Alterations of Oxidative Stress Biomarkers Due to *In Cuero* and Neonatal Exposures of Airborne Manganese

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Erikson, K.M., Dorman, D.C., Fitsanakis, V., Lash, L.H., Aschner, M. (2006) Alterations of oxidative stress biomarkers due to *In utero* and neonatal exposures of airborne manganese. Biol Trace Elem Res 111:199-215

Abstract:
Neonatal rats were exposed to airborne manganese sulfate ($MnSO_4$) (0, 0.05, 0.5, or 1.0 mg Mn/m^3) during gestation (d 0–19) and postnatal days (PNDs) 1–18. On PND 19, 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) and tyrosine hydroxylase (TH) protein levels, metallothionein (MT), TH and GS mRNA levels, and reduced and oxidized glutathione (GSH and GSSG, respectively) levels were determined for all five regions. Mn exposure (all three doses) significantly (p = 0.0021) decreased GS protein levels in the cerebellum, and GS mRNA levels were significantly (p = 0.0008) decreased in the striatum. Both the median and high dose of Mn significantly (p = 0.0114) decreased MT mRNA in the striatum. Mn exposure had no effect on TH protein levels, but it significantly lowered TH mRNA levels in the olfactory bulb (p = 0.0402) and in the striatum (p = 0.0493). Mn exposure significantly lowered GSH levels at the median dose in the olfactory bulb (p = 0.0032) and at the median and high dose in the striatum (p = 0.0346). Significantly elevated (p = 0.0247) GSSG, which can be indicative of oxidative stress, was observed in the cerebellum of pups exposed to the high dose of Mn. These data reveal that alterations of oxidative stress biomarkers resulting from *in utero* and neonatal exposures of airborne Mn exist. Coupled with our previous study in which similarly exposed rats were allowed to recover from Mn exposure for 3 wk, it appears that many of these changes are reversible. It is important to note that the doses of Mn utilized represent levels that are a hundred- to a thousand-fold higher than the inhalation reference concentration set by the United States Environmental Protection Agency.
Index Entries: Rat; manganese; brain; *in utero;* glutathione; glutamine synthetase; metallothionein; tyrosine hydroxylase.

Article:
INTRODUCTION
Recently, our group reported the responses of several markers of oxidative stress as a result of manganese (Mn) exposure in rats *(1–4).* These included neonates, developing male and female rats, and senescent male rats exposed to Mn for periods of time ranging from 3 to 13 wk. The data reported herein explore the effects of *in utero* (gestational days [GDs] 0–18) and neonatal (postnatal days [PNDs] 0–18) Mn inhalation on oxidative stress markers in distinct brain regions of PND 19 rats.

Manganese is an essential trace metal found in all tissues and it is required for normal amino acid, lipid, protein, and carbohydrate metabolism *(5).* Although Mn deficiency is extremely rare in humans, toxicity resulting from overexposure of Mn is more prevalent. The brain appears to be especially vulnerable. Mn neurotoxicity 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 *(6–9).*

Attention to airborne particulate Mn has increased because of the use of the antiknock agent ethylcyclopentadienyl manganese tricarbonyl (MMT) in some unleaded gasolines, although the level of Mn because of MMT in urban areas does not exceed tolerable levels set by the governments of Canada and the United States *(10).* It is speculated that chronic low-level Mn exposure might play a role in the pathogenesis of neurodegenerative disorders, especially in susceptible populations (see ref. *6* for a review).

Oxidative stress has been implicated as a contributing mechanism by which Mn might be cytotoxic *(11).* The oxidation of dopamine by Mn is a potential mechanism by which Mn-induced oxidative stress could occur, especially because manganese can accumulate in dopamine-rich brain regions of rodents and primates (e.g., basal ganglia) following prolonged exposure *(12).* Another possible mechanism is that Mn, through its sequestration in mitochondria *(13),* 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 manganese chloride *(14).* Another laboratory showed evidence suggesting that the ATPase complex is inhibited at very low levels of mitochondrial Mn and that complex I is inhibited only at higher concentrations *(15).* Although trivalent Mn is more effective in inhibiting complex I *(16–18),* the divalent form is by far the predominant species within cells and is largely bound to ATP *(17,19).* Nevertheless, in biological media, Mn of any valence will spontaneously give rise to small amounts of trivalent MN. Interestingly, HaMai et al. *(20)* demonstrated that even trace amounts of trivalent Mn can cause the formation of ROS.

