Manganese Inhalation by Rhesus Monkeys is Associated with Brain Regional Changes in Biomarkers of Neurotoxicity

By: Keith M. Erikson, David C. Dorman, Lawrence H. Lash, and Michael Aschner

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Abstract:

The purpose of this study was to evaluate biochemical markers of neurotoxicity following subchronic manganese sulfate ($MnSO_4$) inhalation. Juvenile rhesus monkeys were exposed to $MnSO_4$ at 0, 0.06, 0.3, or 1.5 mg Mn/m^3 for 65 days. Glutamine synthetase (GS), glutamate transporters (glutamate transporter-1 [GLT-1] and glutamate/aspartate transporter [GLAST]) and tyrosine hydroxylase (TH) protein levels, metallothionein (MT), GLT-1, GLAST, TH and GS mRNA levels, and total glutathione (GSH) levels were assessed in known targets (caudate, globus pallidus, putamen) as well as the cerebellum, frontal cortex, and olfactory cortex. All $MnSO_4$-exposed monkeys had decreased pallidal GS protein, decreased caudate GLT-1 mRNA, decreased pallidal GLAST protein, and increased olfactory cortical TH mRNA levels. Monkeys exposed to $MnSO_4$ at 0.06 or 0.3 mg Mn/m^3 had significantly increased pallidal mRNA levels for GLT-1, GLAST, and TH. Monkeys exposed to $MnSO_4$ at $\geq$ 0.3 mg Mn/m^3 had several alterations including decreased frontal cortical MT mRNA, decreased caudate, globus pallidus, olfactory cortex, and cerebellum GLT-1 protein, decreased olfactory cortex and cerebellum GLAST protein, increased cerebellar GLAST mRNA, and decreased pallidal TH protein levels. Lastly, GSH levels were significantly increased in the frontal cortex and decreased in the caudate of monkeys exposed to the 1.5-mg Mn/m^3 compared to the controls. Overall, as in our previous studies, we observed that increased Mn concentrations due to airborne Mn exposure differentially affects biomarkers in each brain region (e.g., GSH was increased in the frontal cortex and decreased in the caudate despite two- to threefold increases in Mn concentrations in these regions).

Key Words: monkey; manganese; brain; glutamate transporters; glutathione; glutamine synthetase.

Article:

Over the last 5 years, our group has reported brain manganese (Mn) concentrations and responses of several markers of oxidative stress due to Mn exposure in rats. Our prior studies assessed neonatal rats (following combined *in utero* and neonatal Mn exposure), young adult male and female rats, as well as senescent male rats exposed to Mn by inhalation (Dobson et al., 2003; Erikson et al., 2004, 2005, 2006; Weber et al., 2002). But until this study, similar relation-ships between airborne Mn exposure, regional brain Mn concentrations, and brain responses of markers of oxidative stress in nonhuman primates have been largely unexplored. This current study examined the dose-response relationship between sub-chronic Mn inhalation and several markers of oxidative stress and excitotoxicity in the nonhuman primate brain.

While Mn is an essential trace metal necessary for normal cellular functioning (Hurley and Keen, 1987), excessive Mn accumulation in the human central nervous system (CNS) can lead to dysfunction, resulting in development of a neurotoxic syndrome (manganism) that resembles 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). It is speculated that chronic low-level Mn exposure may play a role in the pathogenesis of neurodegenerative disorders, especially in susceptible populations (Aschner et al., 2005).

Oxidative stress has been implicated as a contributing mechanism by which Mn mediates its cytotoxic effects (Aschner, 1997; Taylor et al., 2006). It has been proposed that Mn, through its sequestration in mitochondria (Chen et al., 2001; Galvani et al., 1995), interferes with proper respiration thereby leading to excessive production of reactive oxygen species (ROS). One laboratory reported inhibition of complex I of the electron transport chain after treatment of PC12 cell cultures with $MnCl_2$ (Brouillet et al., 1993). Another laboratory showed evidence suggesting that the ATPase complex is inhibited at very low levels of mitochondrial Mn and that complex I is inhibited at higher concentrations (Gavin et al., 1999).

It has been shown that ROS will interfere with glutamate removal by inhibiting the high-affinity glutamate transporters (Trotti et al., 1998). The ensuing increase in extracellular glutamate levels is excitotoxic to neurons, representing a possible mechanism for Mn neurotoxicity. While there are several glutamate transporters inherent to optimal neuronal function, GLAST (glutamate/aspartate transporter) and glutamate transporter-1 (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 (Erikson et al., 2002b; Hazell and Norenberg, 1997), and GLAST gene expression is significantly decreased due to 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. Such information is critical for more fully evaluating the neurotoxicity of inhaled Mn.

