Airborne Manganese Exposure Differentially Affects End Points of Oxidative Stress in an Age- and Sex-Dependent Manner

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Abstract:
Juvenile female and male (young) and 16-mo-old male (old) rats inhaled manganese in the form of manganese sulfate ($MnSO_4$) at 0, 0.01, 0.1, and 0.5 mg Mn/m^3 or manganese phosphate at 0.1 mg Mn/m^3 in exposures of 6 h/d, 5 d/wk for 13 wk. We assessed biochemical end points indicative of oxidative stress in five brain regions: cerebellum, hippocampus, hypothalamus, olfactory bulb, and striatum. Glutamine synthetase (GS) protein levels, metallothionein (MT) and GS mRNA levels, and total glutathione (GSH) levels were determined for all five regions. Although most brain regions in the three groups of animals were unaffected by manganese exposure in terms of GS protein levels, there was significantly increased protein ($p<0.05$) in the hippocampus and decreased protein in the hypothalamus of young male rats exposed to manganese phosphate as well as in the aged rats exposed to 0.1 mg/m^3 $MnSO_4$. Conversely, GS protein was elevated in the olfactory bulb of females exposed to the high dose of $MnSO_4$. Statistically significant decreases ($p<0.05$) in MT and GS mRNA as a result of manganese exposure were observed in the cerebellum, olfactory bulb, and hippocampus in the young male rats, in the hypothalamus in the young female rats, and in the hippocampus in the senescent males. Total GSH levels significantly ($p<0.05$) decreased in the olfactory bulb of manganese exposed young male rats and increased in the olfactory bulb of female rats exposed to manganese. Both the aged and young female rats had significantly decreased ($p<0.05$) GSH in the striatum resulting from manganese inhalation. The old male rats also had depleted GSH levels in the cerebellum and hypothalamus as a result of the 0.1-mg/m^3 manganese phosphate exposure. These results demonstrate that age and sex are variables that must be considered when assessing the neurotoxicity of manganese.
Index Entries: Rat; manganese; brain; oxidative stress; glutathione; glutamine synthetase; metallothionein.

Article:

INTRODUCTION

Manganese is an essential trace metal that is found in all tissues and is required for normal amino acid, lipid, protein, and carbohydrate metabolism *(1).* Although manganese deficiency is extremely rare in humans, toxicity resulting from manganese imbalance is much more prevalent and the brain appears to be a vulnerable organ to this toxicity (manganese neurotoxicity). This toxicity is associated with occupational overexposures or unusual exposures (e.g., contaminated well water, parenteral nutrition therapy in patients with liver disease) *(2).*

Manganese neurotoxicity most commonly occurs in workers who have been chronically exposed to aerosols or dusts that contain extremely high levels (> 1–5 mg Mn/m^3) of manganese *(3–5).* Currently, the focus on airborne manganese has increased because of the recent approval for the use of a fuel additive in some unleaded gasoline, the antiknock agent methylcyclopentadienyl manganese tricarbonyl (MMT), although the level of manganese resulting from MMT in urban areas does not exceed tolerable levels set by the governments of Canada and the United States *(6).* It has been speculated that chronic low-level manganese exposure might play a role in the pathogenesis of neurodegenerative disorders, especially in susceptible populations. Because of distinct similarities between Parkinson's disease and manganese neurotoxicity (e.g., bradykinesia and wide-

spread rigidity), an intense pursuit of the possible role that manganese has in the etiology of Parkinson's disease is underway.

Oxidative stress has been implicated as a contributing mechanism by which manganese might be cytotoxic *(7).* The oxidation of dopamine by manganese is a potential mechanism invoked for manganese-induced oxidative stress, especially because manganese accumulates in dopamine-rich brain regions of rodents and primates (e.g., basal ganglia) *(8).* Another possible mechanism is that manganese, through its sequestration in mitochondria, interferes with proper respiration, thereby leading to excessive production of reactive oxygen species. One laboratory reported inhibition of complex I of the electron-transport chain after treatment of PC12 cell cultures with $MnCl_2$ *(9).* Another laboratory 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 *(10).* It has been shown that trivalent manganese is more effective in inhibiting complex I *(11–13),* but the divalent form is by far the predominant species within cells and is largely bound to ATP *(12,14).* Nevertheless, in biological media, manganese of any valence 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 formation of reactive oxygen species. Finally, a recent report showed that exposure of 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, the most abundant being phosphate and sulfate *(17,18),* direct exposure of cells to MMT does not represent a toxicologically relevant experimental model.

