Duration of airborne-manganese exposure in rhesus monkeys is associated with brain regional changes in biomarkers of neurotoxicity

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Abstract:
Juvenile (20–24-month-old) rhesus monkeys were exposed to airborne-manganese sulfate ($MnSO_4$) 1.5 mg Mn/m^3 (6 h/day, 5 days/week) for 15 or 33 days, or for 65 days followed by a 45 or 90 days post-exposure recovery period, or air. We assessed biochemical endpoints indicative of oxidative stress and excitotoxicity in the cerebellum, frontal cortex, caudate, globus pallidus, olfactory cortex, and putamen. Glutamine synthetase (GS), glutamate transporters (GLT- 1 and GLAST) and tyrosine hydroxylase (TH) protein levels, metallothionein (MT), GLT-1, GLAST, TH and GS mRNA levels, and total glutathione (GSH) levels were determined for all brain regions. Exposure to Mn significantly decreased MT mRNA in the caudate (vs. air-exposed controls). This depression persisted at least 90 days post-exposure. In contrast, putamen MT mRNA levels were unaffected by Mn exposure. GLT-1 and GLAST were relatively unaffected by short term Mn exposure, except in the globus pallidus where exposure for 33 days led to decreased protein levels, which persisted after 45 days of recovery for both proteins and 90 days of recovery in the case of GLAST. Exposure to 1.5 mg Mn/m^3 caused a significant decrease in GSH levels in the caudate and increased GSH levels in the putamen of monkey exposed for 15 and 33 days with both effects persisting at least 90 days post-exposure. Finally, TH protein levels were significantly lowered in the globus pallidus of the monkeys exposed for 33 days but mRNA levels were significantly increased in this same region. Overall, the nonhuman primate brain responds to airborne Mn in a heterogeneous manner and most alterations in these biomarkers of neurotoxicity are reversible upon cessation of Mn exposure.
Keywords: Brain; Monkey; Air borne; Manganese; Glutathione; Glutamate

Article:
1. Introduction
Excessive manganese (Mn) accumulation in the nervous system can lead to detrimental functions that clinically resemble Parkinson's disease (PD). Mn toxicity is most commonly associated with occupational exposure to aerosols or dusts that contain extremely high levels (> 1–5 mg Mn/m^3) of Mn, consumption of contaminated well water, or parenteral nutrition therapy in patients with liver disease. (Aschner et al., 2005; ATSDR, 2000; Mergler et al., 1994; Pal et al., 1999). Over the last five years, our group has reported brain Mn concentrations and responses of several markers of neurotoxicity following high dose Mn inhalation by pregnant rats and their offspring, young adult rats, and senescent rats (Dobson et al., 2003; Erikson et al., 2004, 2005, 2006; Weber et al., 2002). More recently our group explored the effects of 0.06, 0.3 and 1.5 mg Mn/mm^3 on markers of neurotoxicity in juvenile rhesus monkeys (Erikson et al., 2007). This current study examines how exposure duration influences these markers of neurotoxicity in monkey brain regions with differing susceptibilities to Mn neurotoxicity.

Oxidative stress has been implicated as a contributing mechanism by which Mn mediates its cytotoxicity (Aschner, 1997; Taylor et al., 2006). It has been proposed that Mn, through its sequestration in mitochondria (Galvani et al., 1995), interferes with proper respiration, thereby leading to excessive production of reactive oxygen species (ROS). Increased ROS production can interfere with glutamate removal by inhibiting the high affinity glutamate transporters (Trotti et al., 1998). Increased extracellular glutamate levels are excitotoxic to

neurons and may represent a likely mode of action for Mn neurotoxicity. The glutamate/aspartate transporter (GLAST) and glutamate transporter (GLT-1) are the most prominent astrocytic glutamate transporters (Danbolt, 2001). It has been shown that glutamate uptake is attenuated in astrocytes exposed to Mn (Hazell and Norenberg, 1997; Erikson et al., 2002b) while GLAST gene expression is significantly decreased following Mn exposure (Erikson et al., 2002b). However, little data exist on the in vivo effects of Mn inhalation on glutamate transporter gene expression and protein levels, information that is critical for more fully evaluating the neurotoxicity of inhaled Mn.

In our previous studies we assessed oxidative stress by measuring levels of two antioxidants, namely glutathione (GSH) and metallothionein (MT), as well as glutamine synthetase (GS), a protein that is exquisitely sensitive to oxidative stress (Dobson et al., 2003; Erikson et al., 2004, 2005, 2006, 2007). In this study we evaluated exposure time-dependent changes and recovery after a subchronic Mn inhalation in MT, GLT-1, and GLAST, tyrosine hydroxylase (TH), and GS in select brain regions of rhesus monkeys following high-dose Mn inhalation. We hypothesize that alterations in these markers of neurotoxicity will persist after cessation of Mn exposure, based on our previous study (Erikson et al., 2005).

2. Materials and methods

2. 1. Chemicals

All chemicals were purchased from Sigma Chemical (St. Louis, MO), unless otherwise noted, and were of the highest possible quality.

2.2. Animals and their husbandry

This study was conducted under federal guidelines for the care and use of laboratory animals and was approved by the Chemical Industry Institute of Toxicology Centers for Health Research (CIIT) Institutional Animal Care and Use Committee. Twenty-two male rhesus monkeys purchased from Covance Research Products, Inc. (Alice, TX) were used. Animals were between 20 and 24 months of age at the start of the inhalation exposure.