In our previous inhalation studies, we indirectly assessed oxidative stress by measuring levels of the antioxidants glutathione (GSH) and metallothionein (MT), as well as glutamine synthetase (GS), a protein that is also exquisitely sensitive to oxidative stress. In the present study, we evaluated the effects of Mn exposure on MT, glutamate transporters (GLT- 1 and GLAST), tyrosine hydroxylase (TH), and GS. This study extends our previous observations (Dobson et al., 2003; Erikson et al., 2004, 2005, 2006) by examining the aforementioned endpoints of oxidative stress and cytotoxicity in select brain regions of rhesus monkeys following Mn inhalation.

MATERIALS AND METHODS

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

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 (CIIT) Centers for Health Research (CIIT) Institutional Animal Care and Use Committee. Twenty male rhesus monkeys purchased from Covance Research Products, Inc. (Alice, TX) were used for this phase of the study. Additional endpoints evaluated in this study, but not presented herein, included magnetic resonance imaging of the brain (Dorman et al., 2006b), postexposure clinical chemistry, hematological evaluations, determination of tissue Mn concentrations in the CNS and other organs (Dorman et al., 2006a), and histological evaluation of the respiratory tract (Dorman et al., 2005). 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 (no. 5048) diet from Purina Mills (St Louis, MO) was fed b.i.d. at approximately 2% of the animal's body weight. The diet contained approximately 133 mg Mn/kg diet, and it was calculated that the monkeys were ingesting around 6 mg Mn/kg/day. During nonexposure 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) that were designed to fit within the 8-m3 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 has been published (Dorman et al., 2005).

Experimental design. The in-life portion of this study was performed in accordance with the U.S. Environmental Protection Agency's (USEPA) Good Laboratory Practice Standards for Inhalation Exposure Health Effects Testing (40 CFR Part 79.60). $MnSO_4$ aerosol concentrations of 0.18, 0.92, and 4.62 mg $MnSO_4/m^3$, corresponding to 0.06, 0.3, and 1. 5 mg Mn/m^3, were 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 air (n = 6) or $MnSO_4$ at 0.06 (n = 6), 0.3 (n = 4), or 1.5 mg Mn/m^3 (n = 4) for 65 exposure days/90 total days.

Mn exposures. 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 have been previously described (Dorman et al., 2004, 2005).

Tissue collection. 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, iv, Henry Schein Inc., Port Washington, NY) followed by exsanguinations. Following euthanasia, the brains were removed and divided on the midsagittal 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. Whenever possible, these samples included both white and gray matter areas. All samples were stored in individual plastic vials or bags, frozen in liquid nitrogen, and stored at approximately — 80 °C until chemical analyses were performed.

RNA and protein extraction from brain tissues. Each brain region was treated so that we were able to extract RNA and protein from it. Briefly, the tissue samples had a monophase phenol and guanidine isothiocyanate solution (RNA STAT-60, Tel-Test, Inc., Friendswood, TX) added (1:10 wt:vol 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 sample was centrifuged at 12,000 x g for 10 min. The supernatant was removed and the remaining pellet was resuspended in WANG buffer (25mM N-2-hydroxyethylpiperazine-N′-2- ethanesulfonic acid, pH 7.0, 250mM sucrose, 100uM EDTA, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 1mM dithiothreitol [DTT], 0.2% Triton-X 100). Following sonication of the samples, protein content was determined using the bicinchoninic acid method (Pierce, IL).

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 standard saline citrate (SSC) (1 x SSC = 0.15M sodium chloride, 0.015M 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 100 g/ml denatured salmon sperm DNA, and 20mM 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 overnight as follows: an 800 base pair sequence of rat GS cDNA (a generous gift from Dr Steve Abcouwer, Harvard Medical School, Boston, MA) was used as a template to create an [a^{32}P]-dCTP–labeled probe by random priming (RadPrime DNA Labeling System, Life Technologies, Gaithersburg, MD). GLT-1/GLAST cDNAwas used as a template to create an [a^{32}P]-dCTP–labeled cDNA probe by random priming (RadPrime DNA labeling System, Life Technologies). GLT-1/GLAST cDNAwas kindly provided by Dr Olga Zelenaia (University of Pennsylvannia). The MT-I gene was cloned into the Pst1 site of pBR322. The plasmids were transfected into Escherichia Coli JM109 cells and grown in tetracycline-resistant Luria-Bertani (LB) medium. The cDNA was excised from the plasmid by restriction endonucleases (Pst1). The size of the insert was 450 base pairs. The MT-II gene was cloned into the BamHI/EcoRI site of pUC 13. The plasmids were transfected into E. Coli JM109 cells and grown in ampicillin-resistant LB medium. The cDNA was excised