In our previous studies, we indirectly assessed oxidative stress by measuring levels of antioxidants (glutathione [GSH] or metallothionein [MT]) as well as the abundance of proteins (glutamine synthetase [GS]) that are exquisitely sensitive to oxidative stress. GSH is an ubiquitous antioxidant formed from three amino acids (glutamate, cysteine and glycine) leading to the formation of γ-glutamylcysteinylglycine. It constitutes approx 90% of the intracellular nonprotein thiols and it functions in the conjugation and elimination of toxic molecules, thereby maintaining cellular redox homeostasis *(21).* Alterations in brain GSH metabolism have been linked with oxidative stress and various neurodegenerative diseases *(22).* For example, Sian et al. *(23),* demonstrated decreased GSH levels in the substantia nigra of Parkinson's disease (PD) patients compared to control individuals. Correspondingly, GSH levels are significantly and age-dependently lowered in the striatum of Mn-exposed rats (i.e., older rats have dramatically lower GSH because of Mn exposure, whereas juvenile rats are unaffected) *(3,24).*

The MTs, a class of cysteine-containing intracellular proteins, are highly conserved and widely distributed throughout all cells in an organism. They are important metal-binding proteins, with zinc serving as the primary regulator of MT metabolism in cells *(25–27).* 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 *(28).* Oxidative stress, kainic acid, and 6-hydroxydopamine, a known dopaminergic toxin and ROS generator, induced MT-I gene expression in the brain *(29).* Likewise, compounds that generate free-oxygen species via the redox cycling (e.g., diquat) as well as compounds (e.g., 3-methylindole) that cause lipid peroxidation or that deplete cellular defense mechanisms (e.g., diamide and dimethyl maleate) increase MT in tissue *(30).*

Within the brain, GS is exclusively expressed in astrocytes *(31).* A Mn dependent enzyme, GS catalyzes the formation of glutamine from glutamate. This glutamine is taken up by local glutamatergic or γ-aminobutyric acid-containing (GABA-ergic) neurons where deamination back to glutamate occurs. This process is considered the primary glutamate-recycling pathway in the brain *(32–34).* Inhibition of GS activity can have serious consequences on neuronal functioning (e.g., decreased glutamate and GABA levels; the inability to detoxify ammonia). Given its high susceptibility to oxidation and subsequent rapid degradation, GS serves as an excellent marker for the presence of ROS in the brain *(35).*

This present study extends our previous observations *(1–4)* by examining the aforementioned end points of oxidative stress in select brain regions of rat pups subsequent to *in utero* and neonatal Mn exposure. The rats in

the present study were exposed to similar levels of Mn as those in our last study *(4).* We also analyzed tyrosine hydroxylase (TH) protein and mRNA levels to assess the effect of airborne Mn exposure on catecholamine systems. This is important because a decrease in TH levels could be indicative of altered dopaminergic neuronal functioning and/or cytotoxicity. Finally, examining the impact of airborne Mn exposure in PND 19 rat brains provides a novel developmental aspect of Mn biology, which can further our understanding of the neurological consequences of Mn toxicity.

MATERIALS AND METHODS

Experimental Design

Adult (F0 male and female rats) CD rats (10 rats / gender / Mn 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 up to 14 d during the mating period. Presumed pregnant female rats were continued to receive either air or $MnSO_4$ from GD 0 through GD 19, based on their previous group assignment. Inhalation exposures were not conducted when parturition was expected to occur (i.e., after GD 19). Lactating rats and their pups were then concurrently returned to either air or $MnSO_4$ 1 d after parturition (day of parturition was designated as PND 0) using methods described by Dorman et al. *(36).* Inhalation exposure continued from PND 1 through PND 18 for both dams and pups. Oxidative stress end points were determined in the pups on PND 19.

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% Mn. 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 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, semi-purified AIN-93G certified diet from Bio-Serv (Frenchtown, NJ) formulated to contain approx 10 ppm Mn and 35 ppm iron was given accessible (except during inhalation exposures) throughout the study. Fluorescent lighting was kept on a 12-h light–dark cycle (06:00–18:00). A study day for these exposures was defined as a 6-h exposure, generally from 08:30 to 14:30.

