Persistent Alterations in Biomarkers of Oxidative Stress Resulting from Combined *In Utero* and Neonatal Manganese Inhalation

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Abstract:
Neonatal female and male rats were exposed to airborne manganese sulfate (MnSO4) during gestation and postnatal d 1–18. Three weeks post-exposure, rats were killed and 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. Overall, there was a statistically significant effect of manganese exposure on decreasing brain GS protein levels (p=0.0061), although only the highest dose of manganese (1 mg Mn/m^3) caused a significant increase in GS messenger RNA (mRNA) in both the hypothalamus and olfactory bulb of male rats and a significant decrease in GS mRNA in the striatum of female rats. This highest dose of manganese had no effect on MT mRNA in either males or females; however, the lowest dose (0.05 mg Mn/m^3) decreased MT mRNA in the hippocampus, hypothalamus, and striatum in males. The median dose (0.5 mg Mn/m^3) led to decreased MT mRNA in the hippocampus and hypothalamus of the males and olfactory bulb of the females. Overall, manganese exposure did not affect total GSH levels, a finding that is contrary to those in our previous studies. Only the cerebellum of manganese-exposed young male rats showed a significant reduction (p<0.05) in total GSH levels compared to control levels. These data reveal that alterations in biomarkers of oxidative stress resulting from *in utero* and neonatal exposures of airborne manganese remain despite 3 wk of recovery; however, it is important to note that the doses of manganese utilized represent levels that are 100- fold to a 1000-fold higher than the inhalation reference concentration set by the US Environmental Protection Agency.
Index Entries: Rat; manganese; brain; *in utero;* glutathione; glutamine synthetase; metallothionein.

Article:

INTRODUCTION

Recently, our group has reported the responses of several markers of oxidative stress resulting from manganese exposure in several rat models *(1–3).* These models include young male and female rats as well as senescent male rats exposed to manganese for periods of time ranging from 3 to 13 wk. The data reported herein explore the effects of *in utero* and neonatal manganese inhalation on oxidative stress markers in several rat brain regions. Manganese is an essential trace metal that is found in all tissues and is required for normal amino acid, lipid, protein, and carbohydrate metabolism *(4).* Although manganese deficiency is extremely rare in humans, toxicity resulting from overexposure to manganese is more prevalent and the brain appears to be especially vulnerable. Manganese neurotoxicity is most commonly associated with occupational exposure to aerosols or dusts that contain extremely high levels (> 1–5 mg Mn/m^3) of manganese, consumption of contaminated well water, or parenteral nutrition therapy in patients with liver disease *(5–8).*

Attention to airborne manganese has increased because of the use of the antiknock agent methylcyclopentadienyl manganese tricarbonyl (MMT) in some unleaded gasolines, 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 *(9).* It has been speculated that chronic low-level manganese exposure might play a role in the pathogenesis of neurodegenerative disorders, especially in susceptible populations.

Oxidative stress has been implicated as a contributing mechanism by which manganese might be cytotoxic *(10).* The oxidation of dopamine by manganese is a potential mechanism by which manganese-induced oxidative stress could occur, especially because manganese accumulates in dopamine-rich brain regions of rodents and primates (e.g., basal ganglia) *(11).* 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 MnCl2 *(12).* 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 *(13).* It has been shown that trivalent manganese is more effective at inhibiting complex I *(14–16),* but the divalent form is, by far, the predominant species within cells and is largely bound to ATP *(15,17).* Nevertheless, in biological media, manganese of any valence will spontaneously give rise to infinitesimal amounts of trivalent manganese, and HaMai et al. *(18)* demonstrated that even at trace amounts, trivalent manganese can cause formation of reactive oxygen species.

This present study extends the previous observations by examining the interactions of sex on the reversibility of altered end points of oxidative stress as a result of manganese exposure in select brain regions. The rats in the present study were exposed to levels of manganese similar as those in one of our earlier studies *(1).* Finally, the developmental time-point of the exposure is a novel aspect of this study, furthering the understanding of neurological consequences of manganese exposure.

MATERIALS AND METHODS

Experimental Design

Adult (F0 male and female rats) CD rats (10 rats/ gender/ exposure concentration) were exposed to either air or $MnSO_4$ (0.05, 0.5, or 1 mg Mn/m^3) beginning 28 d prior to breeding and for up to 14 d during the mating period. Presumed pregnant female rats were exposed to either air or $MnSO_4$ from gestation day (GD) 0 through GD 19. Inhalation exposures were not conducted when parturition was expected to occur (i.e., after GD 19). Lactating rats and their pups were then concurrently exposed to either air or MnSO4 beginning 1 d after parturition (day of parturition was designated as postnatal day [PND] 0) using methods described by Dorman et al. *(19).* Inhalation exposure continued from PND 1 through PND 18. Oxidative stress end points were determined in the remaining offspring (whenever possible, n = 1 rat/ sex/ exposure concentration/time-point) on PND 45 ± 1.