from the plasmid by restriction endonucleases (BamHI/EcoRI). The size of the insert was 280 base pairs. The cDNA was run on a 2% agarose gel and purified by binding to diethylaminoethyl membranes (Schleicher & Schuell, NA 45). The probes were [a^{32}P]-dCTP labeled with a random primed DNA labeling kit. TH-^{32}P–labeled TH-specific cDNA probe was used. This is a full-length (1.8 kb) clone isolated from a rat pheochromocytoma. The TH insert was radiolabeled using a commercial nick-translation kit (BRL, Gaithersburg, MD) and [a^{32}P]-dCTP (Amersham Corp., Arlington Heights, IL).

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 3 SSC/0.1% SDS/40mM Tris buffer and probed for 28s rRNA (Barbu and Dautry, 1989).

Western blot analysis. Aliquots of protein (100 mg) were mixed with 5X sample buffer (0.25M Tris, pH 6.8, 10% SDS, 50% glycerol) and 1M DTT, and separated by denaturing SDS-polyacrylamide gel electrophoresis using 5% stacking, and 8% resolving acrylamide gels. Following fractionation, proteins were electrophoretically transferred to a nitrocellulose membrane (Protran BA83, Schleicher and Schuell) in 20% methanol, 0. 1 % SDS, 25mM Tris, and 192mM glycine for 3 h at 60 V. Membranes were then blocked with 5% nonfat powdered milk in TBST (Tris-buffered saline with 0.1% Tween 20, 150mM NaCl, 20mM 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 horseradish peroxidase (HRP)–conjugated anti-rabbit secondary antibody (1:5000). Glutamate transporter (GLAST) protein expression was detected with a rat monoclonal antibody (Alpha Diagnostic International) 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 HRP-conjugated goat anti-mouse (GS protein) or HRP-conjugated goat anti-rabbit (TH protein) secondary antibodies diluted 1:2000 (Kirkegaard and Perry Laboratories, Gaithersburg, 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 (Raytest USA).

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% (vol/vol) perchloric acid containing 1mM bathophenanthroline disulfonic acid and L-γ-glutamyl-L-glutamate. The mixture was vortexed and centrifuged, an aliquot was then removed for high-performance liquid chromatography 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).

Statistical analysis. The data were analyzed using the SPSS system v14.0 (SPSS Inc., Chicago, IL). ANOVA with repeated measures factors (brain regions) and between-groups factors was used to test for interactions between Mn exposure concentration and 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. The statistical analyses were performed on the raw data (optical density) and then converted to percent control for its presentation.

RESULTS

Mn Concentrations

Brain regional Mn concentrations are reported in Dorman et al. (2006a). The following concentrations were measured in control (air-exposed) monkeys: cerebellum = 0.44 mg; caudate = 0.34 mg; frontal cortex = 0.25 mg; globus pallidus = 0.48 mg; olfactory cortex = 0.19 mg; putamen = 0.36 mg. When compared to the air-exposed monkeys, those that were exposed to ≥ 0.06 mg Mn/m^3 experienced significant elevations in Mn concentration in cerebellum (40% higher), globus pallidus (100% higher), olfactory cortex (50% higher), and putamen (60% higher), while increased caudate (75% higher) and frontal cortex (90% higher) Mn concentrations occurred following subchronic exposure to ≥ 0.3 mg Mn/m^3 and 1.5 mg Mn/m^3, respectively. It should be noted that at the highest dose most regions accumulated Mn in a similar fashion (i.e., twofold higher concentration compared to normal) except for the globus pallidus where there was almost a sixfold increase in Mn concentration due to exposure to the 1.5-mg Mn/m^3 dose.

GS Protein and mRNA

The gene expression of GS was relatively unaffected by Mn exposure except in the frontal cortex and olfactory cortex of monkeys exposed to 1.5 mg Mn/m^3 and in the caudate of monkeys exposed to $MnSO_4$ at 0.06 mg Mn/m^3 (Table 1). All $MnSO_4$-exposed animals showed decreased GS protein levels in the globus pallidus. The protein levels of GS were altered in the cerebellum and frontal cortex of monkeys exposed to $MnSO_4$ at 0.3 mg Mn/m^3. The animals exposed to 1.5 mg Mn/m^3 showed altered GS protein in the cerebellum and putamen.

