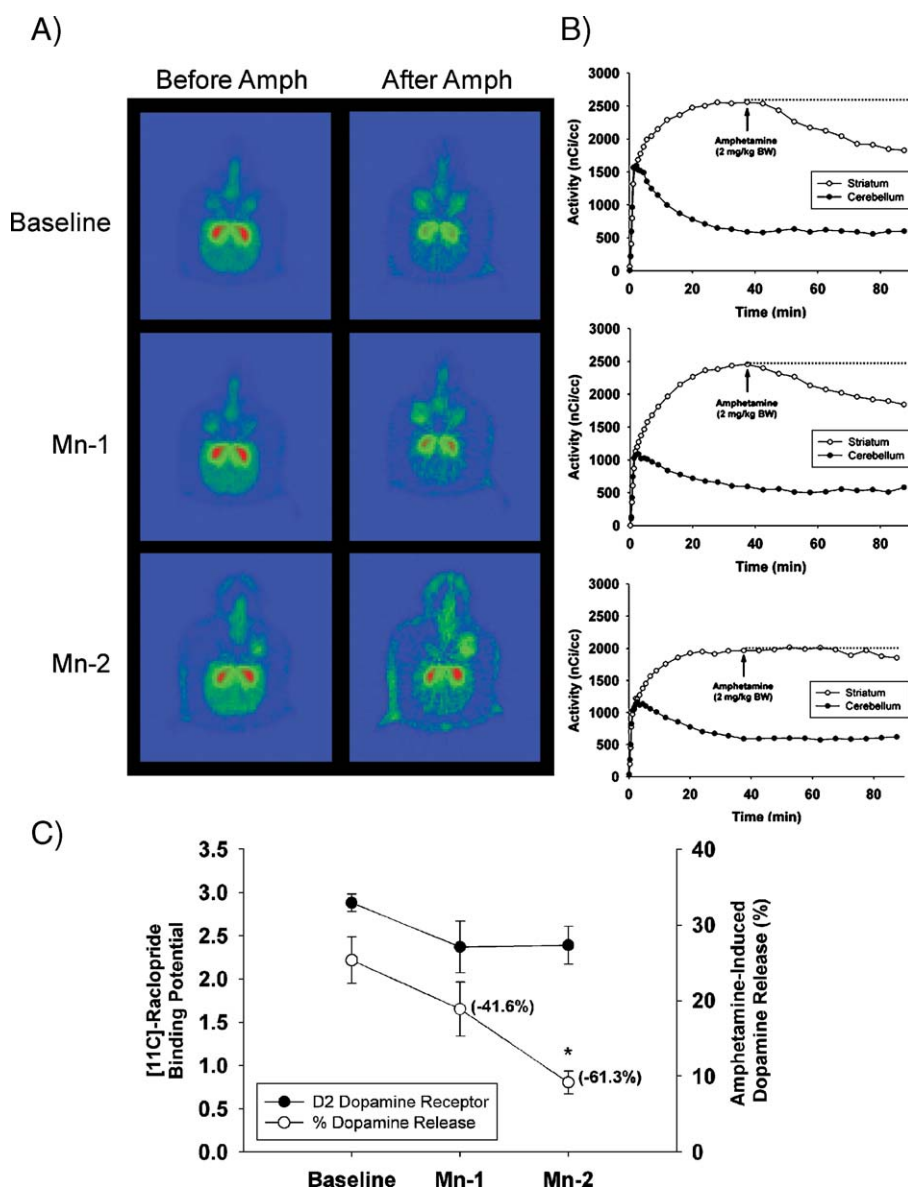


Fig. 2. Effect of chronic Mn exposure on $[^{11}\text{C}]$ -raclopride BP and AMPH-induced dopamine release. (A) Representative pseudocolor trans-axial images of $[^{11}\text{C}]$ -raclopride binding to D2-DAR in the striatum of one animal at baseline and at the Mn-1 and Mn-2 time points. Red areas represent high levels of binding and green areas represent low levels of $[^{11}\text{C}]$ -raclopride binding to D2-DAR. Note the progressive lack of a change in $[^{11}\text{C}]$ -raclopride levels in the striatum after AMPH administration from baseline to Mn-2. (B) $[^{11}\text{C}]$ -raclopride time-activity curves in the striatum and cerebellum before and after AMPH in the same animal as in panel A. Each graph corresponds to the adjacent images in panel A. At baseline (top graph in B), there is a dramatic decrease in $[^{11}\text{C}]$ -raclopride levels in the striatum after AMPH administration. Increasing Mn exposure reduces the effectiveness of AMPH-induced DA released to displace $[^{11}\text{C}]$ -raclopride from the striatum (see middle and lower graph in B). (C) Quantitative data on $[^{11}\text{C}]$ -raclopride BP and AMPH-induced *in vivo* DA release for all animals. For dopamine release, the numbers in parenthesis are the mean percent change from baseline. Each value is the mean \pm SEM of 4 Mn-exposed animals. * $p < 0.05$.