Chemicals

Manganese(II) sulfate monohydrate ($MnSO_4 \cdot H_2O$) (CAS Registry no. 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% manganese. All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted and were of the highest possible quality.

Animals

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 (n=40) and female (n=40) CD rats were purchased from Charles River Laboratories, Inc. (Raleigh, NC). 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.

Breeding Procedures

The parental generation was manganese exposed by inhalation 7 d per week, 6 h per day, prior to breeding (see next section for exposure details). The rats were mated after the daily exposure (female added to male's cage) and separated in the morning for the next exposure day. Each pair was selected randomly within each exposure group for a period of up to 14 d, with no change in mating partner. Females were examined daily after each cohabitation period for a copulation plug or sperm-positive smear, which indicated successful mating, and was designated gestation day 0 (GD 0).

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. $MnSO_4$ atmospheres were generated and characterized using methods described by Dorman et al. *(19).* Nominal $MnSO_4$ exposure concentrations of 0.05, 0.5, and 1 mg Mn/m^3 were used in this study. The target particle size distribution was 1.5–2 µm mass median aerodynamic diameter (MMAD) with a geometric standard deviation (GSD) < 2. Control groups were exposed to HEPA-filtered air only. The overall means (± SD) for the chamber concentrations based on daily optical particle sensor data were 0.001 ± 0.000, 0.157 ± 0.011, 1.50 ± 0.10, and 3.03 ± 0.18 mg/m^3 for the target exposure concentrations of 0, 0.15, 1.53, and 3.10 mg $MnSO_4$/m^3, corresponding to 0, 0.05, 0.5, and 1 mg Mn/m^3, respectively. The particle size distribution was 1.03 µm geometric mean diameter (GMD) and 1.52 GSD, 1.05 µm GMD (GSD = 1.53), and 1.07 µm GMD (GSD = 1.55) for the target concentrations of 0.150, 1.53, and 3.10 mg $MnSO_4$/m^3, respectively.

Tissue Collection

At the termination of exposures, euthanasia (with CO2) 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 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 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 metallothionein (MT) or glutamine synthesase (GS), 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, 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 [α-^{32}P]dCTP-labeled 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 total loaded RNA level, the blots were stripped in 0.1X SSC/0.1%SDS/40 m*M* Tris-HCl buffer and probed for 28s rRNA *(20).*

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* TrisHCl, 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 *(21)* 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 *(22,23)*.

Statistical Analysis

The data were analyzed using the SAS system v8.2 (SAS Institute, Cary, NC). Data were examined for normality of distribution and presence of outliers. Analysis of variance with repeated-measures factors (brain regions) and between-groups factors was used to test for interactions between manganese exposure and sex. When the overall significance resulted in rejection of the null hypothesis ($p < 0.05$), the Tukey's HSD post-hoc test was used to evaluate mean differences.

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

	Brain Region	0.05 mg Mn/m^3	0.5 mg Mn/m^3	1.0 mg Mn/m^3
		(n=8)	(n=8)	(n=8)
MALE	**CB**	88.8±2.2	79.4±2.9	87.6±4.5
	HC	89.5±4.9	94.1±4.2	96.4±3.6
	HYP	80.1±14.5	81.8±10.0	89.1±11.2
	OB	98.2±2.9	90.4±4.7	101±6.2
	ST	94.6±3.3	85.1±3.0	92.3±3.9
FEMALE	**CB**	94.7±5.8	99±6.6	94±3.9
	HC	89.7±3.5	86.2±5.1	87.1±4.3
	HYP	95.7±3.9	95.3±3.8	86.9±3.3
	OB	90.5±2.8	83.9±7.1	90.2±3.1
	ST	90±2.5	91.2±2.9	89.5±3.5

Note: There was a significant overall effect (p=0.0061) of manganese exposure on decreasing GS protein levels compared to control.