TABLE 1
GS Protein and mRNA Levels Following Subchronic $MnSO_4$ Inhalation. Data Are Expressed as Percentage of Reported Control Values. Mean ± SEM

Brain region	Analysis	Control	0.06 mg/mm^3	0.3 mg/mm^3	1.5 mg/mm^3
Caudate	Protein[a]	6645 ± 656	110.7 ± 9	95.7 ± 13	82.2 ± 7
	mRNA[b]	0.262 ± 0.04	68.3 ± 17*	81.6 ± 21	106.1 ± 10
Cerebellum	Protein	675 ± 41	98.2 ± 4	81.9 ± 8*	84.9 ± 7*
	mRNA	5.38 ± 0.42	107.8 ± 4	103.6 ± 4	110.2 ± 8
Frontal cortex	Protein	1492 ± 145	72.1 ± 15	68.4 ± 29*	92.6 ± 21
	mRNA	0.907 ± 0.05	100.4 ± 6	95.7 ± 3	62.5 ± 4*
Globus pallidus	Protein	1329 ± 232	68.8 ± 6*	66.3 ± 17*	55.8 ± 7*
	mRNA	1.006 ± 0.18	123.2 ± 9	97.3 ± 9	85.5 ± 6
Olfactory cortex	Protein	582 ± 36	97.5 ± 5	105.45 ± 8	88.2 ± 2
	mRNA	0.491 ± 0.06	100.7 ± 4	132.1 ± 15	175.6 ± 5*
Putamen	Protein	6487 ± 192	103.3 ± 12	106.8 ± 4	119.8 ± 6*
	mRNA	0.696 ± 0.11	85.8 ± 13	111.9 ± 8	107.6 ± 11

Note. For both Western and northern blots, each brain region was run in triplicate for each animal in each group ($n = 6$ monkeys in the control and 0.06 mg/mm^3 groups and $n = 4$ in the remaining groups).

[a]Protein is reported as optical density.

[b]mRNA is reported as the ratio of mRNA to 28s.

*Denotes statistical significance ($p < 0.05$) compared to control levels.

MT mRNA

The caudate from monkeys exposed to the median dose of Mn (Table 2) showed reduced MT mRNA (vs. air-exposed controls). The cortex from monkeys exposed to ≥ 0.3 mg Mn/ m^3 showed decreased MT mRNA. Finally, the cerebellum, globus pallidus, and olfactory cortex showed altered MT mRNA in monkeys exposed to 1.5 mg Mn/m^3.

TABLE 2
MT mRNA Levels in Monkeys Exposed to $MnSO_4$. Data Are Expressed as Percentage of Reported Control Values. Mean ± SEM

Brain region	Control[a]	0.06 mg/mm^3	0.3 mg/mm^3	1.5 mg/mm^3
Caudate	1.016 ± 0.13	93.7 ± 5	83.8 ± 7*	87.7 ± 12
Cerebellum	6.924 ± 0.56	97.2 ± 12	88.2 ± 3	83.5 ± 7*
Frontal cortex	0.928 ± 0.04	92.3 ± 9	84.9 ± 4*	87.5 ± 6*
Globus pallidus	0.849 ± 0.19	146.4 ± 13	86.6 ± 20	76.4 ± 6*
Olfactory cortex	0.534 ± 0.08	124.2 ± 18	134.1 ± 22	216.1 ± 23*
Putamen	1.007 ± 0.09	85.7 ± 6	91.7 ± 10	96.2 ± 16

Note. For northern blots, each brain region was run in triplicate for each animal in each group ($n = 6$ monkeys in the control and 0.06 mg/mm^3 groups and $n = 4$ in the remaining groups).
[a]mRNA is reported as the ratio of mRNA to 28s.
*Denotes statistical significance ($p < 0.05$) compared to control levels.

GLT-1 Protein and mRNA

GLT- 1 mRNA was significantly decreased in the caudate of monkeys exposed to ≥ 0.06 mg Mn/m^3 (Table 3). GLT- 1 mRNA was also significantly decreased in the globus pallidus and olfactory cortex of monkeys exposed to 0.06 or 0.3 mg Mn/m^3.