All animals were housed in animal rooms or exposure chambers within CIIT's animal facility. This facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. A certified primate chow (#5048) diet from Purina Mills (St. Louis, MO) was fed twice a day (total daily amount fed was approximately 4% of the animal's body weight). During non-exposure periods, domiciliary stainless steel cages (0.4 m^2 x 0.8 m tall) suitable for housing macaque monkeys (Lab Products, Inc., Seaford, DE) were used to individually house monkeys. On each exposure day, animals were transferred to 0.2 m^2 x 0.6 m tall stainless steel cages (Lab Products, Inc., Seaford, DE) that were designed to fit within the 8-m^3 inhalation chambers. Animals were moved back to their domiciliary cages after the end of each 6-h exposure. Additional details concerning the husbandry and health status of these animals have been published (Dorman et al., 2005).

2.3. Experimental design

The in-life portion of this study was performed in accordance with the U.S. Environmental Protection Agency's Good Laboratory Practice (GLP) Standards for Inhalation Exposure Health Effects Testing (40 CFR Part 79.60). A $MnSO_4$ aerosol concentration of 4.62 mg $MnSO_4/m^3$, corresponding to 1.5 mg Mn/m^3, was generated for this study. Control animals were exposed to filtered air. Exposures were conducted for 6 h/day, 5 days/week. Monkeys were exposed to $MnSO_4$ at 1.5 mg Mn/m^3 for 15 (n = 4) or 33 (n = 4 exposure days; or to 1.5 mg Mn/m^3 for 65 exposure days and held for either 45 days (n = 4) or 90 days (n = 4) before evaluation (recovery groups). Control monkeys were exposed to filtered air for 65 exposure days (n = 6). The air-exposed control monkeys were utilized in another study which included magnetic resonance imaging of the brain (Dorman et al., 2006a,b) and involved the short-term (~90 min) use of the injectable anesthetic propofol. The mode of action by which propofol induces anesthesia is not fully understood, although several studies suggest that the compound acts via potentiation of brain gamma-aminobutyric acid ($GABA_A$) receptors (Schwieler et al., 2003). Propofol is a highly lipophilic agent with an extremely fast onset and short duration of action. A mean blood–brain equilibration half-life of less than 3 min has been reported (Kanto and Gepts, 1989). Propofol undergoes rapid redistribution to muscle and fat and other inactive tissue depots with a $t_{1/2}$ of elimination of approximately 30

min in healthy children (Jones et al., 1990). The time interval (~72 h) between propofol administration and euthanasia of the control monkeys was adequate for complete elimination to occur thus minimizing propofol-induced effects on the biomarkers measured in this study. It remains unknown however, whether certain responses seen may indeed be attributed to the anesthetic regimen used; however, finding of similar responses in manganese-exposed rodents (Erikson et al., 2004) without prior propofol anesthesia suggest that the effects seen in this study were induced by the manganese exposure.

2.4. Mn exposures and tissue collection

Manganese (II) sulfate monohydrate ($MnSO_4 \cdot H_2O$) (CAS Registry Number 10034-96-5) was obtained from Sigma– Aldrich Chemical Company, Inc. (Milwaukee, WI). This relatively water-soluble material is a white to pale pink crystalline powder that contains 32.6% Mn. Four 8-m^3 stainless steel and glass inhalation exposure chambers were used. Methods describing chamber monitoring as well as generation and characterization of the $MnSO_4$ aerosol and tissue collection have been previously described (Dorman et al., 2004, 2005; Erikson et al., 2007).

Necropsies were performed the day following the last inhalation exposure (i.e., 12–18 h after termination of the final inhalation exposure). Food was withheld overnight prior to necropsy. Monkeys were anesthetized with ketamine (20 mg/ kg, IM, Fort Dodge Animal Health, Fort Dodge, IA) and euthanized with pentobarbital (80–150 mg/kg, i.v., Henry Schein, Inc., Port Washington, NY) followed by exsanguinations. Following euthanasia, the brains were removed and divided on the mid-sagittal plane with anatomical structures identified using a published atlas (Martin and Bowden, 2000). The following brain structures were used for this study: caudate, putamen, globus pallidus, olfactory cortex, cerebellum, and frontal cortex. All samples were stored in individual plastic vials or bags, frozen in liquid nitrogen, and stored at approximately —80 °C until chemical analyses were per-formed. The brain regions were selected because they are known to be target regions for Mn toxicity based on numerous studies published over the last 7 years.

2.5. RNA and protein extraction from brain tissues

Each brain region was treated to allow RNA and protein extraction. Briefly, the tissue samples had a monophase phenol and guanidine isothiocyanate solution (RNA STAT-60, Tel-Test, Inc., Friendswood, TX) added (1:10, w/v, dilution) and were homogenized, centrifuged at 12,000 x g for 30 min. The clear layer was removed and RNA was isolated from it. The white layer (DNA) was removed from the remaining sample and was centrifuged at 12,000 x g for 10 min. The supernatant was removed and the remaining pellet was resuspended in WANG buffer (25 mM HEPES, pH 7.0, 250 mM sucrose, 100 uM EDTA, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 1 mM dithiothreitol (DTT), 0.2% Triton-X 100). Following sonication of the samples, protein content was determined using the bicinchoninic acid (BCA) method (Pierce, IL).