RESULTS

GS Protein and mRNA

Although manganese exposure had an overall effect on significantly decreasing GS protein in both male and female rats (p=0.0061) (Table 1), GS mRNA levels were affected more selectively (Table 2). For example, GS mRNA levels significantly decreased in the olfactory bulb (lowest and median dose) (p=0.004) and striatum (all three doses) of manganese-exposed F_1 female rats (p=0.012), but these two brain regions were unaffected in F_1 male rats (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)

	Brain Region	0.05 mg Mn/m^3	0.5 mg Mn/m^3	1.0 mg Mn/m^3
		(n=8)	(n=8)	(n=8)
MALE	**CB**	92.1±5.1	94.2±5.9	81.4±9.8
	HC	90.1±1.3	86.2±2.1	100±3.4
	HYP	94.4±2.9	101±3	119±1.9*
	OB	106±7.3	117.4±4.3	132±3.5*
	ST	97.7±3.3	100.1±2.2	99±2.7
FEMALE	**CB**	98.8±2.5	97.6±3.4	100.1±5.1
	HC	96.7±0.6	96.7±0.98	114±3.1
	HYP	100±3.3	102±2.5	105±1.2
	OB	82.1±4.1*	82.5±5.9*	98±2.8
	ST	84.7±3.6*	72.6±5.3*	76.8±1.3*

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

MT mRNA

The MT mRNA levels were significantly decreased in the hippocampus and hypothalamus of F_1 males that were exposed to the lowest and median doses of manganese ($p<0.05$), but those exposed to the highest dose of manganese displayed unchanged MT mRNA in these two regions (Table 3). The F_1 female rats exposed to manganese were unaffected in terms of MT mRNA levels except in the striatum, where there was a significant decrease in mRNA levels across all exposure doses (p=0.002) (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)

	Brain Region	0.05 mg Mn/m^3	0.5 mg Mn/m^3	1.0 mg Mnm^3
		(n=8)	(n=8)	(n=8)
MALE	**CB**	114±8.7	121±10.7	99±7.8
	HC	57±3.9*	52.9±4.1*	94±14.5
	HYP	59.4±9.1*	50±5.5*	80.2±7.5
	OB	103±8.6	108.4±15	120.1±15
	ST	67.1±7.5	123.1±23	85.2±21.2
FEMALE	**CB**	91±4.7	99.4±5.8	102±4.6
	HC	93.4±8.4	93.4±10.4	140±15.6
	HYP	80.2±7.4	96.7±7.1	112±8.9
	OB	120.1±9.9	64.7±8.6	131.4±21
	ST	85.2±6.5*	82.3±6.1*	83.3±6.2*

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

GSH

All three manganese treatments significantly decreased GSH levels in the olfactory bulb of F_1 female rats (p=0.047) but not in the male rats (Table 4). Conversely, all three manganese treatments significantly decreased GSH levels in the cerebellum of the F_1 male rats (p=0.018) but not in their female littermates (Table 4).

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

	Brain Region	0.05 mg Mn/mm^3	0.5 mg Mn/mm^3	1.0 mg Mn/mm^3
		(n=8)	(n=8)	(n=8)
MALE	**CB**	87.1±6.2*	80.1±7.6*	64.7±10*
	HC	93.8±11.2	98.1±4.6	119.1±14
	HYP	74.2±7.4	80.4±14.1	104.6±14
	OB	134.1±32	120.2±23	188.9±44
	ST	83.3±10.9	96.1±19.5	74±10.5
FEMALE	**CB**	116±5.8	101±4.9	122±6.2
	HC	111.7±3.3	108.2±8.4	108.2±8.4
	HYP	82±12.1	87.7±13.3	75.1±15.3
	OB	86.2±5.6*	79.6±8.5*	78.4±11*
	ST	146.4±23	111±24.7	93.8±19.1

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

DISCUSSION

The current inhalation reference concentration (RfC) for manganese, as set by the US Environmental Protection Agency, is 0.05 µg Mn/m^3 *(24).* Thus, the concentrations of manganese used in this study were 1000, 10,000, and 20,000 times this standard for the lowest, median, and highest doses, respectively. Manganese concentrations in the olfactory bulb, striatum, and cerebellum of rats that were exposed similarly to manganese sulfate have been reported *(25).* Dorman and co-workers *(26)* observed increased brain manganese concentrations in PND 14 and PND 19 pups that were exposed to manganese sulfate at > 0.05 mg Mn/m^3. Increased striatal, olfactory bulb, and cerebellum concentrations were approx twofold to threefold higher than those observed in controls. Adult (F_1) rats that were exposed as neonates to $MnSO_4$ had returned to control brain manganese concentrations when assessed at PND 45 ± 1 *(26).* When a similar paradigm of manganese exposure followed by a recovery period was administered to older rats, manganese concentrations in some brain regions, in particular the olfactory bulb, remained elevated when compared to air-exposed rats *(19).* However, in the present study, younger rats that were exposed to manganese sulfate *in utero* and during lactation fully normalized their brain regional manganese concentrations by PND 45 (i.e., 3 wk after exposure sessions ended). It is likely that this ability to normalize brain manganese concentrations reflects the increased requirements of the developing rat brain *(27).*