TABLE 3
GLT-1 Protein and mRNA in Monkeys Following Mn Inhalation. Data Are Expressed as Percentage of Reported Control Values. Mean ± SEM

Brain region	Analysis	Control	0.06 mg/mm^3	0.3 mg/mm^3	1.5 mg/mm^3
Caudate	Protein[a]	1543 ± 63	88.6 ± 9	81.8 ± 10*	76.1 ± 6*
	mRNA[b]	0.874 ± 0.03	88.3 ± 5*	75 ± 10*	84.1 ± 5*
Cerebellum	Protein	415 ± 36	87.7 ± 13	68.3 ± 10*	71.1 ± 14*
	mRNA	5.147 ± 0.21	97.7 ± 8	95.6 ± 4	95 ± 6
Frontal cortex	Protein	1422 ± 128	103 ± 10	88.3 ± 19	88 ± 12
	mRNA	0.908 ± 0.06	97.2 ± 5	95.3 ± 11	100.4 ± 2
Globus pallidus	Protein	1224 ± 60	93.3 ± 2	85.2 ± 1*	78.4 ± 2*
	mRNA	0.669 ± 0.15	153.2 ± 10*	143.3 ± 15*	108.1 ± 16
Olfactory cortex	Protein	967 ± 49	97.5 ± 1	86.1 ± 6*	86.6 ± 2*
	mRNA	1.392 ± 0.36	138.9 ± 5*	124.6 ± 2*	113.2 ± 5
Putamen	Protein	9944 ± 245	101 ± 4	102 ± 3	103 ± 2
	mRNA	0.713 ± 0.02	94.6 ± 5	89.9 ± 9	100.7 ± 6

Note. For both Western and northern blots, each brain region was run in triplicate for each animal in each group ($n = 6$ monkeys in the control and 0.06 mg/mm^3 groups and $n = 4$ in the remaining groups).
[a]Protein is reported as optical density.
[b]mRNA is reported as the ratio of mRNA to 28s.
*Denotes statistical significance ($p < 0.05$) compared to control levels.

GLT-1 protein levels were decreased in the caudate, globus pallidus, cerebellum, and olfactory cortex of monkeys exposed to ≥ 0.3 mg Mn/m^3 (Table 3).

GLAST Protein and mRNA

The globus pallidus of monkeys exposed to > 0.06 mg Mn/m^3 had significantly reduced protein levels of GLAST compared to air-exposed controls (Table 4). GLAST mRNA was also significantly increased in the globus pallidus of monkeys exposed to $MnSO_4$ at 0.06 or 0.3 mg Mn/m^3 , and a similar trend was seen in animals from the highest exposure group. The cerebellum and olfactory cortex had significantly reduced protein

levels of GLAST in monkeys exposed to ≥ 0.3 mg Mn/m^3 (Table 4). These same monkeys displayed increased cerebellar GLAST mRNA levels. The frontal cortex had significantly reduced protein levels of GLAST in monkeys exposed to 1.5 mg Mn/m^3.

TABLE 4
GLAST Protein and mRNA Data in Mn-Exposed Monkeys Are Expressed as Percentage of Reported Control Values. Mean ± SEM

Brain region	Analysis	Control	0.06 mg/mm^3	0.3 mg/mm^3	1.5 mg/mm^3
Caudate	Protein[a]	6967 ± 640	103.8 ± 15	106.7 ± 9	97.5 ± 12
	mRNA[b]	0.734 ± 0.17	85.2 ± 6	82.6 ± 9	95.2 ± 11
Cerebellum	Protein	468 ± 48	89.9 ± 8	70.2 ± 9*	71.3 ± 6*
	mRNA	4.701 ± 0.18	106.1 ± 3	111.9 ± 4*	111.3 ± 4*
Frontal cortex	Protein	1211 ± 343	88.3 ± 31	72.2 ± 26	65.5 ± 13*
	mRNA	0.873 ± 0.06	101.1 ± 7	93.3 ± 2	92.7 ± 3
Globus pallidus	Protein	1198 ± 16	76.3 ± 6*	78.6 ± 1*	70.8 ± 2*
	mRNA	0.530 ± 0.16	171.8 ± 8*	186.8 ± 6*	140.2 ± 16
Olfactory cortex	Protein	389 ± 38	87.7 ± 4	69.5 ± 8*	67.7 ± 10*
	mRNA	1.601 ± 0.39	126.3 ± 5	108.1 ± 2	104.1 ± 4
Putamen	Protein	3987 ± 999	91.3 ± 12	112.1 ± 27	106.5 ± 28
	mRNA	0.786 ± 0.08	94.7 ± 8	93.1 ± 10	94.8 ± 10

Note. For both Western and northern blots, each brain region was run in triplicate for each animal in each group ($n = 6$ monkeys in the control and 0.06 mg/mm^3 groups and $n = 4$ in the remaining groups).
[a]Protein is reported as optical density.
[b]mRNA is reported as the ratio of mRNA to 28s.
*Denotes statistical significance ($p < 0.05$) compared to control levels.