2.6. Northern blot analysis

For Northern blot analysis, 10 μg of RNA were electrophoresed on a 1.2% denaturing agarose gel and transferred onto a positively charged nylon membrane (Nytran SuPerCharge, Schleicher & Schuell, Keene, NH) overnight by capillary transfer in 10x SSC (1x SSC = 0.15M sodium chloride, 0.015 M sodium citrate) buffer. The RNA was immobilized with a UV crosslinker.

For GS, MT, TH, GLT- 1 or GLAST, the blot was prehybridized in 50% deionized formamide, 5x Denhardt's solution, 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 4x SSC 100g/ml denatured salmon sperm DNA, 20 mM Tris pH 8.0 for 1 h at 45 °C. To probe for GS, MT, TH, GLT- 1 or GLAST, the blot was prehybridized in Ultrasensitive Hybridization Buffer (Ambion, Inc., Austin, TX) at 45 °C. The RNA blots were then hybridized as previously published (see Erikson et al., 2007).

Membranes were washed two to three times in 2 x SSC/0.1% SDS at 45 °C for 20 min, and then exposed to Kodak Biomax MR Film, at —80 °C with intensifying screens for 24–36 h. The autoradiograms were quantified by densitometry scanning in conjunction with the TINA v2.09e computer program (Raytest USA,

Inc., Wilmington, NC). To correct for total loaded RNA level, the blots were stripped in 0. 1 x SSC/0.1% SDS/40 mM Tris buffer and probed for 28S rRNA (Barbu and Dautry, 1989).

2.7. Western blot analysis

Aliquots of protein (100 μg) were mixed with 5 x sample buffer (0.25 M Tris, pH 6.8, 10% SDS, 50% glycerol) and 1 M DTT, and separated by denaturing SDS-PAGE using 5% stacking, and 8% resolving acrylamide gels. Following fractionation, proteins were electrophoretically transferred to a nitrocellulose membrane (Protran BA83, Schleicher and Schuell, Keene, NH) in 20% methanol, 0. 1% SDS, 25 mM Tris, and 192 mM glycine for 3 h at 60 V. Membranes were then blocked with 5% non-fat powdered milk in TBST (Tris-buffered saline with 0. 1% Tween 20, 150 mM NaCl; 20 mM Tris) for 1 h. Glutamate transporter (GLT-1) protein expression was detected with a rabbit monoclonal antibody (Alpha Diagnostic International, San Antonio, TX) diluted 1:2500 in TBST and 5% milk overnight, followed by a 1 h incubation with an HRP-conjugated anti-rabbit secondary antibody (1:5000). Glutamate transporter (GLAST) protein expression was detected with a rat monoclonal antibody (Alpha Diagnostic International, San Antonio, TX) diluted 1:2000 in TBST and 5% milk overnight, followed by a 1 h incubation with an HRP-conjugated anti-rat secondary antibody (1:3500). GS proteins were detected with a monoclonal antibody, while TH protein was probed with a polyclonal antibody (both from Chemicon, Temecula, CA). Both antibodies were diluted to 1:2000 followed by incubation with horseradish peroxidase conjugated goat anti-mouse (GS protein) or horseradish peroxidase conjugated goat anti-rabbit (TH protein) secondary antibodies diluted 1:2000 (Kirkegaard and Perry Laboratories, Gaithers-burg, MD) in TBST and 5% milk for 1 h. Protein bands were visualized with the Enhanced Chemiluminescence System (New England Nuclear, Boston, MA) followed by exposure to X-ray film. Western blot reactions were controlled for saturation to assure that differences in protein expression were detectable. This was accomplished by serial dilutions of the samples and by varying the exposure times of the films. Films were digitized and band density was determined using the TINA v2.09e program.

2.8. Total GSH

The GSH assay that we used is described in detail elsewhere (Erikson et al., 2004, 2005, 2006). Briefly, tissue samples (50– 100 mg) were homogenized in 1 ml of 10% (v/v) perchloric acid containing 1 mM bathophenanthroline disulfonic acid (BPDS) and L-γ-glutamyl-L-glutamate. The mixture was vortexed and centrifuged, an aliquot was then removed for HPLC analysis (Fariss and Reed, 1987) on a Waters model 600E multisolvent delivery system using an ion-exchange method with a methanol–acetate mobile phase and gradient elution. The limit of GSH detection was approximately 50 pmol, which equated to approximately 0.4 nmol/mg protein (Lash and Tokarz, 1990; Lash and Woods, 1991).

Table 1
Manganese data are expressed as μg Mn/g tissue

Brain region	Control[a]	15 days	33 days	65 days + 45[b]	65 days + 90[b]
Caudate	0.34 ± 0.02	0.93 ± 0.11*	1.37 ± 0.13*	0.57 ± 0.03	0.43 ± 0.02
Cerebellum	0.44 ± 0.01	0.85 ± 0.06*	0.96 ± 0.05*	0.66 ± 0.04	0.61 ± 0.10
Fr cortex	0.25 ± 0.03	0.36 ± 0.01*	0.52 ± 0.03*	0.26 ± 0.01	0.23 ± 0.01
Glob pallid	0.48 ± 0.04	1.92 ± 0.40*	2.41 ± 0.29*	1.09 ± 0.03*	0.59 ± 0.12
Olf cortex	0.19 ± 0.004	0.43 ± 0.04*	0.45 ± 0.01*	0.26 ± 0.01*	0.21 ± 0.01
Putamen	0.36 ± 0.01	1.01 ± 0.08*	1.50 ± 0.14*	0.58 ± 0.03*	0.44 ± 0.02
Blood	0.010 ± 0.001	0.016 ± 0.006	0.022 ± 0.002*	0.021 ± 0.002*	0.013 ± 0.001

Mean ± S.E.M. (extracted from Dorman et al., 2006a). Each brain region: caudate, cerebellum, frontal cortex (fr cortex), globus pallidus (glob pallid), olfactory cortex (olf cortex) and putamen were run as reported in Dorman et al. (2006a). Asterisk "*" denotes statistical significance ($P < 0.05$) compared to control levels.