Additional details concerning these animals, their husbandry, and Mn exposure has been recently published *(36).* Briefly, the parental generation was Mn-exposed by inhalation 7 d per week, 6 h per day prior to breeding. Rats were exposed whole body in 8-m^3 Hinners-style, stainless steel and glass inhalation exposure chambers using exposure cage rack units. Nominal $MnSO_4$ exposure concentrations of 0.05, 0.5, and 1 mg Mn/m^3 were used in this study. 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 $MnSO4/m^3$, 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 geometric standard deviations (GSD), 1.05 µm GMD (GSD = 1.53), and 1.07 µm GMD (GSD = 1.55) for 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 CO_2 was carried out in accordance with NIH guidelines. The brain areas of interest (cerebellum, olfactory bulb, and striatum) were dissected out, weighed, 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 extracted with a monophase phenol and guanidine isothiocyanate solution (RNA STAT60; Tel-Test, Inc., Friendswood, TX). For Northern blot analysis, 10 µg of RNA were electrophoresed on a 1.2% denaturing agarose gel and transferred onto a positively charged nylon membrane (Nytran SuPerCharge; Schleicher & Schuell, Keene, NH) overnight by capillary transfer in 10X SSC (1X SSC = 0.15 M sodium chloride, 0.015 *M* sodium citrate) buffer. The RNA was immobilized with an ultraviolet (UV) crosslinker.

For GS, MT, or TH, 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 GS, MT, or TH, 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 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-Hcl buffer and probed for 28s rRNA *(37).*

Protein Isolation and Western Blot Analysis

We utilized our standard Western blotting procedure as previously reported *(1–4).* Briefly, 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). 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 and TH protein was probed with a polyclonal antibody (both from Chemicon, Temecula, CA). Both antibodies were diluted to 1 : 2000, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse (GS protein) or horseradish peroxidase-conjugated goat anti-rabbit (TH protein) secondary antibodies diluted 1 : 2000 (Kirkegaard and Perry Laboratories, Gaithersburg, MD) 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 and GSSG Assays

The GSH and oxidized glutathione (GSSG) assay that we used is described in detail elsewhere *(1–4,22,23).* Briefly, 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-y-glutamyl-L-glutamate.

The mixture was vortexed and centrifuged; an aliquot was then removed for high-performance liquid chromatography (HPLC) analysis *(38)* 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 approx 50 pmol, which equated to approx 0.4 nmol/mg protein *(39,40).*

Statistical Analysis

The data were analyzed using the SPSS system v11.5 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) with repeated-measures factors (brain regions) and between-groups factors was used to test for interactions

between Mn exposure and regions. 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.

RESULTS

Manganese Concentrations

Manganese was significantly elevated (twofold to threefold) in the cerebellum, olfactory bulb, and striatum of PND 19 rats exposed to all three doses of Mn compared to control concentrations and was reported by Dorman et al. *(36).*

GS Protein and mRNA

Manganese exposure at low, medium, and high doses significantly decreased GS protein levels only in the cerebellum ($p = 0.0021$) (Fig. 1A). In contrast, GS mRNA levels were significantly lowered in the striatum ($p = 0.0008$) (Fig. 1b) with no significant change in the other regions compared to controls.