“difficult” version of the task at week 28 (6.25 ± 0.20 errors; $p < 0.05$) compared to baseline (Table 1).

Assessment of in vivo DAT, D2-DAR and DA release by PET

Fig. 2 depicts [^{11}C]-raclopride PET images and corresponding TACs in the striatum and cerebellum before and after AMPH at baseline and at the two post-Mn imaging time points in one of the animals. At baseline, AMPH-induced DA release caused a significant downward deflection of the [^{11}C]-raclopride time–activity curve in the striatum (top graph in panel B). This effect was gradually and dramatically decreased with increasing Mn exposure (middle and lower graphs in panel B). The mean \pm SEM of DA release for all animals ($n=4$) showed a 41.6 ± 19.9 percent decrease from baseline at the Mn-1 time point and a 61.3 ± 8.0 percent decrease at the Mn-2 time point (Fig. 2C). The percent decrease in DA release at the Mn-2 time point was significantly different from baseline (Fig. 2C). No change in [^{11}C]-raclopride binding potential, a measure of D2-DAR levels, was measured in the striatum (Fig. 2C). Exposure to Mn produced a small but non-significant decrease in [^{11}C]-methylphenidate binding to DAT relative to baseline (Supplementary Fig. 1).

Post-mortem analysis of dopaminergic neuronal markers

We performed quantitative autoradiography in brain tissue from the same animals undergoing PET studies and compared them to naive controls. Consistent with the PET findings, there was a trend toward a decrease in [^{125}I]-RTI-121 specific binding to DAT in both the caudate and putamen of Mn-exposed animals; however, the differences did not reach statistical significance when compared to naive control animals (Table 2). No significant effect of Mn exposure on [^3H]-raclopride specific binding to D2-DAR was found in the caudate or putamen relative to naive controls (Table 2). We also performed TH immunohistochemistry and analysis of DA and its metabolite HVA as additional methods to assess the integrity of dopaminergic terminals in the striatum. No significant effect of Mn exposure was observed on the level of these markers (Table 2).

Table 2
Post-mortem analysis of caudate and putamen

Neurochemical measurements	Caudate		Putamen	
	Control	Mn-exposed	Control	Mn-exposed
DAT (fmol/mg tissue)	6.8 \pm 0.8	4.7 \pm 0.4	5.4 \pm 0.3	4.4 \pm 0.1
D2-DAR (fmol/mg tissue)	91.8 \pm 1.4	99.9 \pm 4.0	88.3 \pm 1.0	94.5 \pm 2.9
TH immuno (optical units)	79.4 \pm 6.9	78.4 \pm 6.6	85.4 \pm 6.3	88.7 \pm 8.0
DA levels (ng/mg protein)	102.5 \pm 24.2	46.9 \pm 8.9	156.2 \pm 28.4	145.4 \pm 45.9
HVA levels (ng/mg protein)	123.3 \pm 6.7	125.8 \pm 24.8	198.7 \pm 37.6	231.9 \pm 49.9

Each value is the mean \pm SEM of 3 naive control and 4 Mn-exposed animals.