[a] Control Mn is expressed as μg Mn/g tissue wet weight.

[b] Days of recovery after exposure cessation.

2.9. Statistical analysis

The data were analyzed using the SPSS system v14.0 (SPSS, Inc., Chicago, IL). Analysis of variance (ANOVA) with repeated-measures factors (brain regions) and between-groups factors was used to test for interactions between Mn exposure or time and regions. Mauchly's Test of Sphericity was used to test for

violations of sphericity. If violations were found than a Greenhouse-Geisser correction was used. For each parameter tested, there were no significant interactions between Mn exposure time and brain regions, therefore all of our reported results were univariate analysis of variance within each brain region. When the overall significance resulted in rejection of the null hypothesis ($p < 0.05$), Dunnet's procedure was used to evaluate treatment means compared to control means.

3. Results

3.1. Mn concentrations

Brain regional Mn concentrations were reported in Dorman et al. (2006a). Exposure to $MnSO_4$ at 1.5 mg Mn/m^3 for ≥ 15 exposure days led to increased Mn concentrations in all examined brain regions. In the monkeys that were allowed to recover, globus pallidus, putamen, olfactory cortex and blood Mn levels remained elevated after 45 days but returned to control levels by 90 days post-exposure. To aid in evaluating our data, we summarized the tissue Mn levels in Table 1.

3.2. GS protein and mRNA

The gene expression of GS was only altered in the caudate of monkeys exposed to 1.5 mg Mn/m^3 for 33 days (vs. air-exposed controls). The protein levels of GS were altered in the cerebellum, globus pallidus, putamen of monkeys allowed to recover for 45 and 90 days post-exposure, and frontal cortex 90 days post exposure (Table 2).

Table 2
Glutamine synthetase (GS) protein and mRNA data are expressed as percentage of control values, which are shown

Brain region	Analysis	Control[a]	15 days	33 days	65 days + 45[b]	65days + 90[b]
Caudate	Protein	6600 ± 660	100.1 ± 19	104.3 ± 10	116.4 ± 17	117.7 ± 15
	mRNA	0.26 ± 0.04	84.6 ± 13	76.1 ± 3*	70.9 ± 14	84.6 ± 36
Cerebellum	Protein	680 ± 40	94 ± 20	94.7 ± 9	76.1 ± 15*	75.7 ± 13*
	mRNA	5.40 ± 0.40	102.1 ± 5	92.9 ± 3	97.5 ± 11	93.1 ± 5
Fr cortex	Protein	1500 ± 150	103.3 ± 4	109.1 ± 3	92.4 ± 1	85.6 ± 6*
	mRNA	0.91 ± 0.05	94.9 ± 6	96.9 ± 9	85.8 ± 10	85.2 ± 4
Glob pallid	Protein	1300 ± 230	80.4 ± 2	91.7 ± 4	47.5 ± 3*	51.3 ± 15*
	mRNA	1.01 ± 0.18	155 ± 21	159 ± 22	114.4 ± 5	130.1 ± 12
Olf cortex	Protein	580 ± 40	116.8 ± 4	110.9 ± 9	96.6 ± 4	81.5 ± 11
	mRNA	0.49 ± 0.06	174.1 ± 22	132.3 ± 12	156.7 ± 11	151.3 ± 14
Putamen	Protein	6500 ± 200	95 ± 4	93.2 ± 9	103.6 ± 4	85.9 ± 8*
	mRNA	0.70 ± 0.11	91.9 ± 6	85.5 ± 13	100.9 ± 8	118.2 ± 16

Mean ± S.E.M. For both Western and Northern blots, each brain region: caudate, cerebellum, frontal cortex (fr cortex), globus pallidus (glob pallid), olfactory cortex (olf cortex) and putamen was run in triplicate for each animal in each group ($n = 4$ monkeys per dose). Asterisk "*" denotes statistical significance ($P < 0.05$) compared to control levels.

[a] Control protein is expressed as OD and mRNA as ratio of mRNA/28S rRNA for each sample.

[b] Days of recovery after exposure cessation.

Table 3
Metallothionein (MT) mRNA data are expressed as percentage of control values, which are shown

Brain region	Analysis	Control[a]	15 days	33 days	65 days + 45[b]	65 days + 90[b]
Caudate	mRNA	1.02 ± 0.13	103.1 ± 11	82.7 ± 9*	85.4 ± 7*	82.9 ± 12*
Cerebellum	mRNA	6.92 ± 0.56	95.6 ± 9	89.6 ± 8	77.1 ± 12*	68.2 ± 3*
Fr cortex	mRNA	0.93 ± 0.04	99.8 ± 5	86.4 ± 8	92.1 ± 4	90.4 ± 5
Glob pallid	mRNA	0.85 ± 0.19	140.5 ± 35	143.9 ± 14	163.4 ± 18	132.8 ± 18
Olf cortex	mRNA	0.53 ± 0.08	176.6 ± 19	172.1 ± 6*	181.8 ± 41	138.8 ± 16
Putamen	mRNA	1.01 ± 0.09	102.2 ± 9	94.6 ± 6	102.2 ± 23.3	82.6 ± 11.8

Mean ± S.E.M. For Northern blots, each brain region: caudate, cerebellum, frontal cortex (fr cortex), globus pallidus (glob pallid), olfactory cortex (olf cortex) and putamen was run in triplicate for each animal in each group ($n = 4$ monkeys per dose). Asterisk "*" denotes statistical significance ($P < 0.05$) compared to control levels.

