Oxidative Stress Is Induced in the Rat Brain Following Repeated Inhalation Exposure to Manganese Sulfate

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Abstract:
Eight-week-old rats inhaled manganese (Mn) in the form of $MnSO_4$ at 0, 0.03, 0.3, or 3.0 mg Mn/m^3 for 6 h/d for 7 d/wk (14 consecutive exposures). Brain manganese concentrations in these animals were reported by Dorman et al. in 2001, noting the following rank order: olfactory bulb > striatum > cerebellum. We assessed biochemical end points indicative of oxidative stress in these three brain regions, as well as the hypothalamus and hippocampus. Glutamine synthetase (GS) protein levels and total glutathione (GSH) levels were determined for all five regions. GS mRNA and metallothionein (MT) mRNA levels were also evaluated for the cerebellum, hypothalamus, and hippocampus. Statistically significant increases ($p<0.05$) in GS protein were observed in the olfactory bulb upon exposure to the medium and high manganese doses. In the hypothalamus, statistically significant ($p<0.05$) but more modest increases were also noted in the medium and high manganese dose. Total GSH levels significantly ($p<0.05$) decreased only in the hypothalamus (high manganese dose), and MT mRNA significantly increased in the hypothalamus (medium manganese dose). No significant changes were noted in any of the measured parameters in the striatum, although manganese concentrations in this region were also increased. These results demonstrate that the olfactory bulb and hypothalamus represent potentially sensitive areas to oxidative stress induced by exceedingly high levels of inhaled manganese sulfate and that other regions, and especially the striatum, are resistant to manganese-induced oxidative stress despite significant accumulation of this metal.
Index Entries: Manganese; rat; oxidative stress; inhalation, in vivo.

Article:
INTRODUCTION
Manganese-induced neurotoxicity has been found among miners who inhale particulate matter containing manganese. The highest brain manganese levels in these individuals are found in globus pallidus, and there is concomitant neuronal loss in basal ganglia and associated nuclei *(1–3)*. The impact of manganese (Mg) on human health and disease upon low-level chronic exposure to environmental manganese is uncertain. The manganese fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT) is combusted to yield a variety of manganese salts *(4–6)*. The pharmacokinetics and brain accumulation of three MMT combustion products, namely, manganese phosphate ($Mn_5(PO_4)_2[(PO_3)(OH)]_2 \cdot 4H_2O$), manganese sulfate ($MnSO_4$), and manganese tetroxide (Mn_3O_4) were recently evaluated in rats inhaling 0, 0.03, 0.3, or 3 mg/m^3 for 6 h/d on 14 consecutive days *(7)*. The sulfate form of manganese was more efficiently cleared from the rats' lungs and it accumulated at a higher rate in the olfactory bulb and striatum than did the phosphate or tetroxide *(7)*. Thus, the rate of delivery of different forms of manganese to brain regions via inhalation depends, in part, on the solubility of the compounds.

Oxidative stress has been implicated as a contributing mechanism by which manganese can be toxic to cells *(8)*. A potential mechanism invoked for manganese-induced oxidative stress is via the oxidation of dopamine and other catecholamines *(9)*. This is likely because manganese in primates accumulates in dopamine-rich regions,

especially in the basal ganglia. Another possibility is that sequestration of manganese in mitochondria interferes with proper respiration, thereby leading to excessive production of reactive oxygen species. Galvani et al. *(10)* reported inhibition of complex I of the electron transport chain after treatment of PC12 cell cultures with $MnCl_2$. Gavin et al. *(11)* showed evidence suggesting that the ATPase complex is inhibited at very low levels of mitochondrial manganese and that complex I is inhibited only at higher manganese concentrations. In agreement with the earlier suggestion of Archibald and Tyree *(12)* and the findings of Ali et al. *(13),* Chen et al. *(14)* demonstrated that trivalent manganese is more effective at inhibiting complex I, but the divalent form is, by far, the predominant species within cells and is largely bound to ATP. Nevertheless, manganese in any state will spontaneously give rise to infinitesimal amounts of trivalent manganese, and HaMai et al. *(15)* demonstrated that even at trace amounts trivalent manganese can cause the formation of reactive oxygen species (ROS). They also showed evidence that divalent manganese fails to induce oxidative effects. Finally, a recent report showed that exposure of dopaminergic cells to MMT resulted in rapid increases in reactive oxygen species followed by mitochondrially induced apoptosis *(16).* However, because combustion of MMT in cars yields various manganese salts and MMT is unstable in the air and groundwater, direct exposure of cells to MMT does not represent a toxicologically relevant experimental model for inorganic manganese.