Total GSH

Monkeys exposed to 1.5 mg Mn/m^3 had elevated GSH levels in the frontal cortex ($p < 0.05$) (Table 5). There was also a near statistically significant ($p = 0.078$) decrease in putamen GSH levels in these animals. There was no statistically significant alteration in GSH levels in monkeys exposed to $MnSO_4$ at ≤ 0.3 mg Mn/m^3 . However, there was a statistical trend ($p = 0.071$) toward a significant decrease in caudate GSH levels of monkeys exposed to $MnSO_4$ at either 0.06 or 0.3 mg Mn/m^3.

TABLE 5
Total GSH Levels Following Mn Inhalation. Data Are Expressed as Percentage of Reported Control Values. Mean ± SEM

Brain region	Control (nmol/mg prot)	0.06 mg/mm^3	0.3 mg/mm^3	1.5 mg/mm^3
Caudate[a]	2.98 ± 1.69	48.2 ± 49	42.2 ± 34	64.6 ± 34
Cerebellum	12.04 ± 5.0	99.6 ± 13	115.2 ± 11	86.4 ± 46
Frontal cortex	8.25 ± 2.91	78.5 ± 30	83.9 ± 13	215.3 ± 32*
Putamen[a]	16.88 ± 5.9	143.3 ± 36	133.3 ± 23	69.5 ± 21

Note. Each brain region was run in triplicate for each animal in each group ($n = 6$ monkeys in the control and 0.06 mg/mm^3 groups and $n = 4$ in the remaining groups).
[a]Statistical trend ($p = 0.0701$) in caudate and ($p = 0.078$) in putamen of monkeys exposed to the low and median doses.
*Denotes statistical significance ($p < 0.05$) compared to control levels.

TH Protein and mRNA

TH gene expression was significantly increased in the olfactory cortex of monkeys exposed to $MnSO_4$ at ≥ 0.06 mg Mn/m^3 (Table 6). Gene expression was also significantly increased in the globus pallidus of monkeys exposed to $MnSO_4$ at either 0.06 or 0.3 mg Mn/m^3. TH protein levels were significantly decreased in the globus

pallidus of monkeys exposed to ≥ 0.3 mg Mn/m^3, while putamen TH protein levels were significantly decreased in monkeys exposed to 0.3 mg Mn/m^3 (Table 6). Caudate TH protein levels were significantly decreased in monkeys exposed to $MnSO_4$ at 1.5 mg Mn/m^3.

TABLE 6
TH Protein and mRNA Levels Following Subchronic Mn Inhalation. Data Are Expressed as Percentage of Reported Control Values. Mean ± SEM

Brain region	Analysis	Control	0.06 mg/mm^3	0.3 mg/mm^3	1.5 mg/mm^3
Caudate	Protein[a]	759 ± 126	86.9 ± 6	68.4 ± 13	66.1 ± 6*
	mRNA[b]	0.591 ± 0.08	94.5 ± 6	90.7 ± 6	93.5 ± 7
Globus pallidus	Protein	1093 ± 126	76.6 ± 7	66.1 ± 9*	63.9 ± 6*
	mRNA	0.389 ± 0.12	154.88 ± 8*	168.4 ± 4*	121 ± 21
Olfactory cortex	Protein	776 ± 79	107.1 ± 4	111.6 ± 9	118.5 ± 4
	mRNA	0.872 ± 0.18	152.5 ± 8*	134.2 ± 6*	151.4 ± 9*
Putamen	Protein	447 ± 53	91.5 ± 6	79.4 ± 4*	86.9 ± 5
	mRNA	0.372 ± 0.03	85.8 ± 5	111.1 ± 5	107.5 ± 6

For both Western and northern blots, each brain region was run in triplicate for each animal in each group ($n = 6$ monkeys in the control and 0.06 mg/mm^3 groups and $n = 4$ in the remaining groups).
[a]Protein is reported as optical density.
[b]mRNA is reported as the ratio of mRNA to 28s.
*Denotes statistical significance ($p < 0.05$) compared to control levels.