Our laboratory recently reported the effect of manganese exposure (both orally administered and airborne) on oxidative stress end points in young male rats *(19,20).* These rats were exposed to 0, 0.03, 0.3, and 3.0 mg Mn/m^3 manganese sulfate for 14 d, representing doses three times higher than in this current study but for a much shorter duration. This present study extends the previous observations by examining the interactions of sex and/or age and manganese exposure on end points of oxidative stress in select brain regions. It is currently unknown what, if any, sex-specific differences exist in the response to manganese-induced oxidative stress markers. Likewise, the aged brain is also relatively unexplored in terms of assessing manganese-induced oxidative stress. Desole et al. showed that glutathione (GSH) levels decrease in the striatum of senescent male rats exposed to manganese *(21),* but they did not measure metallothionein or glutamine synthetase, or any other markers of oxidative stress. Finally, the rats in the present study were exposed to lower levels of airborne manganese for a longer time period (13 wk) compared to the previous studies (14 d) *(19,20).* Thus, the model used in this study, a chronic, moderate level of manganese exposure, is more representative of the type of manganese exposure that would affect the widest spectrum of the population, although they are hundreds to thousands of times higher than those among the general population of Toronto *(4).*

MATERIALS AND METHODS

Chemicals

Manganese (Mn^{2+}) phosphate, in the mineral form hureaulite $[Mn_5(PO_4)2[(PO_3)(OH)]_2 \cdot 4H_2O]$, was obtained from Alfa Aesar (Ward Hill, MA). The material is a reddish white fine crystalline powder, is 37.7% manganese by weight, and is relatively insoluble in water. Manganese(II) sulfate monohydrate ($MnSO_4 \cdot H_2O$) was obtained from Aldrich Chemical Company, Inc. (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. *(22)* previously detailed animal treatments and exposures. The study was conducted under federal guidelines for the care and use of laboratory animals (National Research Council, 1996) and was approved by the CIIT Institutional Animal Care and Use Committee. Young (6-wk-old) male and female CD rats were purchased from Charles River Laboratories, Inc. (Raleigh, NC). Senescent (≥ 16 mo old) male CD rats were purchased from Charles River Laboratories, Inc. (Canada). All prestudy health screens were negative. 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*. A pelleted, semipurified AIN-93G-certified diet from Bio-Serv (Frenchtown, NJ) formulated to contain approx 10 ppm manganese and 35 ppm iron was given *ad libitum* (except during inhalation exposures) throughout the study. 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.

Manganese Exposures

Rats were exposed whole body in 8-m^3 Hinners-style, stainless-steel and glass inhalation exposure chambers using exposure cage rack units. Hureaulite and $MnSO_4$ atmospheres were generated and characterized using methods described by Dorman et al. *(22).* Nominal $MnSO_4$ exposure concentrations of 0.01, 0.1, and 0.5 mg Mn/m^3 were used in this study. A single hureaulite exposure concentration of 0.1 mg Mn/m^3 was used in this study. The target particle size distribution was 1.5–2 µm mass median aerodynamic diameter with a geometric standard deviation (GSD) < 2. Control groups were exposed to HEPA-filtered air only. All exposures were conducted for 6 h/d, 5 d/wk for 13 wk.

Based on optical particle sensor results, the overall average concentrations (± SD) for the $MnSO_4$ atmospheres were 0.01 ± 0.001, 0.098 ± 0.009, and 0.478 ± 0.042 mg/m^3 for the target concentrations of 0.01, 0.1, and 0.5 mg Mn/m^3, respectively. The geometric mean diameters (GMD) and geometric standard deviations (GSD; μ_g) of the MnSO4 aerosols were determined to be 1.08 µm (μ_g = 1.52), 1.09 µm (μ_g = 1.53), and 1.12 µm (μ_g = 1.56) for the target concentrations of 0.01, 0.1, and 0.5 mg Mn/m^3, respectively. The calculated mass median aerodynamic diameters (MMADs) were 1.85, 1.92, and 2.03 µm for the target $MnSO_4$ concentrations of 0.01, 0.1, and 0.5 mg Mn/m^3, respectively. The overall average concentration (± SEM) for the hureaulite aerosol was 0.099 ± 0.004 mg hureaulite/m^3 for the target concentration of 0.1 mg Mn/m^3. The GMD and calculated MMAD for the hureaulite aerosol were 1.03 µm (μ_g = 1.41) and 1.47 µm, respectively.