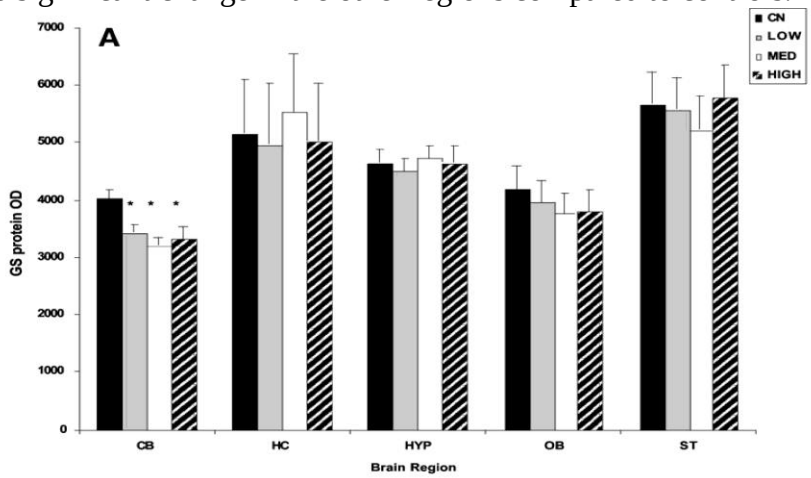

Fig. 1. **(A)** PND 19 rats pups were exposed to 0 mg (CN), 0. 05 mg (LOW), 0. 5 mg (MED), or 1. 0 mg (HIGH) $MnSO_4/m^3$ ($n = 4$–6 rats per group with three replicates each for a total $n = 12$–18). GS protein levels are presented as mean optical density (OD) ± SEM for the cerebellum (CB), hippocampus (HC), hypothalamus (HYP), olfactory bulb (OB), and striatum (ST). Exposure to LOW, MED, and HIGH doses of Mn significantly decreased GS protein in the CB ($p = 0. 0021$) only. *(continues)*

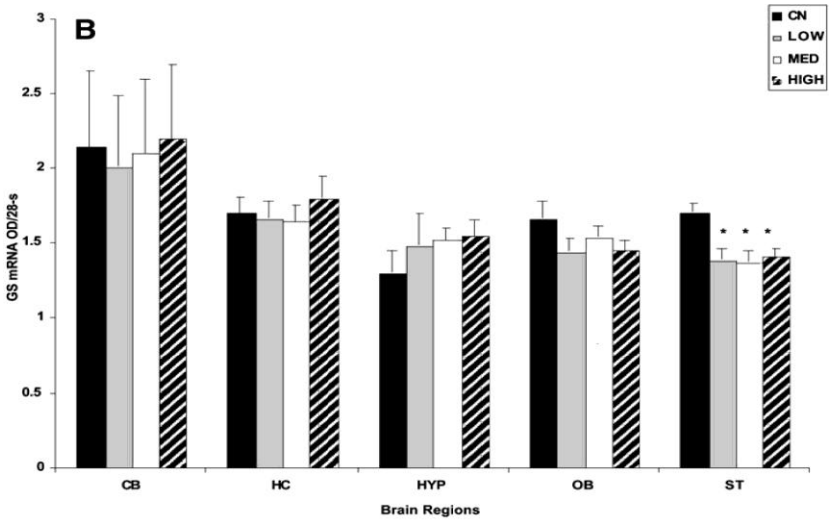

Fig. 1. (continued) **(B)** Treatment groups, sample size, and number per group for each cohort are defined in the legend for part A. GS mRNA levels are presented as mean OD ± SEM for the CB, HC, HYP, OB, and ST. Exposure to LOW, MED, and HIGH doses of Mn significantly decreased GS mRNA in the ST ($p = 0. 0008$) only.

MT mRNA

Treatment with both the medium and high doses of Mn resulted in significantly decreased MT mRNA in the striatum ($p = 0.0114$). Additionally, there was a statistical trend toward lowered MT mRNA in the olfactory bulb related to Mn exposure ($p = 0.0868$) (Fig. 2).