DISCUSSION

The current inhalation reference concentration for Mn, as set by the United States Environmental Protection Agency (U.S. EPA), is 0.05 μg Mn/m^3 (U.S. EPA, 1993). Thus, the concentrations of Mn used in this study were 1000, 6000, and 30,000 times higher than this standard for the low, median, and high exposure concentrations, respectively. However, the doses that we used in this study are well within the doses measured during extreme occupational exposures (> 1–5 mg Mn/m^3) (Aschner et al., 2005). Dorman et al. (2006a,b) previously reported increased brain Mn concentrations for the monkeys used in the present study. Each of the six brain regions studied herein showed significant accumulation of Mn at all three exposure doses, except for the caudate and frontal cortex, which only accumulated Mn at levels significantly higher than controls following exposure to ≥ 0.3 mg Mn/m^3 and 1.5 mg Mn/m^3, respectively (Dorman et al., 2006a). Furthermore, at the highest dose, all of the brain regions experienced a two- to threefold increase in Mn concentration except for the globus pallidus which showed a sixfold increase in Mn concentration compared to control.

The MTs, a class of cysteine-containing intracellular proteins, are highly conserved and widely distributed through-out all cells in an organism. Evidence suggests that MT acts as an antioxidant 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 deplete cellular defense mechanisms (e.g., diamide and dimethyl maleate), increase

MT in tissue (Bauman et al., 1991). Results from our prior rodent studies that examined MT gene expression in brain tissues from Mn-exposed rats have been quite varied. That is, brain regions that avidly acquire Mn following Mn inhalation showed decreased as well as increased MT gene expression (Dobson et al., 2004; Erikson et al., 2005). In primary astrocyte cultures, overnight exposure to 250 or 500mM $MnCl_2$ led to a significant decrease in MT mRNA levels (Erikson et al., 2002b). In the present study, monkeys exposed to 0.3 mg Mn/m^3 had decreased MT mRNA in the frontal cortex and caudate, whereas animals exposed to 1.5 mg Mn/m^3 had lowered MT mRNA levels in the frontal cortex, globus pallidus, and cerebellum and increased MT

gene expression in the olfactory cortex. Finally, in the putamen of the exposed monkeys, there was no significant alteration in MT gene expression due to Mn inhalation. Data from the present study largely corroborates our previous work where changes in MT gene expression were found to be rather insensitive to high-dose Mn exposure.

Like MT, GS also 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). This Mn-dependent enzyme catalyzes the formation of glutamine from glutamate and is exclusively expressed in the CNS in astrocytes (Martinez-Hernandez et al., 1977). Glutamine is taken up by local glutamatergic or g-amino-butyric acid-containing (GABAergic) neurons where deamination to glutamate occurs. This process is considered the primary glutamate-recycling pathway in the brain (Ottersen et al., 1992; Van den Berg and Garfinkel, 1971; Westergaard et al., 1995). Unlike our previous studies (Erikson et al., 2005, 2006), MnSO4 inhalation did not uniformly affect GS gene expression (i.e., only the olfactory cortex showed a significant increase in monkeys exposed to 1.5 mg Mn/m^3) ; however, GS protein levels were altered in most of the brain regions of monkeys exposed to the highest $MnSO_4$ concentration. The effect of Mn exposure on GS protein concentration differed among brain regions. For example, the cerebellum of monkeys exposed to $MnSO_4$ at 1.5 mg Mn/m^3 had a 16% decrease in GS protein levels while concentrations in the putamen increased approximately 20% (vs. air-exposed controls). Inhibition of GS activity can have serious consequences on neuronal functioning through impaired detoxification of ammonia or due to decreased glutamate or g-aminobutyric acid levels.

Increased extracellular glutamate concentrations can also contribute to neurotoxicity with glutamate excitotoxicity being postulated as a triggering biochemical event for manganism. Glutamate levels have been shown to be elevated in the basal ganglia of Mn-exposed rats (Erikson et al., 2002a; Garcia et al., 2006). As mentioned earlier, astrocytes are the primary neural cell involved in glutamate metabolism. Specifically, astrocytes clear glutamate from the extracellular space with the glutamate transporters, GLT-1 and/or GLAST. Upon intracellular trans-port, glutamate is aminated within the astrocyte to glutamine via GS (Danbolt, 2001). Glutamine can then be transported to neurons where it can undergo deamination back to glutamate for use as a neurotransmitter or in protein synthesis. As mentioned previously, this process is referred to as the glutamate-recycling pathway and is critically dependent upon normally functioning GLT- 1 and GLAST proteins localized in astrocytes.