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 –80°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 analysis, 10 µg of RNA were electrophoresed on a 1.2% agarose denaturing gel and transferred onto a positively charged nylon membrane (Nytran SuPerCharge; Schleicher & Schuell, Keene, NH) overnight by capillary transfer in 10X SSC (1X SSC = 0.15 *M* sodium chloride, 0.015 *M* sodium citrate) buffer. The RNA was immobilized with an ultraviolet (UV) crosslinker.

For metallothionen (MT) or glutamine synthetase (GS), the blot was prehybridized in 50% deionized formamide, 5X Denhardt's solution, 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 4X SSC of 100 g/mL denatured salmon sperm DNA, and 20 m*M* Tris-HCl, pH 8.0 for 1 h at 45°C. To probe for MT or GS, the blot was prehybridized in Ultrasensitive Hybridization Buffer (Ambion, Inc., Austin, TX) at 45°C. The RNA blots were then hybridized overnight with 10^5 cpm/mL of a [α-^{32}P]dCTPlabeled random primed cDNA probe (approx 1×10^8 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 the total loaded RNA level, the blots were stripped in 0.1X SSC/0.1% SDS/40 mM Tris buffer and probed for 28S rRNA *(23).*

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 µL 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 of 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*) were 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 sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) 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 Trisbuffered saline with Tween (TBST: 0.1% Tween, 150 m*M* NaCl, 20 m*M* TrisHCl) containing 0.1% gelatin (type B from bovine skin; Sigma, St. Louis, MO). GS proteins were detected with a monoclonal antibody (Chemicon, Temecula, CA) diluted to 1 : 2000, followed by incubation with a horseradish peroxidase conjugated goat anti-mouse 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 chromatography (HPLC) analysis *(24)* 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 *(25,26).*

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 ($p < 0.05$), we utilized Dunnett's procedure to determine which manganese exposure levels differed from controls within each parameter analyzed. All analyses were performed using GraphPad InStat version 3.02 for Windows (GraphPad Software, San Diego, CA).

RESULTS

GS Protein

The GS protein levels were significantly elevated in the hippocampus and decreased in the hypothalamus of young males exposed to manganese phosphate (*see* Table 1). In old males, GS protein levels were significantly decreased upon exposure to the low and medium doses of $MnSO_4$ in the hippocampus and only the medium dose of $MnSO_4$ in the hypothalamus. Female rats exposed to the medium dose of $MnSO_4$ had a statistically significant decrease GS protein levels in the hippocampus and increased levels in the olfactory bulb of females exposed to the high dose of $MnSO_4$ (*see* Table 1).

Table 1
GS Protein Levels Expressed as Percent of Control ± SD in Cerebellum (CB), Hippocampus (HC), Hypothalamus (HYP), Olfactory Bulb (OB), and Striatum (ST)

		Low Dose	Med Dose	High Dose	Med Dose
		$MnSO_4$	$MnSO_4$	$MnSO_4$	$MnPO_4$
MALE	CB	101±8.9	121.4±16.2	99.2±17.6	100.6±11.1
	HC	98.6±6.2	127.8±16.2	130.8±10.7	171.3±14*
	HYP	112.1±14.7	102.1±18.2	92.5±14.2	20.2±5.2*
	OB	96.2±16.4	123.9±12.4	115.4±3.5	115.9±52.1
	ST	111.7±5.4	85.8±10.4	96.7±4	106.9±3.1
FEMALE	CB	108.9±8.9	113.6±4.4	85.9±21.1	88.8±19.2
	HC	102.1±1.7	80.7±9.4*	104.8±3.4	92.3±1.9
	HYP	104.8±4.3	111.3±9.5	98.5±1.6	89.9±7.9
	OB	118.9±3.8	106.6±4.5	127.8±2.7*	113.9±6.5
	ST	99.5±3.8	98.9±0.4	91.9±1.2	95.1±0.4
OLD MALE	CB	107.8±8.8	101.9±6.8	120.9±1.9	110.3±7.8
	HC	81.2±4.2*	78.4±8.5*	89.1±9.5	86.3±3.1
	HYP	90.4±11.7	57.9±4.3*	83.2±8.2	79.1±6.9
	OB	86.3±5.1	92.1±11.7	85.8±6.4	90.9±4.1
	ST	103.1±8.1	85.3±15.2	88.8±5.6	77.8±14

Note: The asterisk (*) indicates a statistically significant difference from control, $p<0.05$.