Manganese exposure studies do not always yield results consistent with induction of oxidative stress. Manganese administered orally to developing CD rats (0, 25, or 50 mg/kg/d of $MnCl_2$ from postnatal day [PND] 1 to PND 49) was correlated with increased spontaneous motor activity on PND 21, but ROS levels were only elevated in the cerebellum and not in the striatum, the primary target site in primates *(17).* Addition-ally, experiments by Ball et al. *(18)* demonstrated that six metal com-pounds, including iron sulfate, copper sulfate, vanadyl sulfate, cobalt sulfate, nickel sulfate, and zinc sulfate, are able to form ROS (measured by malondialdehyde formation) in aqueous extracts of National Institute of Standards and Technology (NIST) ambient particulate matter and diesel engine particles. Manganese, however, did not. Furthermore, a study by Oubrahim et al. *(19)* confirmed that low cellular concentrations of divalent manganese are actually protective against oxidative stress. This is consistent with the well-known requirement for manganese as a cofactor for the mitochondrial superoxide dismutase enzyme, which has tremendous physiological antioxidant properties.

We previously analyzed the end points of oxidative stress in brains of neonatal rats exposed orally to $MnCl_2$ *(20).* The susceptibility of neonatal animals to manganese neurotoxicity may be higher compared with adults because of the incomplete development of the blood–brain barrier and their inability to excrete manganese before weaning. However, the only significant oxidative difference noted in these animals was an increase in glutathione (GSH) levels in the cortex *(20).* Additionally, the manganese concentrations required for optimal central nervous system (CNS) development in neonatal rat brain are known to be higher than are found in adult rat brain *(21)* and, therefore, the interpretation of these data as evidence for increased susceptibility to manganese neurotoxicity is questionable. Blood concentrations of manganese are also threefold higher in neonatal rats than in adults, and this undoubtedly contributes to the higher level of manganese uptake into the brain *(22,23).*

In contrast to the previous work, the present study was conducted in adult rats exposed to manganese by inhalation. After 14 consecutive days of manganese sulfate inhalation exposures, GSH, glutamine synthetase (GS) protein, and mRNA and metallothionein (MT) mRNA levels were measured in the cerebellum, striatum, olfactory bulb, hypothalamus, and hippocampus as indicators of potential oxidative stress in the brain.

MATERIALS AND METHODS

Chemicals

Manganese(II) sulfate monohydrate ($MnSO_4 \cdot H_2O$) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Manganese sulfate is a relatively water-soluble, pale pink, crystalline powder that contains 32% manganese. All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted and were of the highest possible quality.

Animals

Dorman et al. *(7)* previously detailed animal treatments and exposures. The study was conducted under federal guidelines for the care and use of laboratory animals *(24)* and was approved by the CIIT Institutional Animal Care and Use Committee. Six-week-old male Crl : CD (SD)BR rats were purchased from Charles River Laboratories, Inc. (Raleigh, NC) and ear-tagged for identification purposes. Animals were acclimated for approx 2 wk in a HEPA-filtered, mass-air-displacement room maintained at 18.5–21.5°C and 40–60% relative humidity in CIIT's AAALAC-accredited animal facility. Rats were individually housed in suspended stainless-steel cages (Lab Products, Inc., Seaford, DE) with an automatic watering system that provided reverse-osmosis-purified water ad libitum. Fluorescent lighting was kept on a 12-h light–dark cycle (0600–1800). A study day for these exposures was defined as a 6-h exposure, generally from 0830 to 1430. Animals were given NIH-07 rodent chow (Zeigler Bros., Gradners, PA) ad libitum except during exposure times. Manganese concentrations in this diet are approx 100–150 ppm.