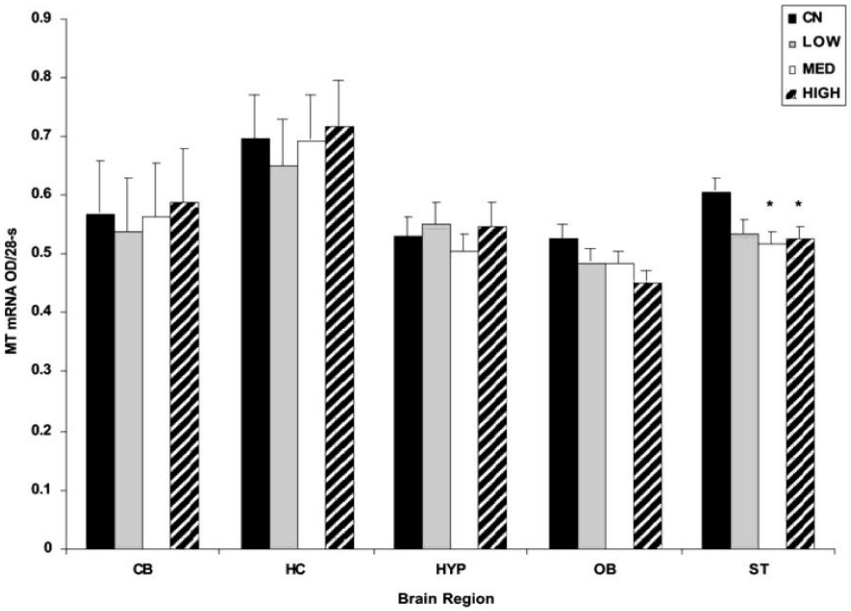

Fig. 2 Treatment groups, sample size, and number per group for each cohort are defined in the legend for Fig. 1A. MT mRNA levels are presented as mean OD ± SEM for the CB, HC, HYP, OB, and ST. Exposure to MED and HIGH doses of Mn significantly decreased MT mRNA in the ST ($p = 0.0114$) and a trend toward significance in the OB ($p = 0.0868$).

TH Protein and mRNA

Whereas TH protein levels were not significantly affected by Mn exposure (Fig. 3A), TH mRNA levels were significantly decreased in the olfactory bulb of pups exposed to the low and medium doses ($p = 0.0402$). Additionally, the TH mRNA levels in the striatum of pups exposed to low, median, and high doses of airborne Mn were also significantly lower compared to controls ($p = 0.0493$) (Fig. 3B).

GSH, GSSG, and GSH/GSSG Ratio

Manganese exposure at the median dose caused a significant decrease in GSH concentrations in the olfactory bulb ($p = 0.0032$) and striatum ($p = 0.0346$). However, pups exposed to the high dose of airborne Mn showed a significant decrease ($p = 0.0346$) in GSH concentrations only in the striatum (Fig. 4A). GSSG levels of Mn-exposed pups did not statistically differ from the control pups, except in the cerebellum of rats exposed to the highest dose, where levels were twofold higher than control concentrations ($p = 0.0247$) (Fig. 4B). GSH / GSSG ratio, an indication of the redox status of the cells in the respective brain sections, was significantly lower in the hippocampus and striatum of pups exposed to both medium and high doses of $MnSO_4$ ($p = 0.0027$ and $p = 0.0164$, respectively) compared to controls. Treatment with all three doses of Mn resulted in a decrease in GSH / GSSG in the olfactory bulb compared to controls ($p = 0.0295$) (Fig. 4C).

DISCUSSION

The current inhalation reference concentration (RfC) for Mn, as set by the US Environmental Protection Agency (US EPA) is 0.05 µg Mn/m^3 *(41).* The concentrations of Mn used in this study were 1000, 10,000, and 20,000 times higher than this standard for the lowest, median, and highest doses, respectively. Dorman and co-workers *(36)* observed increased brain Mn concentrations in PND 14 and PND 19 pups exposed to $MnSO_4$ at ≥0.05 mg Mn/m^3 *in utero* and during lactation. By PND 19, increased striatal, olfactory bulb, and cerebellum concentrations were approximately twofold to threefold higher than those observed in controls. Interestingly, their regional brain Mn concentrations had fully normalized by PND 45 (i.e., 3 wk after the exposure sessions ended) *(36).* It is likely that normalized brain Mn concentrations reflected the increased requirements of the developing rat brain and ongoing elimination *(42).* Thus, younger animals might appropriately utilize the additional Mn burden during a critical window in development. If the exposure ceases before this developmental period ends, it might be within the capacity of the nervous system to compensate for the increased metal amount.