GS mRNA

The GS mRNA levels were significantly decreased in the cerebellum of all young males that were exposed to manganese, in the olfactory bulb upon exposure to the low and medium doses of $MnSO_4$, and in the hippocampus upon exposure to the high dose of $MnSO_4$ (*see* Table 2). Old males that were exposed to the low dose of $MnSO_4$ had a statistically significant decrease in GS mRNA in the hypothalamus, whereas those exposed to the high dose of $MnSO_4$ had a statistically significant decrease in GS mRNA in the hippocampus. The female rats exposed to manganese phosphate had a statistically significant increase in GS mRNA levels in the hypothalamus (*see* Table 2).

Table 2
GS mRNA Levels Expressed as Percent of Control ± SD in Cerebellum (CB), Hippocampus (HC), Hypothalamus (HYP), Olfactory Bulb (OB), and Striatum (ST)

		Low Dose	Med Dose	High Dose	Med Dose
		$MnSO_4$	$MnSO_4$	$MnSO_4$	$MnPO_4$
MALE	CB	64.2±1.3*	32.5±3.1*	70±15.3*	71.5±10*
	HC	80.8±29.4	73.7±45.5	28.4±8.8*	88.4±14
	HYP	74.5±35.2	74.5±31.8	73.4±11.9	63.3±13.5
	OB	70.6±9.7*	54.9±8.1*	83.3±18.7	89.5±12.1
	ST	86.1±37.9	86.5±19.3	95.9±34.5	95.1±4.2
FEMALE	CB	28.9±13.3	68.7±56.2	149.9±8.3	74.7±24.4
	HC	90.3±6.7	90.2±6.8	96.9±4.4	99.3±7.9
	HYP	116.5±14.6	115.4±13.6	90.4±9	120.7±4.8*
	OB	98.9±6.3	100.3±12.9	96.7±18.5	115.5±14.5
	ST	69.7±13.6	85.6±13.3	90.1±4.8	101.9±23.9
OLD MALE	CB	82.4±10.3	112.6±14.2	114.7±3.1	93.9±13.6
	HC	87.4±6.7	85.2±5.5	44.7±0.9*	77.9±16.4
	HYP	67.2±1.5*	100.5±2.8	101.5±6.7	101.9±14.9
	OB	101.3±14.7	110.1±5.1	114.8±11.1	92.5±3.4
	ST	99.3±11.1	87.1±4.6	108.6±9.1	104.9±5.5

Note: The asterisk (*) indicates a statistically significant difference from control, $p<0.05$.

MT mRNA

The MT mRNA levels were significantly decreased in the cerebellum of all young males that were exposed to manganese, in the olfactory bulb upon exposure to the low and medium doses of $MnSO_4$, and in the hippocampus upon exposure to the high dose of $MnSO_4$ (Table 3). In the old males, MT mRNA was significantly decreased in the olfactory bulb and hippocampus upon exposure to the medium dose of manganese phosphate and medium and high doses of $MnSO_4$, respectively. Finally, the female rats exposed to the high dose of $MnSO_4$ had significantly decreased MT gene expression in the hypothalamus (*see* Table 3).

Table 3
MT mRNA Levels Expressed as Percent of Control ± SD in Cerebellum (CB), Hippocampus (HC), Hypothalamus (HYP), Olfactory Bulb (OB), and Striatum (ST)