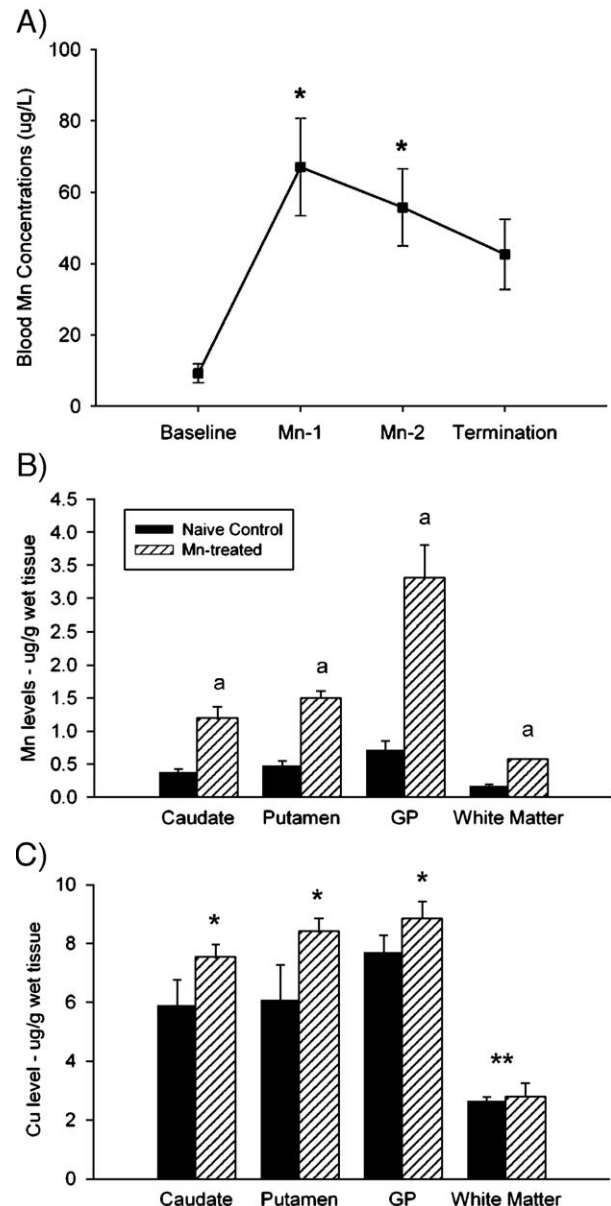


Fig. 3. Whole blood and brain tissue metals analysis. (A) Whole blood Mn concentrations at various time points during the exposure period. Each value is the mean \pm SEM of $n=4$ Mn-exposed animals except at baseline where $n=3$. * $p < 0.05$ from baseline levels. (B) Effect of Mn exposure on brain Mn concentrations. Each value is the mean \pm SEM of 3 naive controls and 4 Mn-exposed animals. a=significantly different from control ($p < 0.05$) within a brain region. (C) Effect of Mn treatment on brain Cu concentrations. *Significant Mn treatment effect $F_{1,3}=8.38$; $p=0.009$. **Significant brain region effect $F_{1,3}=29.7$; $p=0.0001$ where white matter is significantly different from all other regions regardless of treatment. GP=globus pallidus.

Metal analysis in whole blood and brain tissue

Whole blood samples were obtained under fasting conditions on the day of the PET studies and at termination of the studies. The results show a significant effect ($F_{3,11}=4.73$; $p=0.024$) of Mn exposure on whole blood Mn concentrations (Fig. 3A). No significant effect of Mn exposure was observed on whole blood Cu, Fe or Zn concentrations (data not shown). The mean \pm SEM of whole blood Mn concentrations in $\mu\text{g/l}$ was as follows:

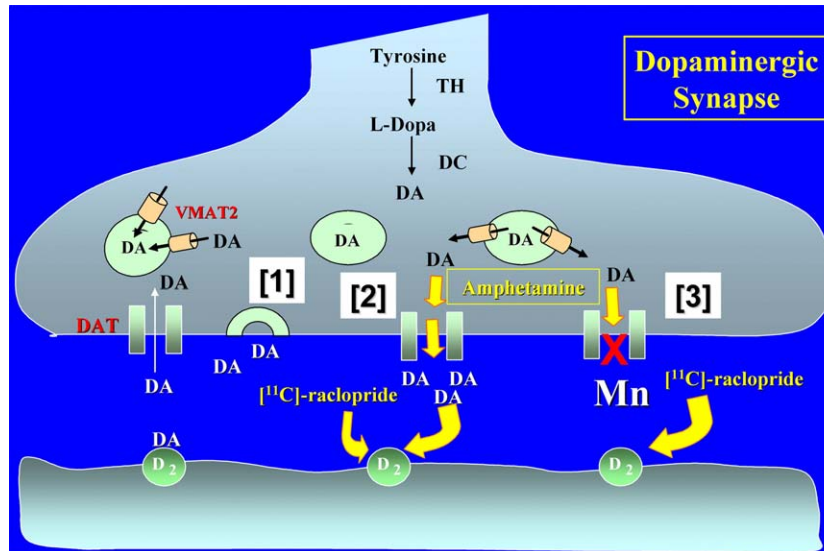


Fig. 4. Schematic of a dopaminergic synapse under normal physiological conditions [1], the effect of amphetamine [2] and the effect of manganese (Mn) on amphetamine-induced dopamine (DA) release. Arrow thickness indicates level of [¹¹C]-raclopride binding to D2-dopamine receptors (D2). Under normal physiological conditions [1], DA is released into the synapse and interacts with dopamine receptors. Dopaminergic neurotransmission is terminated by the re-uptake of dopamine into the pre-synaptic element by dopamine transporters (DAT) and dopamine is re-packaged into vesicles by vesicular monoamine transporter type-2 (VMAT-2). With the administration of amphetamine [2], there is a reversal of the vesicular and plasmalemmal membrane transporters producing a massive release of dopamine into the synapse that is able to effectively compete with [¹¹C]-raclopride for binding to D2-dopamine receptors. This causes a dramatic decrease in [¹¹C]-raclopride binding in the striatum. In animals treated with manganese (Mn), there is abrogation of the amphetamine-induced dopamine release into the synapse allowing a greater amount of [¹¹C]-raclopride binding to D2-dopamine receptors. TH=tyrosine hydroxylase; DC=DOPA decarboxylase.