At the end of the acclimation period, animals were randomly assigned to inhalation-treatment groups based on their prestudy body weight. Mean initial body weights within each treatment group were not statistically different. The animals (n = 12 rats/ concentration) were exposed to $MnSO_4$ inhalation for 6 h/d, 7 d/wk for 14 consecutive exposure days.

Manganese Exposures

Nominal exposure concentrations were 0.092, 0.92, and 9.2 mg $MnSO_4/m^3$, corresponding to 0.03, 0.3, and 3 mg Mn/m^3. Target nominal particle size (MMAD) was approx 1.5–2 pm with a geometric standard deviation (σg) <2. Control groups were exposed to HEPA-filtered air only. Rats were exposed in stainless-steel wire cage units contained within eight Hazelton 1-m^3 stainless-steel and glass inhalation exposure chambers. Prior to the animals being placed in the 1-m^3 chambers, each chamber was checked for uniformity of distribution of the test compound aerosol by measuring its concentration at nine positions within the chamber. Animal positions within the 1-m^3 exposure chambers were rotated once during the experiment to minimize experimental error resulting from any undetected differences in the environment or manganese aerosol concentration.

The airflow through each 1-m^3 chamber was maintained at 200–250 L/min to provide 12–15 air changes per hour during the exposures. The temperature and humidity in each 1-m^3 inhalation chamber was measured every 30 min using a thermistor (PreCon, Memphis, TN) and a humidity probe (OMEGA Engineering, Inc., Stamford, CT). The temperature was maintained at a target of 22.2°C, and relative humidity was maintained at 30–70% with a target of 50%.

The $MnSO_4$ atmospheres were generated as a dry powder using a Wright Dust Feeder with an air-delivery pressure of 25 psi. The $MnSO_4$ was packed into a Wright Dust Feeder generation cup at a pressure of 2000 psi. A Trost Airjet Mill (Garlock Corp., Newton, PA) was also required to generate the $MnSO_4$ aerosol. The aerosol was delivered into stainless-steel prechambers (fitted with ^{85}Kr discharging units) of approx 38-L that allowed settling of larger particles (>10 μm). The aerosol then went through a polyvinyl chloride distribution line tuned to allow equal residence times of particles in the pathway to each 1-m^3 exposure chamber. The aerosol was diluted to the appropriate concentration in the inlet airstream to each 1-m^3 inhalation chamber. The aerosol in the prechamber and in each 1-m^3 inhalation chamber was monitored continuously using an optical particle sensor (Real-time Aerosol Monitor, Model RAM-S; MIE Inc., Bedford, MA) precalibrated for a standard dust (Arizona road dust). The average aerosol mass concentration was determined using gravimetric filter samples. Flow through the filter samplers was started 15 min after the beginning of the exposure and stopped at the end of the exposure. The filter sampler flow was controlled with critical flow orifices. The particle size distribution was measured with a time-of-flight aerosol spectrometer (TSI APS Model 3320) and a microorifice uniform deposit cascade impactor (MOUDI, MSP Corp., Minneapolis, MN).

Tissue Collection

At the termination of exposures, euthanasia (with CO_2) was carried out in accordance with NIH guidelines. The brain areas of interest were dissected out and weighed, and then placed in high-purity linear polyethylene vials, frozen in liquid nitrogen, and stored at –70°C until analysis.