		Low Dose $MnSO_4$	Med Dose $MnSO_4$	High Dose $MnSO_4$	Med Dose $MnPO_4$
MALE	CB	68.4±2.5*	36.1±2.9*	73.4±14*	76.1±11*
	HC	91.9±33.9	88.3±61.6	22.6±5.8*	88.8±49.7
	HYP	138.1±118	105.8±43	118.1±58	160.5±25
	OB	69.2±9.2*	49.5±8.3*	81.9±18.3	87.7±12.6
	ST	130.7±48	97.2±24	151.9±19	101.8±50
FEMALE	CB	126.7±11	97.6±13.9	82.2±2.7	80.1±6.9
	HC	102.4±6.7	111.1±6	92.8±4.5	100.3±5
	HYP	101.7±9	85.2±1.1	57.6±20*	81.6±7.4
	OB	103.5±11	81.1±11.3	74.8±5.1	81.1±12.9
	ST	98.2±9.6	79.1±8.7	80.6±12	95.4±7.1
OLD MALE	CB	85.6±13.2	84.6±14.7	107.1±12	87.9±8.6
	HC	81.1±7.7	63.6±3.2*	79.7±2.5*	49.3±3.3*
	HYP	87.3±14.1	110.4±5	151.4±8	93.9±11.7
	OB	88.5±22.9	88.1±15.6	93.9±6.9	63.3±1.1*
	ST	94.5±0.3	111.7±16	131.9±13	91±15.2

Note: The asterisk (*) indicates a statistically significant difference from control, $p<0.05$.

GSH

All four manganese treatments significantly decreased GSH levels in the olfactory bulb of young male rats (*see* Table 4). Female rats exposed to all levels of manganese had significant reductions in GSH levels in the striatum compared to controls, but had significantly elevated levels of GSH in the olfactory bulb in those exposed to the medium and high doses of MnSO4. Old male rats exposed to low, medium, and high doses of MnSO4 had a statistically significant decrease in GSH levels in the striatum, whereas the high dose of MnSO4 and manganese phosphate exposure caused a statistically significant decrease in GSH levels in the cerebellum. Finally, in the senescent male rats, the manganese phosphate treatment caused significantly decreased GSH levels in the hypothalamus (*see* Table 4).

DISCUSSION

The current inhalation reference concentration (RfC) for manganese, as set by the United States Environmental Protection Agency, is 0.05 μg Mn/m^3 *(27).* Thus, the concentrations of manganese used in this study were 200, 2000, and 10,000 times this standard for the low, medium (both sulfate and phosphate), and high doses, respectively. The accumulation of manganese in three brain regions from exposed rats was assessed, and the relative concentrations revealed that the olfactory bulb accumulated the most manganese, followed by the striatum, and then the cerebellum for young male and female rats, as well as the senescent males *(22).* Although the pattern of manganese accumulation was similar, there was a marked difference in brain regional manganese concentrations among the three groups *(22).*

Table 4
GSH Levels Expressed as Percent of Control ± SD in Cerebellum (CB), Hippocampus (HC), hypothalamus (HYP), Olfactory Bulb (OB), and Striatum (ST)

		Low Dose $MnSO_4$	Med Dose $MnSO_4$	High Dose $MnSO_4$	Med Dose $MnPO_4$
MALE	CB	94.8±10.6	82.4±31.6	92.8±17.6	63.9±14.1
	HC	76.7±6.1	136.4±40.1	75.1±25.6	153.1±31.1
	HYP	141.6±21.5	162.7±74.7	95.5±10.8	95.5±25.5
	OB	19.6±20*	15.6±2.3*	22.1±5.8*	23.3±0.8*
	ST	60.4±47.1	96.3±42.5	97.6±8.8	64.4±18.1
FEMALE	CB	155.6±11.9	106.8±35.8	108.1±34.5	73.1±41.9
	HC	85.5±4.9	89.9±14.1	80.4±6.4	91.4±11.8
	HYP	83.9±10.6	86.7±15.3	98.9±40.4	123.1±43.8
	OB	218.7±63	201.1±39*	209.9±44*	63.95±29.5
	ST	44.6±8.6*	32.1±7.2*	22.2±7.1*	36.3±15*
OLD MALE	CB	88.4±3.4	76.9±12.3	54.7±8.3*	58.1±15*
	HC	85.5±6.8	85.6±8.5	101.3±32.2	97.2±5.3
	HYP	108.9±43.8	94.1±9.2	77.4±10.5	60.3±4.6*
	OB	72.2±20.1	74.2±30.7	68.8±15.9	90.7±19.1
	ST	65.2±10*	28.1±14*	76.8±6.9*	73.6±14.9

Note: The asterisk (*) indicates a statistically significant difference from control, $p<0.05$.