Baseline: 9.2 ± 2.7 ($n=3$; range: 5.1–14.2 $\mu\text{g/l}$). One animal had a baseline Mn value of 62.4 $\mu\text{g/l}$. This value was determined by statistical methods to be an outlier and removed from the average baseline value); Mn-1 time point: 67.1 ± 13.7 ($n=4$; range: 42.9–106.1 $\mu\text{g/l}$); Mn-2 time point: 55.7 ± 10.8 ($n=4$; range: 29.4–73.7 $\mu\text{g/l}$); at termination: 42.6 ± 9.9 ($n=4$; range: 14.6–58.6 $\mu\text{g/l}$).

Post-mortem brain tissue from Mn-exposed and control animals was also analyzed for metal content. There was a significant increase in Mn concentrations (Treatment effect: $F_{1,20}=7.06$; $p=0.015$) in all brain regions examined (Fig. 3B). We also found a significant effect of Mn exposure ($F_{1,20}=8.38$; $p=0.009$) on brain Cu concentrations (Fig. 3C) with no significant effect on Fe or Zn concentrations (data not shown). The Mn-induced increase in brain Cu concentrations was primarily driven by increases in basal ganglia structures with little or no increase in white matter (Fig. 3C). Cu concentrations in white matter were significantly lower relative to all other regions irrespective of treatment (Fig. 3C).

Discussion

In the present study, we describe behavioral and neuroimaging abnormalities resulting from chronic low level Mn exposure in non-human primates. We found that Mn exposure produced subtle deficits on behavioral rating scores, activity levels and fine motor function that were significantly different from baseline at approximately the same time at which a maximal effect on *in vivo* DA release was measured in the striatum. The novel finding that Mn exposure progressively decreased *in vivo* DA release provides a potential link to the

subtle deficits in motor function observed in the same animals. An important observation from these studies is that whole blood Mn concentrations during the exposure period were within the upper range of concentrations reported in children or adults receiving environmental, medical or occupational exposures (See Gulson et al., 2006 and table within). It is important to note that, in a study by Takser et al. (2003) referenced in the Gulson et al. (2006) manuscript, they found that approximately 50% of 112 cord blood samples obtained at birth in a French population had Mn concentrations greater than 40 $\mu\text{g/l}$.

In the PET studies that assess *in vivo* DA release, AMPH is used to elicit the release of DA resulting in the acute reduction of striatal [¹¹C]-raclopride reflecting the competition of the released DA with [¹¹C]-raclopride for binding to D2-DAR. This is a commonly used technique to examine *in vivo* DA release by PET and it is shown to be correlated with extracellular DA concentrations measured by *in vivo* microdialysis (Laruelle, 2000). The progressive decrease in AMPH-evoked DA release produced by Mn was present in the absence of a change in DAT or D2-DAR levels measured by PET in the same animals. Post-mortem analysis confirmed the lack of a Mn effect on markers of dopamine terminal integrity (Table 2). Together, these findings suggest that, at this level and duration of Mn exposure, there is dopaminergic terminal dysfunction in the absence of terminal loss in the striatum. To our knowledge, this is the first report of a Mn-induced decrease on *in vivo* DA release in the absence of terminal loss and suggests a novel mechanism of Mn effects on nigrostriatal dopaminergic system function.

What are potential mechanism(s) by which Mn exposure may produce a decrease in AMPH-induced DA release? AMPH has multiple actions on DA nerve terminals (Seiden et al.,

1993). It is known to reverse the DAT-mediated transport of DA resulting in the efflux of DA from a cytosolic pool to the extracellular space (Cadoni et al., 1995; Koob and Nestler, 1997; Seiden et al., 1993; Sulzer et al., 1995). AMPH can also redistribute DA from vesicles to the cytosol that is then available for release (Sulzer et al., 1995). Based on this knowledge, there are several potential explanations to our findings. One possibility is that Mn exposure decreased cytosolic and/or vesicular DA levels reducing the amount of DA available for release. This is not a likely explanation since we found no significant differences in DA or HVA concentrations in the striatum of Mn-exposed animals relative to controls. It should be noted, however, that the mean DA concentration in the caudate of Mn-treated animals was lower than the mean value for controls (Table 2). However, the differences did not reach statistical significance due to a high degree of variability.