RNA Isolation and Northern Blot Analysis

The RNA tissue samples were homogenized and total RNA was extracted with a monophase phenol and guanidine isothiocyanate solution (RNA STAT-60; Tel-Test, Inc., Friendswood, TX). For Northern blot analysis, 10 µg of RNA was electrophoresed on a 1.2% agarose denaturing gel and transferred onto a positively charged nylon membrane (Nytran SuPer-Charge; Schleicher & Schuell, Keene, NH) overnight by capillary transfer in 10X SSC (1X SSC = 0.15 *M* sodium chloride, 0.015 *M* sodium citrate) buffer. The RNA was immobilized with a ultraviolet (UV) crosslinker.

For MT or GS, the blots were prehybridized in 50% deionized for-mamide, 5X Denhardt's solution, 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 4X SSC 100 g/mL denatured salmon sperm DNA, 20 m*M* Tris-HCl, pH 8.0, for 1 h at 45°C. To probe for MT or GS, the blot was pre-hybridized in Ultrasensitive Hybridization Buffer (Ambion, Inc., Austin, TX) at 45°C. The RNA blots were then hybridized overnight with 105 cpm/mL of [α-32P]dCTP-labeled random primed cDNA probe (approx 1 x 10^8 cpm/µg; RadPrime DNA Labeling System, Gibco-BRL, Life Technologies, Rockville, MD). Membranes were washed two to three times in 2X 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 0.1X SSC/0.1%SDS/40 m*M* Tris buffer and probed for 28s rRNA *(25).*

Protein Isolation and Western Blot Analysis

Tissue lysates were centrifuged for 10 min at 10,000*g* to remove cellular debris, and the protein content of the resultant supernatant was determined with the bicinchoninic acid method (Pierce Chemical, Rockford, IL). An aliquot of 100 µg of protein was concentrated from the imidazole lysis buffer by organic extraction. Sample volumes were brought up to 400 µL with water and an equal volume of methanol (400 µL) was added, followed by 100 µL of chloroform. Samples were vortexed for 20 s and centrifuged at 14,000*g* for 3 min. The upper layer was removed and discarded. An additional 300 gL of methanol was added to each sample and they were again vortexed and centrifuged. The supernatant was removed and the pellet was air-dried. Each pellet was then dissolved in 100 µL 2% SDS and heated to 65°C.

Five microliters of 5X loading buffer (50% glycerol; 10% SDS, 0.25 *M* Tris-HCl, pH 6.8) and dithiothreitol (DTT) (final concentration 100 m*M*) was added to the extracted proteins and the samples were boiled for 10 min. Bromophenol blue (1 µL of a 50% [w/v] solution) was added and proteins were resolved by denaturing SDS-PAGE (polyacrylamide gel electrophoresis) with a 5% stacking and 8% resolving acrylamide gels in a 0.1% SDS, 25 m*M* Tris-HCl, 192 m*M* glycine buffer. Following fractionation, proteins were electrophoretically transferred to a nitrocellulose membrane (Protran, BA83; Schleicher & Schuell, Keene, NH) in 20% methanol, 0.1% SDS, 25 m*M* Tris-HCl, and 192 m*M* glycine for 3 h at 60 V.

Membranes were blocked with 5% low-fat powdered milk in Tris-buffered saline with Tween (TBST; 0.1% Tween, 150 m*M* NaCl, 20 mM Tris-HCl) containing 0.1% gelatin (type B from bovine skin, Sigma, St. Louis, MO). GS proteins were detected with a monoclonal antibody (Chemicon, Temec-ula, CA) diluted to 1 : 2000 followed by incubation with a horseradish-peroxidase-conjugated secondary antibody diluted to 1 : 2000 (Kirkegaard and Perry Laboratories, Gaithersburg, MD), both in TBST and 5% milk for 1 h. Protein bands were visualized with the Renaissance enhanced chemiluminescence system (New England Nuclear, Boston, MA). The autoradiograms were quantified by densitometry scanning in conjunction with the TINA v2.09e computer program (Raytest USA, Inc., Wilmington, NC).