[a] Control mRNA is expressed as ratio of mRNA/28S rRNA for each sample.

[b] Days of recovery after exposure cessation.

3.3. MT mRNA

The caudate from monkeys exposed to 1.5 mg Mn/m^3 for 33 days as well as those allowed 45 and 90 days recovery (Table 3) showed reduced MT mRNA. The cerebellum showed reduced MT mRNA in monkeys exposed to 1.5 mg Mn/m^3 after 45 and 90 days of recovery while the olfactory cortex showed increased MT mRNA after 33 days of exposure.

3.4. GLT-1 protein and mRNA

GLT- 1 mRNA was significantly altered in the caudate of monkeys exposed to 1.5 mg Mn/m^3 for 15 and 33 days (Table 4). Monkeys recovering from exposure for 45 and 90 days showed altered mRNA levels in the cerebellum, globus pallidus (45 days only) and frontal cortex (90 days only). Alerted GLT- 1 protein levels were found in the globus pallidus at 33 exposure days and 45 days after exposure and in the olfactory cortex 90 days after exposure (Table 4).

3.5. GLAST protein and mRNA

The cerebellum had significantly decreased GLAST mRNA levels in monkeys exposed to 1.5 mg Mn/m^3 and then allowed to recover for 90 days (Table 5). Levels were increased in globus pallidus of monkeys exposed for 33 days and in the olfactory cortex of monkeys recovered for 45 days (Table 5). GLAST protein levels were unaffected by Mn exposure in all brain regions except the globus pallidus where protein was significantly lower in the monkeys exposed for 33 days and those recovered for 45 and 90 days (Table 5).

3.6. Total GSH

The monkeys exposed to the high dose of Mn had lowered GSH levels in the caudate after 15 and 33 days exposure and this effect persisted despite 45 and 90 days recovery (Table 6). In the frontal cortex and putamen, 33 days exposure to Mn led to significantly increased GSH levels and this higher level persisted in the frontal cortex after 45 days of recovery and in the putamen after 45 and 90 days of recovery.

Table 4
Glutamate transporter (GLT-1) protein and mRNA data are expressed as percentage of control values, which are shown

Brain region	Analysis	Control[a]	15 days	33 days	65 d + 45[b]	65d + 90[b]
Caudate	Protein	1540 ± 60	113.7 ± 5	107.3 ± 11	102.2 ± 14	113.3 ± 16
	mRNA	0.87 ± 0.03	69.4 ± 15*	68.2 ± 8*	96.2 ± 11	114.7 ± 16
Cerebellum	Protein	415 ± 36	78.6 ± 67	70.6 ± 25	91.2 ± 19	99.7 ± 32
	mRNA	5.15 ± 0.21	102.5 ± 16	88.6 ± 8	74.9 ± 10*	69.3 ± 5*
Fr cortex	Protein	1420 ± 130	96.5 ± 14	91.3 ± 9	106.2 ± 6	96.9 ± 8
	mRNA	0.91 ± 0.06	104.7 ± 3	83.9 ± 8	83.7 ± 14	77.4 ± 5*
Glob pallid	Protein	1220 ± 60	89.3 ± 4	72.6 ± 6*	80.9 ± 3*	84.5 ± 14
	mRNA	0.67 ± 0.15	122.1 ± 18	178.2 ± 33	106.2 ± 16	154.8 ± 38
Olf cortex	Protein	970 ± 49	95.8 ± 2	84.1 ± 6	86.9 ± 4	76.8 ± 5*
	mRNA	1.39 ± 0.36	146.8 ± 16	130.3 ± 13	175.7 ± 9	144.3 ± 8
Putamen	Protein	9900 ± 250	87.4 ± 9	78.7 ± 24	83.3 ± 6	77.5 ± 7
	mRNA	0.71 ± 0.02	97.1 ± 13	91.6 ± 4	104.8 ± 11	99.6 ± 11

Mean ± S.E.M. For both Western and Northern blots, each brain region: caudate, cerebellum, frontal cortex (fr cortex), globus pallidus (glob pallid.), olfactory cortex (olf cortex) and putamen was run in triplicate for each animal in each group ($n = 4$ monkeys per dose). Asterisk "*" denotes statistical significance ($P < 0.05$) compared to control levels.

[a] Control protein is expressed as OD and mRNA as ratio of mRNA/28S rRNA for each sample.

[b] Days of recovery after exposure cessation.