A second possibility is that chronic Mn exposure produced a progressive net increase in basal levels of extracellular DA reducing the dynamic range by which AMPH elicits an increase in extracellular DA reducing the ability to compete with [¹¹C]-raclopride for D2-DAR binding. Mn has been shown to both stimulate DA release (Drapeau and Nachsen, 1984; Powls et al., 1996) and inhibit DA uptake (Chen et al., 2006). Although there is no current *in vivo* data to support this interpretation, future studies by our group will examine the degree to which extracellular levels of DA are altered in the striatum of Mn-exposed non-human primates.

Chronic exposure to Mn may also interfere with mechanism (s) by which AMPH produces an increase in DA release by reversing DAT flux. Studies have shown that the stimulating effect of AMPH on DA release requires a functional DAT (Jones et al., 1998) and DAT antagonists abrogate AMPH-induced DA release (Fisher and Cho, 1979; Raiteri et al., 1979; Scarponi et al., 1999). This is a potential explanation since there is evidence that Mn inhibits the binding of the cocaine analog [³H]-WIN 35,428 to DAT and decreases [³H]-DA uptake into rat striatal synaptosomes (Chen et al., 2006). Therefore, it is possible that the effects of chronic Mn exposure on *in vivo* DA release may be due to the ability of Mn to act as a DAT antagonist (see Fig. 4).

What are the functional implications of a decrease in AMPH-evoked and DAT-mediated *in vivo* DA release by chronic Mn exposure? It is known that, in the striatum, DA is released from DA axon terminals via an exocytotic, calcium-dependent mechanism. The function of DAT is to remove DA from the synapse back into the pre-synaptic terminal (see Fig. 4). On the other hand, in dendrites of substantia nigra dopaminergic neurons, a different role for DAT has been suggested. Stimulation of subthalamic glutamatergic fibers innervating the substantia nigra produces the release of DA from dopaminergic somatodendrites via a DAT-mediated and calcium-independent process (Falkenburger et al., 2001). This carrier-mediated somatodendritic release of DA stimulates DA autoreceptors to reduce the excitability of DA neurons (Blakely, 2001; Falkenburger et al., 2001). Thus, one possible effect of inhibiting carrier-mediated DA release in Mn-exposed non-human primates may be to alter the excitability

of nigrostriatal dopaminergic neurons and their activity to projection areas.

Another potential effect of an inhibition of DAT-mediated DA release by Mn exposure may be to alter DA compartmentalization enhancing the probability of DA oxidation, generation of reactive oxygen species and neuronal injury (Hastings et al., 1996; Stokes et al., 1999). We also found that chronic Mn exposure produced an increase in brain Cu concentrations (primarily in basal ganglia structures, Fig. 3C) providing an environment conducive for DA oxidation and the generation of reactive oxygen species. Cu is a redox active metal and Cu–DA complexes and quinone formation have been identified and implicated in the neuropathology of PD (Paris et al., 2001). The accumulation of Mn in basal ganglia and the resulting increase in Cu concentrations may have important implications to changes in motor function observed in Mn-exposed animals since increased levels of these metals in the basal ganglia have been observed in primary adult-onset dystonia (Becker et al., 1999), a symptom that is prominent in Mn-induced parkinsonism.

In summary, we show that non-human primates chronically exposed to levels of Mn that produced subtle deficits in motor function have an apparently intact but dysfunctional nigrostriatal DA system. The decreased *in vivo* DA release documented in these animals may represent an early event that may progress to neuronal injury and degeneration with protracted Mn exposures.

Acknowledgments

This work was supported by NIEHS grant # ES10975 (TRG). We wish to thank Debbie VanKempen for excellent technical assistance and care of the animals during the neuroimaging studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.expneurol.2006.06.015.