Specifically, the old male and young female rats had a significant increase in olfactory bulb manganese concentrations as a result of manganese exposure, but only the aged males exposed to the high dose of $MnSO_4$ had statistically increased manganese concentrations in the striatum and cerebellum and not the female rats *(22).* Exposure to the 0.1-mg Mn/m^3 level of manganese phosphate caused a significant increase in manganese concentrations in the olfactory bulb of young and old males, but not female rats. Furthermore, this exposure did not cause significant increases in manganese concentration in the striatum or cerebellum in any of the tested groups. Manganese concentrations of the hypothalamus and hippocampus were not measured in this study *(22).*

One can detect the presence of oxidants by measuring species that are known to increase or decrease in response to oxidative stress. This includes ubiquitous antioxidants, such as GSH, as well as species that are more specific to particular tissue types (e.g., glutamine synthetase [GS]). In the central nervous system (CNS), GS is localized exclusively in astrocytes *(28),* where it has a critical role in amino acid metabolism. GS metabolizes glutamate that is removed by astrocytes from the extracellular space to glutamine, and the latter is recycled to neurons as part of the glutamate–glutamine cycle *(29).* GS is highly susceptible to oxidation and subsequent rapid degradation; therefore, it serves as an excellent marker for the presence of reactive oxygen species in the brain *(30).*

The results of this study showed that GS protein levels increased considerably in the hippocampus of manganese phosphate-exposed young male rats (*see* Table 1). Conversely, manganese exposure caused a dramatic decrease in GS protein in the hypothalamus of these rats (*see* Table 1). Although manganese concentration was not assessed in these two brain regions, there was no effect of increased manganese on GS protein levels in the cerebellum and olfactory bulb of young male rats in which manganese concentrations increased because of exposure *(22).* Female rats exposed to the high dose of $MnSO_4$ had significantly increased GS protein in the olfactory bulb even without concomitant increases in manganese levels, whereas the aged male rats exposed to the medium dose of $MnSO_4$ had a statistically significant decrease in GS protein in the hippocampus and hypothalamus (*see* Table 1). Analogous to the young male rats, there was no correlation between the brain regions that accumulated manganese in the old rats and the noted alterations in GS protein levels in these rats. Corroborating earlier findings *(19,20),* the GS mRNA levels (*see* Table 2) measured in this

study was not reflected in GS protein (i.e., brain regions that had increased mRNA levels did not have increased protein levels).

The MTs are a class of highly conserved proteins known to bind metals, but in recent years, evidence has shown that they might also have some important antioxidant properties *(31).* It has been suggested that MTs can neutralize reactive oxygen species through oxidative release of zinc from MT thiolate clusters *(32).* In vitro experiments demonstrated that MTs had a greater ability at scavenging oxygen radicals when compared with other sulfhydryl-containing molecules *(33).* Furthermore, when two different mitochondrial-specific reactive oxygen species generators were applied to cultured cells, MTs increased more than GSH, Mn-SOD (superoxide dismutase), catalase, and other well-known antioxidants *(34).* As with GS mRNA levels, we found that MT mRNA levels were significantly decreased in the cerebellum, hippocampus, and olfactory bulb of exposed young male rats (*see* Tables 2 and 3, respectively). The female rats had significantly decreased MT mRNA levels in the hypothalamus because of the high dose of $MnSO_4$, whereas old male rats had a dose-dependent decrease in MT mRNA in the hippocampus (*see* Table 3).