Table 5
Glutamate–aspartate transporter (GLAST) protein and mRNA data are expressed as percentage of control values, which are shown

Brain region	Analysis	Control[a]	15 days	33 days	65 day + 45[b]	65 day + 90[b]
Caudate	Protein	7000 ± 600	104.1 ± 19	100.7 ± 18	98.8 ± 18	113.3 ± 7
	mRNA	0.73 ± 0.17	91.5 ± 7	107.9 ± 22	115.6 ± 8	121.2 ± 16
Cerebellum	Protein	470 ± 50	87.7 ± 32	95.5 ± 19	73.3 ± 15	95.1 ± 50
	mRNA	4.70 ± 0.18	104.2 ± 17	104.8 ± 8	89.7 ± 16	74.6 ± 2*
Fr cortex	Protein	1200 ± 340	80.3 ± 19	83.2 ± 33	99.5 ± 19	89.4 ± 13
	mRNA	0.87 ± 0.06	103.8 ± 4	98 ± 13	99.7 ± 2	97.8 ± 4
Glob pallid	Protein	1200 ± 20	81.5 ± 6	71.7 ± 6*	75.9 ± 4*	66.5 ± 2*
	mRNA	0.53 ± 0.16	142.2 ± 11	196.8 ± 6*	133.3 ± 10	128.1 ± 12
Olf cortex	Protein	390 ± 40	117.1 ± 21	109.4 ± 11	85.8 ± 4	96.8 ± 12
	mRNA	1.60 ± 0.39	156.5 ± 14	132.5 ± 9	164.1 ± 8*	103.1 ± 8
Putamen	Protein	4000 ± 1000	104.5 ± 4	92.6 ± 7	95.3 ± 9	89.7 ± 15
	mRNA	0.79 ± 0.08	89.1 ± 9	108.2 ± 8	92.8 ± 9	87.4 ± 17

Mean ± S.E.M. For both Western and Northern blots, each brain region: caudate, cerebellum, frontal cortex (fr cortex), globus pallidus (glob pallid), olfactory cortex (olf cortex) and putamen was run in triplicate for each animal in each group ($n = 4$ monkeys per dose). Asterisk "*" denotes statistical significance ($P < 0.05$) compared to control levels.
[a] Control protein is expressed as OD and mRNA as ratio of mRNA/28S rRNA for each sample.
[b] Days of recovery after exposure cessation.

Table 6
Total GSH data are expressed as percentage of control values, which are shown

Brain region	Control (nmol/mg prot)	15 days	33 days	65 days + 45[a]	65 days + 90[a]
Caudate	2.98 ± 1.69	52.5 ± 32*	45.1 ± 41*	24.1 ± 34*	36.8 ± 63*
Cerebellum	12.04 ± 5.00	69.9 ± 39	69.5 ± 18	80.7 ± 21	86.7 ± 11
Fr cortex	8.25 ± 2.91	154.4 ± 28	240.8 ± 28*	173.4 ± 21*	132.6 ± 21
Putamen	16.88 ± 5.90	91.6 ± 33	226.9 ± 11*	182.6 ± 29*	179.8 ± 42*

Each brain region: caudate, cerebellum, frontal cortex (fr cortex), and putamen was run in triplicate for each animal in each group ($n = 4$ monkeys per dose). Asterisk "*" denotes statistical significance ($P < 0.05$) compared to control levels.
[a] Days of recovery after exposure cessation.

3. 7. TH protein and mRNA

TH protein levels were significantly decreased in the globus pallidus of monkeys exposed to Mn for 33 days as well as those undergoing 45 and 90 days recovery (Table 7). The group that recovered for 45 days also displayed decreased TH protein in the olfactory cortex, whereas the 90 days recovery group had decreased TH protein in the putamen (Table 7). Mn had very little effect on mRNA levels except in the globus pallidus of monkeys exposed for 33 days and recovered for 45 days. (Table 7)

Table 7
TH-protein and mRNA data are expressed as percentage of control values, which are shown

Brain region	Analysis	Control[a]	15 days	33 days	65 days + 45[b]	65 days + 90[b]
Caudate	Protein	760 ± 130	110.9 ± 2	103.8 ± 3	107.1 ± 7	105.3 ± 1
	mRNA	0.59 ± 0.08	103.3 ± 9	85.6 ± 7	87.1 ± 7	75.4 ± 10
Glob pallid	Protein	1100 ± 130	85.6 ± 7	74.1 ± 8*	74.5 ± 5*	67.8 ± 13*
	mRNA	0.39 ± 0.12	149.1 ± 22	192.3 ± 19*	169.8 ± 9*	144.9 ± 12
Olf cortex	Protein	780 ± 80	119.8 ± 15	108.8 ± 8	77.9 ± 4*	97.3 ± 10
	mRNA	0.87 ± 0.18	131.9 ± 15	131.7 ± 10	165.1 ± 11	148.1 ± 13
Putamen	Protein	450 ± 50	93.5 ± 2	94 ± 5	97.1 ± 2	82.1 ± 3*
	mRNA	0.37 ± 0.03	118.2 ± 6	110.6 ± 5	98.2 ± 10	108.6 ± 11

Mean ± S.E.M. Each brain region: caudate, globus pallidus (glob pallid), olfactory cortex (olf cortex) and putamen was run in triplicate for each animal in each group ($n = 4$ monkeys per dose).
Asterisk "*" denotes statistical significance ($P < 0.05$) compared to control levels.
[a] Control protein is expressed as OD and mRNA as ratio of mRNA/28S rRNA for each sample.
[b] Days of recovery after exposure cessation.