References

- Aschner, M., 2000. Manganese: brain transport and emerging research needs. *Environ. Health Perspect.* 108, 429–432.
- Becker, G., Berg, D., Rausch, W.-D., Lange, H.K., Riederer, P., Reiners, K., 1999. Increased tissue copper and manganese content in the lentiform nucleus in primary adult-onset dystonia. *Ann. Neurol.* 46, 260–263.
- Blakely, R.D., 2001. Dopamine's reversal of fortune. *Science* 293, 2407–2409.
- Brooks, D.J., Frey, K.A., Marek, K.L., Oakes, D., Paty, D., Prentice, R., Shults, C.W., Stoessl, A.J., 2003. Assessment of neuroimaging techniques as biomarkers of the progression of Parkinson's disease. *Exp. Neurol.* 184, S68–S79.
- Cadoni, C., Pinna, A., Russi, G., Consolo, S., Di Chiara, G., 1995. Role of vesicular dopamine in the *in vivo* stimulation of striatal dopamine transmission by amphetamine: evidence from microdialysis and Fos immunohistochemistry. *Neuroscience* 65, 1027–1039.
- Calne, D.B., Chu, N.S., Huang, C.C., Lu, C.S., Olanow, W., 1994. Manganism and idiopathic parkinsonism: similarities and differences. *Neurology* 44, 1583–1586.