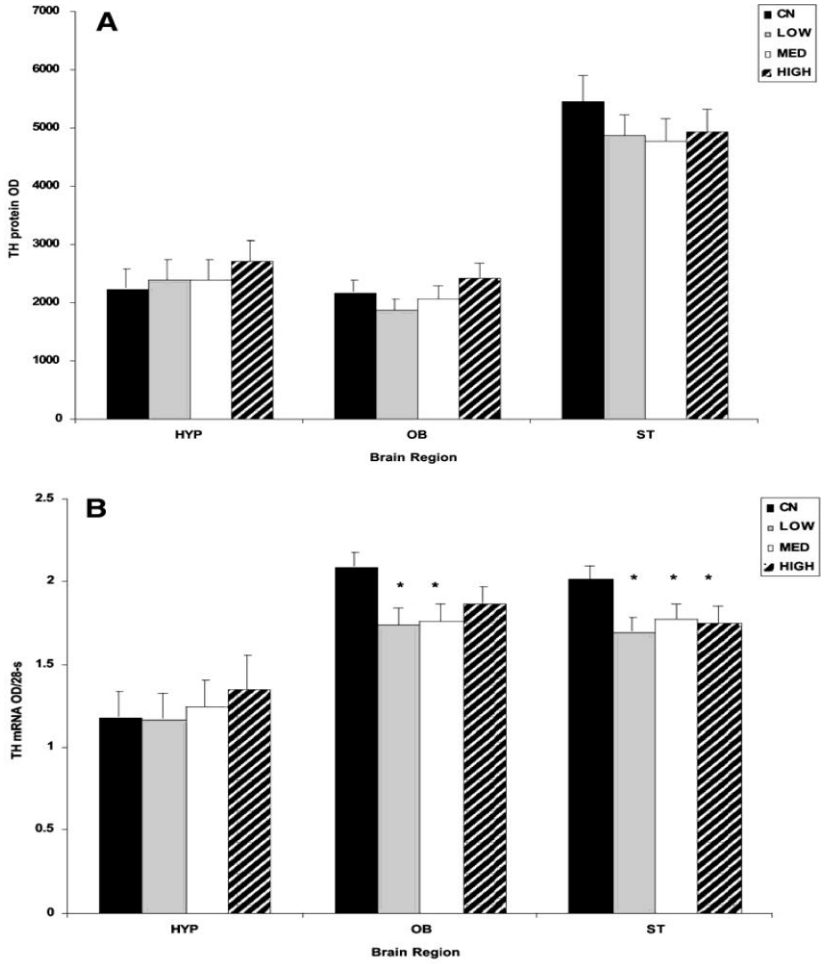

Fig. 3. Treatment groups, sample size, and number per group for each cohort are defined in the legend for Fig. 1a. **(A)** Tyrosine hydroxylase (TH) protein levels are presented as are mean OD ± SEM for the HYP, OB, and ST. No statistically significant differences were observed. **(B)** TH mRNA levels are presented as mean OD ± SEM for the HYP, OB, and ST. TH mRNA levels were significantly decreased in pups exposed to the LOW and MED doses (OB [$p = 0.0402$]) and additionally to the HIGH dose (ST [$p = 0.0493$]) compared to the CN group.

Our previous study showed alterations in oxidative stress markers in specific brain regions (e.g., significantly lowered MT and GS mRNA in the hippocampus and striatum) of developing male and female rats after 3 wk of recovery from *in utero* and neonatal exposures to airborne Mn *(4).* Furthermore, these changes occurred in the absence of increased brain Mn concentrations and were in contrast to our earlier studies conducted on adult rats *(1–3).* The question remains whether the increased Mn observed at PND19 is associated with alterations in oxidative stress markers.

One can indirectly detect the presence of oxidants by measuring species known to increase or decrease in response to ROS production. These species include ubiquitous antioxidants, such as GSH, as well as biomarkers that are more specific to particular tissue types (e.g., GS and TH). In the central nervous system (CNS), GS is localized exclusively in astrocytes *(43),* where it plays a critical role in amino acid metabolism. However, it can also be utilized as a marker for oxidative stress in the brain *(35).* Here, levels of GS protein decreased significantly in the cerebellum of Mn exposed rat pups (Fig. 1A), a region where persistent alterations remained even after brain Mn levels were normalized. Specifically, in rats exposed to the median dose of Mn, there was a decrease in GS protein levels compared to nonexposed rats *(4),* and this decrease occurred despite normal Mn concentrations in this brain region.