Glutathione is a ubiquitous antioxidant formed from three amino acids (glutamate, cysteine, and glycine [γ-glutamylcysteinylglycine]). It constitutes approx 90% of the intracellular nonprotein thiols *(35)* and functions in conjugation and elimination of toxic molecules, thereby maintaining cellular redox homeostasis *(35).* In this study, GSH levels were significantly lowered in the striatum of female and old male rats upon manganese exposure (*see* Table 4). This effect was absent in the young male rats, which is consistent with our previous study *(19).* Our data support the findings of a previous study that showed decreased GSH levels in the striatum of aged rats exposed to manganese chloride *(21).* It has been reported that there is an age-dependent decrease in dopaminergic neurons that occurs with aging *(36).* Therefore, there are fewer "reserve" neurons in the striatum of old rats to maintain GSH homeostasis during manganese exposure. Another possibility is that GSH peroxidase activity is increased under these conditions, which could cause decreased tissue GSH levels. This effect is a plausible explanation for the statistically significant decrease in GSH in the striatum of manganese-exposed young female rats, a brain region that is reportedly unaffected by manganese in young male rats *(19,20).* It has been shown that sex hormones regulate GSH-dependent enzymes in rats (e.g., GSH peroxidase activity is increased in brain tissue when progesterone levels are high) *(37).* This paradoxical effect of manganese exposure is potentially the results of differences in sex hormone levels between the females and males *(36).*
In conclusion, we report for the first time the consequences of airborne manganese exposure on end points of oxidative stress in young female and old male rats. The data suggest an effect of manganese on end points of oxidative stress that are both age dependent and sex dependent. The striatum is a prime example of this observation, for both this study and previous studies *(19,20)* have shown that manganese exposure in young male rats has no effect on markers of oxidative stress. However, both female and aged rats responded to the same manganese exposure with a significant reduction in striatal GSH levels. These data illustrate the importance of considering the gender and age of subjects when assessing the consequences of manganese exposure.

REFERENCES:

1. L. S. Hurley and C. L. Keen, Manganese, in *Trace Elements in Human Health and Animal Nutrition,* E. Underwood and W. Mertz, eds., Academic, New York, pp. 185–223 (1987).
2. M. Aschner, K. M. Erikson, and D. C. Dorman, Manganese dosimetry: species differences and implications for neurotoxicity, *Crit. Rev. Toxicol.,* in press.
3. ATSDR (Agency for Toxic Substances and Disease Registry), Toxicological profile for manganese, U.S. Department of Health And Human Services Public Health Service (available at http://www.atsdr.cdc.gov/toxprofiles/tp151.html), September 2000.
4. D. Mergler, G. Huel, R. Bowler, et al., Nervous system dysfunction among workers with long-term exposure to manganese, *Environ. Res.* 64,151–180 (1994).
5. P. K. Pal, A. Samii, and D. B. Calne, Manganese neurotoxicity: a review of clinical features, imaging and pathology, *Neurotoxicology* 20, 227–238 (1999).