- Carson, R.E., Breier, A., DeBartolomeis, A., Saunders, R.C., Su, T.P., Schmall, B., Der, M.G., Pickar, D., Eckelman, W.C., 1997. Quantification of amphetamine-induced changes in [¹¹C]-raclopride binding with continuous infusion. *J. Cereb. Blood Flow Metab.* 17, 437–447.
- Chen, M.-K., Lee, J.-S., McGlothlan, J.L., Furukawa, E., Adams, R.J., Alexander, M., Wong, D.F., Guilarte, T.R., 2006. Acute manganese administration alters dopamine transporter levels in non-human primate striatum. *Neurotoxicology* 27, 229–236.
- Davis, J.M., 1999. Inhalation health risks of manganese: an EPA perspective. *Neurotoxicology* 20, 511–518.
- Dietz, M.C., Ihrig, A., Wrazidlo, W., Bader, M., Jansen, O., Triebig, G., 2001. Results of magnetic resonance imaging in long-term manganese dioxide-exposed workers. *Environ. Res.* 85, 37–40.
- DiMonte, D.A., 2003. The environment and Parkinson's disease: is the nigrostriatal system preferentially targeted by neurotoxins? *Lancet Neurol.* 2, 531–538.
- Drapeau, P., Nachsen, D.A., 1984. Manganese fluxes and manganese-dependent neurotransmitter release in presynaptic nerve endings isolated from rat brain. *J. Physiol.* 348, 493–510.
- Endres, C.J., Kolachana, B.S., Saunders, R.C., Su, T., Weinberger, D., Breier, A., Eckleman, W., Carson, R.E., 1997. Kinetic modeling of [¹¹C]-raclopride combined PET-microdialysis studies. *J. Cereb. Blood Flow Metab.* 17, 932–942.
- Eriksson, H., Tedroff, J., Thuomas, K.-A., Aquilonius, S.-M., Hartvig, P., Fasth, K.-J., Bjurling, P., Langstrom, B., Hedstrom, K.-G., Heilbronn, E., 1992. Manganese induced brain lesions in *Macaca fascicularis* as revealed by positron emission tomography and magnetic resonance imaging. *Arch. Toxicol.* 66, 403–407.
- Erikson, K.M., Syversen, T., Steinnes, E., Aschner, M., 2004. Globus pallidus: a target brain region for divalent metal accumulation associated with dietary iron deficiency. *J. Nutr. Biochem.* 15, 335–341.
- Falkenburger, B.H., Barstow, K.L., Mintz, I.M., 2001. Dendrodendritic inhibition through reversal of dopamine transport. *Science* 293, 2465–2470.
- Fisher, J.F., Cho, A.K., 1979. Chemical release of dopamine from striatal homogenates: evidence for an exchange diffusion model. *J. Pharmacol. Exp. Ther.* 208, 203–209.
- Gorell, J.M., Johnson, C.C., Rybicki, B.A., Peterson, E.L., Kortsha, G.X., Brown, G.G., Richardson, R.J., 1999. Occupational exposure to manganese, copper, lead, iron, mercury and zinc and the risk of Parkinson's disease. *Neurotoxicology* 20, 239–248.
- Gulson, B., Mizon, K., Korsch, M., Stauber, J., Davis, J.M., Louie, H., Wu, M., Swan, H., 2006. Changes in manganese and lead in the environment and young children associated with the introduction of methylcyclopentadienyl manganese tricarbonyl in gasoline-preliminary results. *Environ. Res.* 100, 100–114.
- Hastings, T.G., Lewis, D.A., Zigmond, M.J., 1996. Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. *Proc. Natl. Acad. Sci.* 93, 1956–1961.
- Huang, C.-C., Chu, N.-S., Lu, C.-S., Chen, R.-S., Calne, D.B., 1998. Long-term progression in chronic manganese—Ten years follow-up. *Neurology* 50, 698–700.
- Huang, C.-C., Weng, Y.-H., Lu, C.-S., Chu, N.-S., Yen, T.-C., 2003. Dopamine transporter binding in chronic manganese intoxication. *J. Neurol.* 250, 1335–1339.
- Jankovic, J., 2005. Searching for a relationship between manganese and welding and Parkinson's disease. *Neurology* 64, 2021–2028.
- Jones, S.R., Gainetdinov, R.R., Wightman, R.M., Caron, M.G., 1998. Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. *J. Neurosci.* 18, 1979–1986.
- Josephs, K.A., Ahlskog, J.E., Klos, K.J., Kumar, N., Fealey, R.D., Trenerry, M. R., Cowl, C.T., 2005. Neurologic manifestations in welders with pallidal MRI T1 hyperintensity. *Neurology* 64, 2033–2039.
- Kaiser, J., 2003. Manganese: a high octane dispute. *Science* 300, 926–928.
- Kim, Y., Kim, K.S., Yang, J.S., Park, I.J., Kim, E., Jin, Y., Kwon, K.-R., Chang, K.H., Kim, J.-W., Park, S.-H., Lim, H.S., Cheong, H.K., Shin, Y.C., Park, J., Moon, Y., 1999. Increase in signal intensities on T1-weighted magnetic resonance images in asymptomatic manganese-exposed workers. *Neurotoxicology* 20, 901–908.
- Kim, Y., Kim, J.-M., Kim, J.-W., Yoo, C.-I., Lee, C.R., Lee, J.H., Kim, H.K., Yang, S.O., Chung, H.K., Lee, D.S., Jeon, B., 2002. Dopamine transporter density is decreased in parkinsonian patients with a history of manganese exposure: what does it mean? *Mov. Dis.* 17, 568–575.
- Koob, G.F., Nestler, E.J., 1997. The neurobiology of drug addiction. *J. Neuropsychiatry Clin. Neurosci.* 9, 482–497.
- Lammertsma, A.A., Hume, S.P., 1996. Simplified reference tissue model for PET receptor studies. *NeuroImage* 4, 153–158.
- Laruelle, M., 2000. Imaging synaptic neurotransmission with in vivo binding competition techniques: a critical review. *J. Cereb. Blood Flow Metab.* 20, 423–451.
- Lu, C.-S., Huang, C.-C., Chu, N.-S., Calne, D.B., 1994. Levodopa failure in chronic manganese. *Neurology* 44, 1600–1602.
- Mena, I., Marin, O., Fuenzalida, S., Cotzias, G.C., 1967. Chronic manganese poisoning: clinical picture and manganese turnover. *Neurology* 17, 128–136.
- Nader, M.A., Daunais, J.B., Moore, T., Nader, S.H., Moore, R.J., Smith, H.R., Friedman, D.P., Porrino, L.J., 2002. Effects of cocaine self-administration on striatal dopamine synthesis in rhesus monkeys: initial and chronic exposure. *Neuropsychopharmacology* 27, 35–46.
- Newland, M.C., Ceckler, T.L., Kordower, J.H., Weiss, B., 1989. Visualizing manganese in the primate basal ganglia with magnetic resonance imaging. *Exp. Neurol.* 106, 251–258.
- Olanow, C.W., 2004. Manganese-induced parkinsonism and Parkinson's disease. *Ann. N. Y. Acad. Sci.* 1012, 209–223.
- Pal, P.K., Samii, A., Calne, D.B., 1999. Manganese neurotoxicity: a review of clinical features, imaging and pathology. *Neurotoxicology* 20, 227–238.
- Paris, I., Dagnino-Subiabre, A., Marcelain, K., Bennett, L.B., Caviedes, P., Caviedes, R., Olea Azar, C., Segura-Aguilar, J., 2001. Copper neurotoxicity is dependent on dopamine-mediated copper uptake and one-electron reduction of aminochrome in a rat substantia nigra neuronal cell line. *J. Neurochem.* 77, 519–529.
- Pirker, W., Djamshidian, S., Asenbaum, S., Gerschlag, W., Tribl, G., Hoffmann, M., Brucke, T., 2002. Progression of dopaminergic degeneration in Parkinson's disease and atypical parkinsonism: a longitudinal β -CIT SPECT study. *Mov. Dis.* 17, 45–53.
- Powls, D.A., O'Brien, K.J., Harrison, S.M., Jarvie, P.E., Dunkley, P.R., 1996. Mn²⁺ can substitute for Ca²⁺ in causing catecholamine secretion but not for increasing tyrosine hydroxylase phosphorylation in bovine adrenal chromaffin cells. *Cell Calcium.* 19, 419–429.
- Racette, B.A., McGee-Minnich, L., Moerlein, S.M., Mink, J.W., Videen, T.O., Perlmutter, J.S., 2001. Welding-related parkinsonism: clinical features, treatment, and pathophysiology. *Neurology* 56, 8–13.
- Racette, B.A., Tabbal, S.D., Jennings, D., Good, L., Perlmutter, J.S., Evanoff, B., 2005a. Prevalence of parkinsonism and relationship to exposure in a large sample of Alabama welders. *Neurology* 64, 230–235.
- Racette, B.A., Antenor, J.A., McGee-Minnich, L., Moerlein, S.M., Videen, T.O., Kotagal, V., Perlmutter, J.S., 2005b. [¹⁸F]-FDOPA PET and clinical features in Parkinsonism due to manganese. *Mov. Dis.* 20, 492–496.
- Raiteri, M., Cerrito, F., Cervoni, A.M., Levi, G., 1979. Dopamine can be released by two mechanisms differentially affected by the dopamine transport inhibitor nomifensine. *J. Pharmacol. Exp. Ther.* 208, 195–202.
- Sadek, A.H., Rauch, R., Schulz, P.E., 2003. Parkinsonism due to manganese in a welder. *Int. J. Toxicol.* 22, 393–401.
- Scarponi, M., Bernardi, G., Mercuri, N.B., 1999. Electrophysiological evidence for a reciprocal interaction between amphetamine and cocaine-related drugs on rat midbrain dopaminergic neurons. *Eur. J. Neurosci.* 11, 593–598.
- Schneider, J.S., Kovelowski, C.J., 1990. Chronic exposure to low doses of MPTP: I. Cognitive deficits in motor asymptomatic monkeys. *Brain Res.* 519, 122–128.
- Schneider, J.S., Yuwiler, A., 1989. GM1 ganglioside treatment promotes recovery of striatal dopamine concentrations in the mouse model for MPTP-induced parkinsonism. *Exp. Neurol.* 105, 177–183.
- Seiden, L.S., Sabol, K.E., Ricaurte, G.A., 1993. Amphetamine: effects on