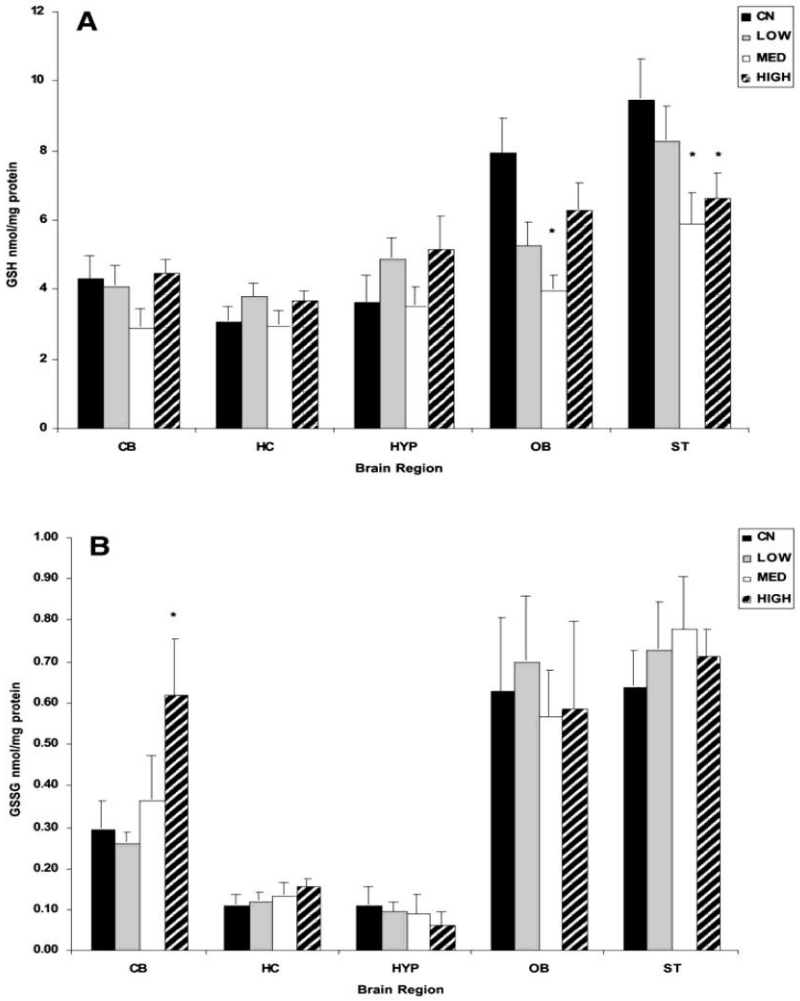

Fig. 4 Treatment groups, sample size, and number per group for each cohort are defined in the legend for Fig. 1A. **(A)** GSH levels are presented as mean nmol GSH/mg protein ± SEM for CB, HC, HYP, OB, and ST. Exposure to the MED dose of Mn significantly lowered GSH in the OB ($p = 0.0032$) and in the ST; both the MED and HIGH doses significantly decreased GSH ($p = 0.0346$). **(B)** GSSG levels are presented as mean nmol GSSG/mg protein for CB, HC, HYP, OB, and ST. Mn exposure affected brain GSSG levels minimally, with the only significant increase observed in the CB in rats exposed to the HIGH dose ($p = 0.0247$). *(continues)*

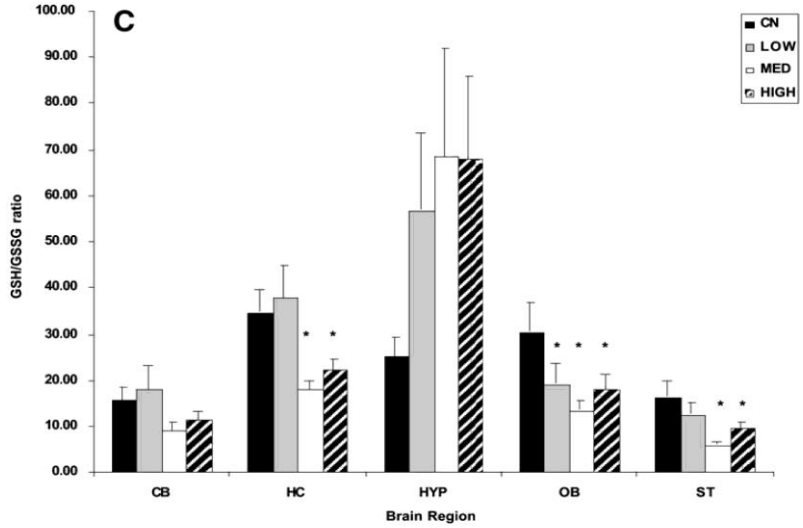

Fig. 4 (continued) **(C)** GSH/GSSG ratio was calculated and the data are mean values ± SEM for the CB, HC, HYP, OB, and ST. Exposure to the MED and HIGH doses of Mn led to a signicantly lower ratio in the HC and ST ($p = 0.0027$ and $p = 0.0164$, respectively) compared to controls. Treatment with all three doses of Mn when compared to CN resulted in a decreased ratio in the OB ($p = 0.0295$).