4. Discussion

Most human exposures remain below the current (RfC) inhalation reference concentration (0.05 μg Mn/m^3) for inhalable manganese set by the U.S. Environmental Protection Agency (Clayton et al., 1999; Loranger and Zayed, 1997; Pellizzari et al., 1999; Zayed et al., 1999). Average levels of manganese in ambient air are approximately 5 and 33 ng Mn/m^3 in nonurban and urban air, respectively (ATSDR, 2000) although air concentrations may be higher near ferromanganese or silicomanganese industries. The daily intake of manganese from the ambient air is estimated to be <2 μg Mn/day in the general population (Lynam et al., 1999; Zayed et al., 1999). The monkeys in this study were part of a larger study reported in Dorman et al. (2006a) in which they were exposed to a range of Mn exposure concentrations, the highest exceeding the RfC 30,000. This highest dose, 1.5 mg Mn/m^3 was used in this study in order to keep the number of monkeys used to a minimum, yet allowing us to effectively evaluate the duration of exposure on the markers of neurotoxicity. It should be noted that we did not select this dose to mimic a human exposure paradigm, but to ensure Mn accumulation in the brain regions at shorter duration periods.

Dorman et al. (2006a,b) observed increased brain Mn concentrations in these monkeys and reported it elsewhere. In the six brain regions studied herein, all showed significant accumulation of Mn following 15 and 33 days of exposure (Dorman et al., 2006a). In monkeys allowed to recover from Mn exposure for 45 and 90 days, all of these brain regions (except frontal cortex) had elevated Mn levels (40–120% above control values) (Table 1). It should be noted though that increased brain Mn concentrations in half of the brain regions of the group allowed to recover for 45 days and all of the brain regions of the 90 days recovery group were not statistically different from the control group; but, the elevated Mn may be biologically relevant for several biomarkers remained altered even after 45 and 90 days of recovery (e.g., GS protein).

Within the brain, GS is exclusively expressed in astrocytes (Martinez-Hernandez et al., 1977). A Mn-dependent enzyme, GS catalyzes the formation of glutamine from glutamate. Glutamine is taken up by local glutamatergic or γ-aminobutyric acid-containing (GABAergic) neurons where deamination to glutamate occurs. This process is considered the primary glutamate-recycling pathway in the brain (Van den Berg and Garfinkel, 1971; Westergaard et al., 1995; Ottersen et al., 1992). Inhibition of GS activity can have serious consequences on neuronal functioning (e.g., the inability to detoxify ammonia). GS serves as an excellent marker for the presence of ROS in the brain, because it is highly susceptible to oxidation and subsequent rapid degradation (Stadtman, 1992). GS protein levels were decreased in most of the brain regions of monkeys exposed to the high concentration of airborne Mn which persisted even after recovery (e.g., cerebellum, frontal cortex, globus pallidus and putamen) (Table 2). These data corroborate our other recent monkey study (Erikson et al., 2007), where we found that at a lower dose of airborne Mn (0.3 mg Mn/mm^3) globus pallidus, cerebellum and frontal cortex all displayed decreased GS protein and unchanged GS mRNA levels. Taken together, we speculate that the loss of protein is likely due to increased degradation caused by increased oxidation and not by decreased synthesis because mRNA levels were relatively unaffected. We would like to point out that since GS is found exclusively in astrocytes and that we used dissected tissue containing several cell types (e.g., neurons and several glial cells), that some of our heterogeneous findings may be due to the cellular diversity of the tissue.

The MTs, a class of cysteine-containing intracellular metal-binding proteins, are highly conserved and widely distributed throughout all cells in an organism. The MT act as antioxidants by neutralizing ROS both systemically and in the brain. In situ hybridization studies demonstrate that bacterial endotoxin induces MT gene expression (Itano et al., 1991). Oxidative stress, kainic acid, and 6-hydroxydopamine, a known dopaminergic toxin and ROS generator, induced MT-I gene expression in the brain (Shiraga et al., 1993). Likewise, compounds that generate free oxygen species via redox cycling (e.g., diquat), along with compounds that cause lipid peroxidation (e.g., 3- methylindole) or that deplete cellular defense mechanisms (e.g., diamide and dimethyl maleate) increase tissue MT expression (Bauman et al., 1991). The effect of inhaled Mn on rat brain MT gene expression have been quite varied, that is brain regions that avidly acquire Mn during airborne exposure showed decreased as well as increased MT gene expression (Dobson et al., 2003; Erikson et al., 2005). In primary astrocyte cultures, overnight exposure to 250 and 500 μM $MnCl_2$ led to a significant decrease in MT mRNA levels. Interestingly, the caudate of monkeys exposed for 33 days to 1.5 mg Mn/m^3 had a significant

decrease in MT mRNA levels which remained after the monkeys recovered from Mn exposure for 45 and 90 days of Mn-exposed monkeys compared to controls. In the globus pallidus and olfactory cortex, Mn exposure caused increased MT expression albeit not statistically significant due to small sample size and large error (note: olfactory cortex of 33 days exposure group was significant). Finally, in the putamen of the exposed monkeys, there was no significant alteration in MT gene expression due to Mn inhalation, corroborating our previous findings (Erikson et al., 2007) that even though brain regions accumulate Mn to a similar degree, the effects on biomarkers of neurotoxicity vary greatly.