This study presents novel data that show alterations in oxidative stress markers in specific brain regions of developing male and female rats after 3 wk of recovery from *in utero* and neonatal exposures to airborne manganese. Furthermore, these changes in brain regional oxidative stress markers occurred in the absence of increased brain manganese concentrations and were in contrast to our earlier studies conducted in adult rats *(1–3).*

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 biomarkers that are more specific to particular tissue types (e.g., glutamine synthetase). In the central nervous system, GS is localized exclusively in astrocytes *(28),* where it has a critical role in amino acid metabolism. Glutamine synthetase 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).* Glutamine synthetase is highly susceptible to oxidation and subsequent rapid degradation and, therefore, it serves as an excellent marker for the presence of reactive oxygen species in the brain *(30).*

Although GS protein levels decreased in most brain regions of manganese exposed male and female rats, only the cerebellum in the male rats exposed to the median dose had a statistically relevant decrease in GS protein levels compared to nonexposed rats (Table 1) and this decrease occurred despite normal manganese concentrations in this brain region. This is in contrast to our previous studies in which the effects of manganese exposure in older animals of both sexes were more selectively affected [e.g., hippocampus only in male rats and hypothalamus in female rats *(3)*]. Corroborating earlier findings *(1–3),* the GS mRNA levels (Table 2) measured in this study was not reflected in altered amounts of GS protein (i.e., brain regions that had increased mRNA levels did not have increased protein levels). In fact, there were more statistically significant changes in GS mRNA than there were in the protein levels, which is in contrast to our prior studies *(1–3).* There were significant increases in GS mRNA levels in the hypothalamus and olfactory bulb of male rats recovering from the highest manganese exposure, whereas GS mRNA levels were significantly decreased in the striatum of female rats recovering from the median and high exposures (Table 2). Sex differences in the response to manganese exposure were exemplified in the striatum, where the females had significant decreases in GS mRNA but the males were unaffected in this brain region.

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).* In vitro experiments demonstrated that MTs had a greater ability at scavenging oxygen radicals when compared with other sulfhydryl-containing molecules *(32)* and they increased more than glutathione (GSH), Mn-SOD (superoxide dismutase) catalase, and other well-known antioxidants *(33).* We found that MT mRNA levels were significantly decreased in the hippocampus, hypothalamus, and striatum in males recovering from the lowest manganese dose and in the hippocampus and hypothalamus in those recovering from the median dose manganese exposure (Table 3). The only statistically relevant change in the females was in the olfactory bulb of female rats recovering from the median dose (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 *(34)* and functions in conjugation and elimination of toxic molecules, thereby maintaining cellular redox homeostasis *(34).* We previously reported that GSH levels were significantly lowered in the striatum of female and old male rats upon manganese exposure *(3)* and that this effect was absent in the young male rats, which was consistent with our other study *(1).* These data are consistent with the findings of a previous study that showed decreased GSH levels in the striatum of aged rats exposed to manganese chloride *(35).* In this current study, the only statistically significant effect of manganese exposure appeared in the cerebellum of male rats recovering from the highest manganese exposure level (Table 4). This relative lack of an effect of manganese exposure on GSH in these animals likely reflects the reversibility of manganese exposure and/or the plasticity of the developing nervous system in terms of GSH metabolism. Although this is speculative, it is consistent with the critical role GSH plays in the maintenance of redox homeostasis.

In conclusion, we report for the first time the consequences of *in utero* and early developmental airborne manganese exposure on end points of oxidative stress in developing female and male rats. These alterations are not associated with increased manganese as in our prior studies, but these changes persist even after brain regional manganese concentrations return to control levels. Furthermore, the reversibility of these alterations in markers of oxidative stress caused by manganese exposure is influenced by the sex of the rat. Unlike our previous studies *(1–3),* manganese exposure in young male rats had more of an impact on oxidative stress end points than it did in female rats. We cannot determine whether this difference is the result of a lack of reversibility in the male rats or whether markers of oxidative stress in the female rat are less affected by manganese exposure. It is noteworthy that these effects occurred at doses of manganese 10–20 thousand times higher than the current RfC. We are presently undertaking a study in which we will specifically examine some of these reversibility issues.

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