Corroborating earlier findings *(1–4)*, the GS mRNA levels measured in this study were not reflected in altered amounts of GS protein (i.e., brain regions that had increased mRNA levels did not have increased protein levels). There was a significant decrease in GS mRNA levels only in the striatum (Fig. 1B), which is contradictory to what one would predict knowing that there were many brain regions with alterations in GS mRNA resulting from a similar exposure paradigm *(4)*. However, considering the plasticity of the neonatal nervous system, it is perhaps not surprising that airborne Mn results in mixed effects on GS protein and mRNA levels.

The MTs are a class of highly conserved proteins known to bind metals. In recent years, evidence indicates that they also respond to oxidative stress in the same manner as other well-known antioxidants (e.g., GSH, Mn-SOD) *(44,45)*. We found that MT mRNA levels were significantly decreased in the striatum of rat pups exposed to all three doses of Mn (Fig. 2). As with GS, the effects of neonatal Mn exposure on MT mRNA were isolated to this one brain region in contrast to our other studies *(1,3,4)*.

Tyrosine hydroxylase is the rate-limiting enzyme for catecholamine synthesis and is often used as a marker for dopaminergic neurons. Because Mn accumulates in dopamine-rich brain regions, especially those associated with subsequent neurotoxicity, we sought to specifically examine the effects of airborne Mn on TH protein and mRNA levels. Gestational and postnatal Mn exposure had no effect on TH protein levels (Fig. 3A), but led to decreased levels of TH mRNA in the olfactory bulb and striatum (Fig. 3B). We did not look at TH protein or mRNA levels in the ventral mid-brain, the brain region in which dopamine cell bodies reside (nigrostriatal, mesocortical, and mesolimbic dopamine tracts). These areas would likely have the highest levels of TH in the brain, but to remain consistent with our previous studies *(1–4)*, we did not include this region. We did measure hypothalamic TH protein and mRNA levels, a brain region containing dopamine cell bodies for the tuberoinfundibular system. Here, we observed no effect of Mn exposure on TH levels in this region. This could be the result of the fact that the hypothalamus is not normally associated with Mn neurotoxicity. Overall, these data suggest that the developing nervous system is resistant to the effects of Mn exposure in the context of alterations in TH levels, suggesting that dopamine-containing neurons are spared.

Glutathione is an ubiquitous tripeptide antioxidant (γ-glutamylcysteinylglycine). It is critical for maintaining cellular redox homeostasis and is oxidized to GSSG in the presence of ROS. Thus, one can use the GSH:GSSG ratio to measure cellular redox homeostasis. Here, a lower ratio indicates increased oxidative stress. We have reported, as well as others, that GSH levels were significantly lowered in the striatum of female and old male rats following Mn exposure *(3,24)*, and this is consistent with our current study. Our data here suggest that brain regions that accumulated the most Mn, the striatum and olfactory bulb, also demonstrated significantly decreased GSH levels (Fig. 4A). Furthermore, the increase in oxidative stress is confirmed by the GSH / GSSG ratio in the striatum and olfactory bulb, where Mn exposure significantly decreased this ratio (Fig. 4C). This ratio is determined by calculating changes in the concentrations of GSH, or GSSG, or both. As with the case of the striatum and olfactory bulbs presented here, it is likely that the decreased ratios are the result of lowered GSH concentrations (Fig. 4A) and relatively unaltered GSSG levels (Fig. 4B).

In conclusion, we report for the first time the consequences of *in utero* and early developmental airborne Mn exposure on end points related to oxidative stress in rat pups aged PND 19. Unlike our previous studies *(1–4)*, where there was no clear regional effect, the striatum was impacted significantly here by early exposure to Mn. Increased Mn accumulation in this region was associated with decreases in GS, MT, and TH mRNA, along with significantly lowered GSH levels. It is noteworthy that these effects occurred at doses of Mn 10–20 thousand times higher than the current RfC and that these changes are reversible upon cessation of Mn exposure *(4)*.

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