Glutamate excitotoxicity has been implicated as a triggering event for manganism. Glutamate levels have been shown to be elevated in the basal ganglia of Mn-exposed rats (Garcia et al., 2006; Erikson et al., 2002a). Astrocytes are the primary cells in the brain which dictate glutamate metabolism. In fact, the mechanism by which the brain handles ammonia is related to this role. Specifically, astrocytes clear glutamate from the extracellular space with GLT- 1 and/or GLAST. Upon intra-cellular transport, glutamate is aminated to glutamine via GS (see Danbolt, 2001 for comprehensive review). Glutamine can then be transported to neurons where it can undergo deamination forming glutamate for use as a neurotransmitter or protein synthesis. This glutamate-recycling pathway is critically dependent upon normally functioning astrocytic GLT-1 and GLAST proteins.

Attenuated glutamate uptake in Mn-exposed astrocytes has been reported by several groups (Hazell and Norenberg, 1997; Erikson et al., 2002b). Altered glutamate uptake has been linked to Mn dose-dependent decreases in GLAST expression (Erikson et al., 2002a). GLT-1 has also been shown to be affected by Mn exposure as well, (Mutkus et al., 2005) indicating another possible mechanism for Mn-induced alterations in glutamate uptake. Overall, GLAST protein and gene expression were relatively unaffected in the six brain regions that we analyzed, with the exception of globus pallidus which had significantly increased mRNA and decreased protein levels in the monkeys exposed for 33 days and decreased protein in those allowed to recover for 45 and 90 days (Table 4). In contrast, GLT- 1 was more broadly (i.e., more brain regions) affected by high dose Mn exposure. This regional effect on mRNA expression levels was observed in the monkeys that were repeatedly exposed (15 and 33 exposure days) to Mn, returning to normal levels after Mn exposure ended and the monkeys were allowed to recover. It is worth mentioning that while glutamate levels were not measured in these animals, similarly treated monkeys were found to have unaltered brain regional glutamate concentrations due to Mn exposure (Struve et al., 2007), suggesting that altered transporter levels is not affecting glutamate concentrations.

Glutathione (GSH) is a ubiquitous antioxidant formed from three amino acids, glutamate, cysteine and glycine, leading to formation of γ-glutamylcysteinylglycine. Alterations in brain GSH metabolism have been linked with oxidative stress and various neurodegenerative diseases including PD (Gegg et al., 2003). Sian et al. (1994) demonstrated decreased GSH levels in the substantia nigra of PD patients compared to presumed normal individuals. Correspondingly, GSH levels are significantly and age-dependently lowered in the striatum of Mn-exposed rats (i.e., older rats have dramatically lower GSH due to Mn exposure, whereas juvenile rats are unaffected) (Desole et al., 1995; Erikson et al., 2004).

This study showed that in two regions of the basal ganglia, similar increases in Mn concentrations led to opposite responses in GSH metabolism. Specifically, monkeys exposed to Mn for 15 or 33 days had significantly lower levels of total GSH in the caudate compared to controls (Table 5). This significant decrease remained even after 45 and 90 days of recovery from Mn exposure. In contrast, Mn exposure led to a significant increase in total GSH in the putamen where it stayed elevated even 90 days after cessation of Mn exposure. To our knowledge, this is the first report of regional differences in GSH levels within the basal ganglia of Mn-exposed monkeys, for our previous study in which monkeys were exposed to lower doses of airborne Mn yielded no effect on GSH levels (Erikson et al., 2007).

Tyrosine hydroxylase is the rate-limiting enzyme for catecholamine synthesis, and is often used as a marker for dopaminergic neurons. We sought to specifically examine the effects of airborne Mn on TH protein and mRNA

levels. Manganese exposure (33 days) decreased TH protein levels and increased TH mRNA levels in the globus pallidus; these alterations remained after 45 and 90 days post-exposure (Table 6). Globus pallidus is not known as a dopamine-rich region, however dopaminergic projections exist (Porritt et al., 2000). We were surprised that TH levels in neither the caudate nor putamen were affected by Mn exposure given that our previous study found that airborne-Mn exposure led to decreased TH levels in caudate, putamen and globus pallidus (Erikson et al., 2007). However, our prior study utilized a longer exposure period, so it appears that during a shorter exposure period to airborne-Mn, the brain region that is most susceptible to Mn accumulation (globus pallidus) is the brain region where we observe a change in TH levels.

In conclusion, non-human primates exposed to airborne Mn respond both similarly and differently in terms of alterations in biomarkers of neurotoxicity across brain regions when compared to rats. Both species accumulate Mn across brain regions in a dose-dependent manner and both species display the ability to normalize Mn levels after cessation of airborne-Mn exposure (Dorman et al., 2005, 2006a). Similarly, both rats and monkeys exposed to airborne Mn show significant alterations in GSH levels particularly in the striatum (Erikson et al., 2004, 2005, 2006) (Table 6), an area known to exhibit heightened sensitivity to Mn. There was a heterogeneous response to Mn exposure and cessation, in that some of these alterations were reversible upon cessation of manganese exposure (GLT- 1 and TH protein levels in caudate, Tables 4 and 7, respectively) while others were not (MT mRNA and GSH levels in caudate, Tables 3 and 6, respectively). This varied response to Mn cessation displayed within the caudate may be due to the assortment of cells represented in our use of dissected brain regions and that we may have observed more homogeneous results if we had used isolated cell cultures or tissue slices. Finally, an overall finding from both of our monkey studies is that when the duration of Mn exposure is 65 days (Erikson et al., 2007) several brain regions display alterations in biomarkers of neurotoxicity; whereas after 15–33 days exposure most of the outcomes we measured were altered primarily in the globus pallidus, putamen and caudate emphasizing the increased sensitivity to Mn inherent to these brain regions.

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