Attenuated glutamate uptake in Mn-exposed astrocytes has been reported (Erikson et al., 2002b; Hazell and Norenberg, 1997). Altered glutamate uptake by astrocytes has been linked to Mn dose-dependent decreased GLAST expression (Erikson et al., 2002b). More recently, GLT- 1 has also been shown to be affected by Mn exposure as well, indicating possible mechanisms for the Mn-associated decrease in glutamate uptake (Mutkus et al., 2005). In the present study, reductions in glutamate transporter protein levels were one of the more consistent findings seen. Reduced GLAST protein levels were seen in the globus pallidus of monkeys exposed to $\geq$ 0.06 mg Mn/m^3. Reduced GLAST protein levels were also seen in the cerebellum and olfactory cortex of monkeys exposed to $\geq$ 0.3 mg Mn/m^3, while similar reductions were seen in the frontal cortex of monkeys exposed to the highest $MnSO_4$ level. In contrast, increased GLAST mRNA levels were seen in some groups of Mn-exposed monkeys. Significantly higher GLAST mRNA levels (vs. control animals) were seen in the globus pallidus following exposure to > 0.06 mg Mn/m3 and in the cerebellum following exposure to $\geq$ 0.3 mg Mn/m^3. The caudate, cerebellum, globus pallidus, and olfactory cortex of monkeys exposed to $\geq$ 0.3 mg Mn/m^3 had significantly lower protein levels of GLT-1 compared to the control animal. The caudate also showed decreased GLT-1 mRNA in monkeys exposed to $\geq$ 0.06 mg Mn/m^3 and increased GLT- 1 mRNA in animals exposed to 0.06 and 0.3 mg Mn/m^3 in the globus pallidus and olfactory cortex, respectively (Table 3).

Overall, Mn inhalation appears to affect the gene and protein expression of both glutamate transporters in the caudate, globus pallidus, and cerebellum of exposed monkeys. We can infer from this finding that Mn may alter glutamate homeostasis in the basal ganglia, which may contribute to excitotoxicity. While glutamate levels measured in these regions were un-affected by Mn exposure (Struve et al., unpublished data), it should be noted that this measurement reflects tissue levels of total glutamate. It does not allow for a distinction between intra-

versus extracellular glutamate concentrations, which would more accurately reflect any consequences of altered GLT- 1 and GLAST transporter levels.

GSH is a ubiquitous intracellular antioxidant formed from the amino acids glutamate, cysteine, and glycine leading to the formation of g-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 unaffected individuals. Correspondingly, GSH levels are significantly and age-dependently lowered in the striatum of Mn-exposed rats (Erikson et al., 2004). Older rats have dramatically lower GSH levels following high-dose Mn exposure (Desole et al., 1995), whereas concentrations seen in juvenile rats are unaffected (Erikson et al., 2004). Our present study showed that in two brain regions, similar increases in Mn concentrations can lead to opposite GSH metabolism responses. Specifically, there was decreased GSH levels in the caudate of exposed monkeys (albeit, not statistically significant; $p = 0.071$), while increased GSH levels occurred in the frontal cortex of monkeys exposed to 1.5 mg Mn/m^3.

Since Mn accumulates in dopamine-rich brain regions, especially those associated with subsequent neurotoxicity, we sought to examine the effects of Mn inhalation on dopaminergic neurons. In particular, we examined TH protein and mRNA levels following Mn inhalation since TH is the rate-limiting enzyme for catecholamine synthesis and is often used as a marker for dopaminergic neurons. Mn exposure decreased TH protein levels in the caudate and globus pallidus of monkeys exposed to ≥ 0.3 mg Mn/m^3. TH mRNA levels were also altered in the olfactory cortex and globus pallidus in Mn-exposed monkeys, but not the caudate which is in contrast to our observations in rodents (Erikson et al., 2006), where TH mRNA levels were altered while protein levels remained unaffected. Overall, as with the rodent data, our current data suggest that increased Mn levels in the striatum is not directly affecting dopamine-containing neurons which is consistent with the neurochemistry data reported by Struve et al. (un-published data).

In conclusion, when compared to our previous studies, nonhuman primates exposed subchronically to MnSO4 respond both similarly and differently in terms of alterations in biomarkers of neurotoxicity across different brain regions. These monkeys accumulate Mn in a dose- and region-dependent manner and display the ability to normalize brain Mn levels after cessation of the Mn exposure (Dorman et al., 2005, 2006a). We found that GS and GLAST gene expression and protein levels in monkeys were relatively unaffected by Mn exposure. However, GLT-1 mRNA and protein levels were significantly altered in the caudate of Mn-exposed monkeys. MT mRNA, which in rats is both increased and decreased due to Mn exposure, was generally reduced in Mn-exposed monkeys. Finally, TH protein levels were lowered in the caudate and globus pallidus of Mn-exposed monkeys, whereas gene expression was increased in the primate olfactory cortex. Our studies over the past few years have established that the effects of Mn inhalation on biomarkers of neurotoxicity vary greatly among different mammalian brain regions even when similar Mn concentrations are achieved within these regions.

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