GSH Level Determination

Tissue samples (50–100 mg) were homogenized in 1 mL of 10% (v/v) perchloric acid containing 1 m*M* bathophenanthroline disulfonic acid (BPDS) and L-γ-glutamyl-L-glutamate. The mixture was vortexed and centrifuged, and an aliquot was removed for high-performance liquid chromatographic (HPLC) analysis *(26)* with 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 approx 50 pmol, which equated to approx 0.4 nmol/mg protein *(27,28)*.

Statistical Analysis

To determine statistical significance between experimental groups, one-way analysis of variance (ANOVA) was used. When the overall significance resulted in rejection of the null hypothesis ($p < 0.05$), the source of the variance was determined with the Student–Newman–Keuls posttest. All analyses were performed using GraphPad InStat version 3.02 for Windows (GraphPad Software, San Diego, CA). Unless otherwise noted, all data are presented as mean ± SEM values.

RESULTS

GS Protein and mRNA Expression

A statistically significant ($p<0.05$) increase in GS protein was observed in rats' olfactory bulbs at the medium (0.3 mg/m^3) and high (3 mg/m^3) doses of manganese (*see* Table 1). There was also a modest, but statistically significant ($p<0.05$) increase in GS in the hypothalamus at all doses. GS protein levels were significantly decreased in cerebellum at the highest manganese dose, despite a significant increase in the GS mRNA (*see* Table 1).

Table 1

GS Protein and mRNA Levels[a]

Endpoint	Cerebellum	Striatum	Olfactory Bulb	Hypothalamus	Hippocampus
GS Protein Levels					
Control	100.00 ± 3.54	100.00 ± 3.17	100.00 ± 7.24	100.00 ± 2.01	100.00 ± 12.41
Low Mn Dose	101.64 ± 1.89	100.57 ± 1.17	134.32 ± 10.44	119.92 ± 1.80*	121.75 ± 5.92
Med. Mn Dose	103.11 ± 3.57	97.52 ± 1.92	144.05 ± 10.78*	113.08 ± 2.05*	114.45 ± 7.75
High Mn Dose	86.33 ± 1.25*	102.72 ± 1.75	160.15 ± 8.23*	113.11 ± 1.82*	102.05 ± 11.34
GS mRNA Levels					
Control	100.00 ± 14.45	ND	ND	100.00 ± 3.21	100.00 ± 14.28
Low Mn Dose	177.67 ± 10.84*	ND	ND	85.02 ± 20.17	107.72 ± 16.42
Med. Mn Dose	82.90 ± 19.32	ND	ND	114.83 ± 22.71	62.30 ± 7.82
High Mn Dose	172.58 ± 5.50*	ND	ND	105.65 ± 14.96	67.51 ± 8.53

Note: Values represent percentage of control mean ± SEM.

**** p< 0.05 versus respective controls.***

[a]Given degradation in the tissue, MT mRNA levels in olfactory bulb or striatum could not be determined.

Table 2

Total GSH Levels

Endpoint	Cerebellum	Striatum	Olfactory Bulb	Hypothalamus	Hippocampus
GSH Levels					
Control	100.00 ± 6.58	100.00 ± 12.80	100.00 ± 21.85	100.00 ± 15.70	100.00 ± 20.43
(nmol/mg prot.)	10.66 ± 1.06	1.84 ± 0.35	7.00 ± 3.28	0.10 ± 0.03	4.61 ± 1.78
Low Mn Dose	60.55 ± 4.21	60.53 ± 28.04	123.00 ± 60.86	67.35 ± 5.15	129.57 ± 14.32
(nmol/mg prot.)	6.45 ± 0.45	1.11 ± 0.52	8.61 ± 4.26	0.07 ± 0.10	5.97 ± 0.66
Med. Mn Dose	105.72 ± 3.00	93.16 ± 27.94	181.71 ± 33.57	53.60 ± 14.53	105.71 ± 21.61
(nmol/mg prot.)	11.27 ± 0.32	1.71 ± 0.51	12.72 ± 2.35	0.05 ± 0.01	4.87 ± 1.00
High Mn Dose	113.81 ± 18.98	76.63 ± 32.34	95.14 ± 59.86	26.80 ± 3.33*	136.15 ± 32.29
(nmol/mg prot.)	12.13 ± 2.02	1.41 ± 0.60	6.66 ± 4.19	0.03 ± 0.003	6.28 ± 1.49