6. E. D. Pellizzari, C. A. Clayton, C. E. Rodes, et al., Particulate matter and manganese exposures in Indianapolis, Indiana, *J. Expo. Anal. Environ. Epidemiol.* 11, 423–440 (2001).
7. M. Aschner, Manganese neurotoxicity and oxidative damage, in *Metals and Oxidative Damage in Neurological Disorders*, J. R. Connor, ed. Plenum, New York, pp. 77–93 (1997).
8. W. N. Sloot, J. Korf, J. F. Koster, et al., Manganese-induced hydroxyl radical formation in rat striatum is not attenuated by dopamine depletion or iron chelation in vivo, *Exp. Neurol.* 138,236–245 (1996).
9. P. Galvani, P. Fumagalli, and A. Santagostino, Vulnerability of mitochondrial complex I in PC12 cells exposed to manganese, *Eur. J. Pharmacol.* 293, 377–383 (1995).
10. C. E. Gavin, K. K. Gunter, and T. E. Gunter, Manganese and calcium transport in mitochondria: implications for manganese toxicity, *Neurotoxicology* 20, 445–453 (1999).
11. F. S. Archibald and C. Tyree, Manganese poisoning and the attack of trivalent manganese upon catecholamines, *Arch. Biochem. Biophys.* 256, 638–650 (1987).
12. S. F. Ali, H. M. Duhart, G. D. Newport, G. W. Lipe, et al., Manganese-induced reactive oxygen species: comparison between Mn^{+2} and Mn^{+3}, *Neurodegeneration* 4, 329–334 (1995).
13. J. Y. Chen, G. C. Tsao, Q. Zhao, et al., Differential cytotoxicity of Mn(II) and Mn(III): special reference to mitochondrial [Fe–S] containing enzymes, *Toxicol. Appl. Pharmacol.* 175,160–168 (2001).
14. K. K. Gunter, L. M. Miller, M. Aschner, et al., XANES spectroscopy: a promising tool for toxicology: a tutorial, *Neurotoxicology* 23,127–146 (2002).
15. D. HaMai A. Campbell, and S. C. Bondy, Modulation of oxidative events by multivalent manganese complexes in brain tissue, *Free Radical Biol. Med.* 31, 763–768 (2001).
16. V. Anantharam, M. Kitazawa, J. Wagner, et al., Caspase-3-dependent proteolytic cleavage of protein kinase C delta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl, *J. Neurosci.* 22,1738–1751 (2002).
17. D. R. Lynam, J. W. Roos, G. D. Pfeifer, et al., Environmental effects and exposures to manganese from use of methylcyclopentadienyl manganese tricarbonyl (MMT) in gasoline, *Neurotoxicology* 20,145–150 (1999).
18. J. Zayed, B. Hong, and G. L'Esperance, Characterization of manganese-containing particles collected from the exhaust emissions of automobiles running with MMT additive, *Environ. Sci. Technol.* 33,3341–3346 (1999).
19. A. W. Dobson, S. Weber, D. C. Dorman, et al., Inhaled manganese sulfate and measures of oxidative stress in rat brain, *Biol. Trace Elemement Res.* 93,113–126 (2003).
20. Weber, D. C. Dorman, L. H. Lash, et al., Effects of manganese (Mn) on the developing rat brain: oxidative-stress related endpoints, *Neurotoxicology* 23,169–175 (2002).
21. M. S. Desole, G. Esposito, R. Mighelli, et al., Cellular defence mechanisms in the striatum of young and aged rats subchronically exposed to manganese, *Neuropharmacology* 34,289–295 (1995).
22. D. C. Dorman, B. E. McManus, M. W. Marshall, et al., Old age and gender influence pharmacokinetics of inhaled manganese sulfate and manganese phosphate in rats, *Toxicol. Appl. Pharmacol.* 197,113–124 (2004).
23. V. Barbu and F. Dautry, Northern blot normalization with a 28S rRNA oligonucleotide probe, *Nucleic Acids Res.* 17, 7115 (1989).
24. M. W. Fariss and D. J. Reed, High-performance liquid chromatography of thiols and disulfides: dinitrophenol derivatives, *Methods Enzymol.* 143,101–109 (1987).
25. L. H. Lash and J. J. Tokarz, Oxidative stress in isolated rat renal proximal and distal tubular cells, *Am. J. Physiol.* 259, F338-F347 (1990).
26. L. H. Lash and E. B. Woods, Cytotoxicity of alkylating agents in isolated rat kidney proximal and distal tubular cells, *Arch. Biochem. Biophys.* 286,46–56 (1991).
27. J. M. Davis, Inhalation health risks of manganese: an EPA perspective, *Neurotoxicology* 20,511–518 (1999).
28. A. Martinez-Hernandez, K. P. Bell, and M. D. Norenberg, Glutamine synthetase: glial localization in the brain, *Science* 195,1356–1358 (1977).
29. U. Sonnewald, N. Westergaard, and A. Schousboe, Glutamate transport and metabolism in astrocytes, *Glia* 21, 56–63 (1997).
30. E. R. Stadtman, Protein oxidation and aging, *Science* 257,1220–1224 (1992).
31. M. Aschner, The functional significance of brain metallothioneins, *FASEB J.* 10, 1129–1136 (1996).

32. P. Hainut and J. Milner, Redox modulation of p53 conformation and sequence-specific DNA binding in vitro, *Cancer Res.* 53, 4469–4473 (1993).
33. S. Hussain, W. Slikker, Jr. , and S. F. Ali, Role of metallothionein and other antioxidants in scavenging superoxide radicals and their possible role in neuroprotection, *Neurochem. Int.* 29,145–152 (1996).
34. M. Kondoh, Y. Inoue, S. Atagi, et al., Specific induction of metallothionein synthesis by mitochondrial oxidative stress, *Life Sci.* 69, 2137–2146 (2001).
35. A. Meister and M. E. Anderson, Glutathione, *Annu. Rev. Biochem.* 52, 711–760 (1983).
36. Y. Chu, K. Kompoliti, E. J. Cochran, et al., Age-related decreases in Nurr-1 immunoreactivity in the human substantia nigra, *J. Comp. Neurol.* 450, 203–214 (2002).
37. S. B. Pajovic, Z. S. Saicic, M. B. Spasic, et al., Effects of progesterone and estradiol benzoate on glutathione dependent antioxidant enzyme activities in the brain of female rats, *Gen. Physiol. Biophys.* 18,35–44 (1999).