- catecholamine systems and behavior. *Annu. Rev. Pharmacol. Toxicol.* 32, 639–677.
- Shinotoh, H., Snow, B.J., Hewitt, K.A., Pate, B.D., Doudet, D., Nugent, R., Perl, D.P., Olanow, W., Calne, D.B., 1995. MRI and PET studies of manganese-intoxicated monkeys. *Neurology* 45, 1199–1204.
- Shinotoh, H., Snow, B.J., Chu, N.S., Huang, C.C., Lu, C.S., Lee, C., Takanashi, H., Calne, D.B., 1997. Presynaptic and postsynaptic striatal dopaminergic function in patients with manganese intoxication: a positron emission tomography study. *Neurology* 48, 1053–1056.
- Stokes, A.H., Hastings, T.G., Vrana, K.E., 1999. Cytotoxic and genotoxic potential of dopamine. *J. Neurosci. Res.* 55, 659–665.
- Strazielle, C., Lalonde, R., Amdiss, F., Botez, M.I., Hebert, C., Reader, T.A., 1998. Distribution of dopamine transporters in basal ganglia of cerebellar ataxic mice by [¹²⁵I]-RTI-121 quantitative autoradiography. *Neurochem. Int.* 32, 61–68.
- Sulzer, D., Chen, T.-K., Lau, Y.Y., Kristensen, H., Rayport, S., Ewing, A., 1995. Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and promotes reverse transport. *J. Neurosci.* 15, 4102–4108.
- Takser, L., Mergler, D., Hellier, G., Sahuquillo, J., Huel, G., 2003. Manganese, monoamine metabolite levels at birth, and child psychomotor development. *Neurotoxicology* 24, 667–674.
- Thobois, S., Jahanshahi, M., Pinto, S., Frackowiak, R., Limousin-Dowsey, P., 2004. PET and SPECT functional imaging studies in Parkinsonian syndromes: from lesions to its consequences. *NeuroImage* 23, 1–16.
- Watabe, H., Endres, C.J., Carson, R.E., 1998. Modeling methods for the determination of dopamine release with [¹¹C]raclopride and constant infusion. *NeuroImage* 7, A57.
- Wolters, E.C., Huang, C.C., Clark, C., Peppard, R.F., Okada, J., Chu, N.S., Adam, M.J., Ruth, T.J., Li, D., Calne, D.B., 1989. Positron emission tomography in manganese intoxication. *Ann. Neurol.* 26, 647–651.