Note: Values represent percentage of control mean ± SEM except where otherwise indicated. * p< 0.05 versus respective controls

Tyrosine hydroxylase protein levels were also evaluated in the striatum and olfactory bulb, but no significant differences were found (data not shown).

GSH Levels

The only brain region with a statistically significant ($p<0.05$) change in GSH levels was the hypothalamus (*see* Table 2). There was a 30% decrease in total GSH levels at the lowest dose, 50% decrease at the medium dose, and 75% decrease at the highest dose in the hypothalamus of manganese exposed as compared to control rats (*see* Table 2). However, only the 3- mg/m^3 dose was statistically significant when compared to controls.

MT mRNA Levels

The hypothalamus was the only region in which the 0.3-mg/m^3 manganese dose yielded (threefold) a statistically significant ($p<0.05$) increase in MT mRNA. Other regions had decreased MT mRNA because of manganese exposure, but these were not statistically significant, and they should be viewed with caution given the large variability (*see* Table 3).

Table 3
***MT mRNA Levels*[a]**

Endpoint	Cerebellum	Striatum	Olfactory Bulb	Hypothalamus	Hippocampus
MT mRNA Levels					
Control	100.00 ± 14.54	ND	ND	100.00 ± 15.00	100.00 ± 18.22
Low Mn Dose	125.90 ± 26.85	ND	ND	229.85 ± 31.87	113.29 ± 30.57
Med. Mn Dose	84.53 ± 11.93	ND	ND	278.86 ± 63.30*	65.54 ± 21.15
High Mn Dose	94.98 ± 20.93	ND	ND	189.82 ± 17.82	38.51 ± 10.05

Note: Values represent percentage of control mean ± SEM.
**** p< 0.05 versus respective controls***
[a] Given degradation in the tissue, MT mRNA levels in olfactory bulb or striatum could not be determined.

DISCUSSION

The current inhalation reference concentration for manganese, as set by the United States Environmental Protection Agency, is 0.05 μg Mn/m^3. Thus, the concentrations of manganese used in this study were 600, 6000, and 60,000 times this standard for the low, medium, and high doses, respectively. The accumulation of manganese in three brain regions from exposed rats was assessed, and the relative concentrations were olfactory bulb > striatum > cerebellum *(7).* The control concentration of manganese in olfactory bulb was less than 1 μg/g tissue, and upon exposure to the highest manganese dose, it increased to almost 4.5 μg/g. Striatal differences were similar, with less than 1 μg/g in control animals and approx 3.25 μg/g tissue in animals receiving the highest manganese exposure. The cerebellar concentrations of manganese did not change upon manganese exposure *(7),* and the hypothalamic and hippocampal manganese levels were not analyzed.

Oxidative stress can be measured through detection of many different chemical species. Some methods, such as direct detection by the fluorescence-producing reaction of dichlorofluorescein (DCF) with oxygen radicals can provide useful information in any type of tissue. Ali et al. *(13)* demonstrated that both manganese injected in vivo and exposure of brain regions to manganese in vitro resulted in detection of ROS by DCF fluorescence. The limitation of this technique is that living cells are required for study. Another rationale behind quantitation of oxidative stress is measurement of products directly damaged by the ROS. This damage can be found in every class of macromolecule, but DNA, lipid, and protein oxidation are most often investigated. The techniques for detection of these molecules are varied, and the inconsistency between methods has been widely debated. Artifactual formation of damage during sample preparation is one issue that may contribute to this problem.

Another approach to detecting the presence of oxidants is to measure species that are known to increase or decrease in response to such stress. This includes ubiquitous antioxidants, such as glutathione, as well as ones that are more specific to particular tissue types or oxidative agents. In the CNS, GS is localized exclusively in

astrocytes *(29),* where it has a critical role in amino acid metabolism. GS metabolizes glutamate that is removed from the extracellular space by astrocytes to glutamine, and the latter is recycled to neurons as part of the glutamate–glutamine cycle *(30).* GS is highly susceptible to oxidation and subsequent rapid degradation, and, therefore, it serves as an excellent marker for the presence of reactive oxy-gen species in the brain *(31).* The results of this study showed that GS protein levels increased considerably in olfactory bulb and to a lesser extent in hypothalamus (*see* Table 1). This may suggest lowered availability of glutamine for neuronal use as a with neurotransmitter homeostasis within these regions. However, one GS molecule requires eight manganese ions for optimal activity *(32).* Therefore, slight increases in manganese concentrations also enhance the activity of GS *(33).* It is possible that the elevated manganese levels also stimulate additional GS synthesis. No changes in GS protein levels were noted in the striatum, even though manganese accumulation was high following the inhalation exposures.

The MTs are a class of highly conserved proteins, which are classically known to be upregulated in response to metal exposure. They contain cysteine and are known to bind metals, but in recent years, evidence has shown that they may also have some important antioxidant properties. Hainut and Milner *(34)* suggested that MTs can neutralize ROS through oxidative release of zinc from MT thiolate clusters. There is good evidence of MT upregulation in response to hyperoxia in the lung *(35).* In vitro experiments on the ability of several sulfhydrylcontaining molecules to scavenge oxygen radicals demonstrated that MTs are superior to others *(36).* Furthermore, Zhou et al. *(37)* found that MT-overexpressing transgenic mice were less susceptible to alcohol-induced oxidative liver injury. Also, when two different mitochondrial-specific ROS generators were applied to cultured cells, MTs increased more than GSH, Mn-SOD (super-oxide dismutase) catalase, and other well-known antioxidants *(38).* This study found that MT mRNA was increased in hypothalamus, where we also noted an increase in GS protein and a decrease in GSH (*see* Table 2).

The data presented in these studies suggest that the olfactory bulb, previously found to have the highest accumulation of manganese after inhalation exposure *(7),* had the most changes in biochemical end points. This was not the case in the striatum, where there was a threefold increase in manganese concentration as a result of inhalation exposure, but no indication of oxidative stress *(7).* The hypothalamus, for which manganese lev-els after inhalation exposures were not measured, was observed to have changes in all of the oxidant indicators we analyzed.

In summary, the present study determined whether oxidative stress could occur in the rat brain following inhalation of the sulfate form. The preliminary results indicate significant changes in the levels of GS protein in the olfactory bulb and hypothalamus upon exposure to the medium and high manganese doses. GSH levels decreased and MT mRNA increased in the hypothalamus (medium manganese dose), and there were no apparent changes in striatal tissue in any of the measured parameters. Given that the hypothalamus does not represent a target area for manganese toxicity in primates *(39),* it cannot be unequivocally stated at this point that our data support the ability of manganese to induce oxidative stress in this area. The effect of manganese on oxidative-stress-related end points (GSH content and GS) in the olfactory bulb is consistent with recent observations on the prominence of the olfactory pathway in manganese delivery to the brain in the rodent *(40–42),* but given the interspecies differences in nasal and brain anatomy and physiology and the uncertainty about the role of olfactory pathways in the absorption of manganese in humans, the relevance of this finding to primates has yet to be determined. Finally, the lack of changes in any of the measured parameters in the striatum indicates that manganese inhalation, even at very high doses, does not induce oxidative stress in this brain region.

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