Brain Accumulation and Toxicity of Mn(II) and Mn(III) Exposures

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Concern over the neurotoxic effects of chronic moderate exposures to manganese has arisen due to increased awareness of occupational exposures and to the use of methylcyclopentadienyl manganese tricarbonyl, a manganese-containing gasoline antiknock additive. Little data exist on how the oxidation state of manganese exposure affects toxicity. The objective of this study was to better understand how the oxidation state of manganese exposure affects accumulation and subsequent toxicity of manganese. This study utilized a rat model of manganese neurotoxicity to investigate how ip exposure to Mn(II)-chloride or Mn(III)-pyrophosphate at total cumulative doses of 0, 30, or 90 mg Mn/kg body weight affected the brain region distribution and neurotoxicity of manganese. Results indicate that Mn(III) exposures produced significantly higher blood manganese levels than equimolar exposures to Mn(II). Brain manganese concentrations increased in a dose-dependent manner, with Mn(III) exposures producing significantly higher (> 25%) levels than exposures to Mn(II) but with no measurable differences in the accumulation of manganese across different brain regions. Gamma amino butyric acid concentrations were increased in the globus pallidus (GP) with manganese exposure. Dopamine (DA) levels were altered in the GP, with the highest Mn(II) and Mn(III) exposures producing significantly different DA levels. In addition, transferrin receptor and H-ferritin protein expression increased in the GP with manganese exposure. These data substantiate the heightened susceptibility of the GP to manganese, and they indicate that the oxidation state of manganese exposure may be an important determinant of tissue toxicodynamics and subsequent neurotoxicity.

Key Words: GABA; dopamine; oxidation state; neurotoxicity; PIXE; brain region.

Metal speciation (i.e., oxidation state and ligand environment) is important in determining the functionality and toxicity of trace metals in biological systems (Finney and O’Halloran, 2003; Thompson and Orvig, 2003). This may be particularly true for manganese, which is an essential trace element in biology (Finley and Davis, 1999; Underwood, 1977; Yocum and Pecoraro, 1999) that exhibits complex reduction-oxidation (redox) chemistry (Klewicki and Morgan, 1998; Silva and Williams, 1991). Concern over possible neurotoxic effects associated with chronic exposure to moderate levels of manganese in mixed oxidation states may be justified by recent studies reporting that workers in the manganese alloy industry are exposed to manganese aerosols of mixed oxidation states (Mn(0), Mn(II), Mn(III), and Mn(IV)) (Thomassen et al., 2001) and by the growing use of methylcyclopentadienyl manganese tricarbonyl, a manganese-containing antiknock additive in gasoline currently in use in a number of developed countries (e.g., Canada, Australia) and proposed for use in the United States (Cooper, 1984; Ressler et al., 2000).

Elevated occupational exposures to manganese are known to cause significant neurotoxicity (Cook, 1974). Furthermore, epidemiologic studies have suggested a relationship between elevated environmental manganese exposure and an increased risk for parkinsonian disturbances (Barbeau, 1984; Corrigan et al., 1998; Gorell et al., 1997; Rybicki et al., 1993; Yamada et al., 1986), an association that has also been supported by numerous laboratory studies (Brown and Taylor, 1999; Gavin et al., 1999; Gwiazda et al., 2002; Olanow et al., 1996; Witholt et al., 2000). While the exact mechanisms underlying the neurotoxic effects of manganese remain unclear, these studies collectively suggest that elevated environmental exposures to manganese may be sufficient to exacerbate the emergence of neurological diseases (Gwiazda et al., 2002; Witholt et al., 2000). In light of these observations, there is a need to further examine the role of manganese oxidation state and its ability to mediate the uptake, retention, and resulting toxicity of manganese.

The mechanisms of manganese neurotoxicity may vary with exposure dose and speciation (Roels et al., 1997); examples of the former exist in the literature. The postmortem tissues of workers occupationally exposed to manganese exhibit decreases in dopamine (DA) in the basal ganglia (Shinotoh et al., 1997; Wolters et al., 1989; Yamada et al., 1986), and some of these observations have been reproduced in nonhuman primate models (Bird et al., 1984; Eriksson et al., 1987; Neff et al., 1969; Olanow et al., 1996; Shinotoh et al., 1995). Based on these and other studies, DA metabolism in the basal ganglia...
(e.g., striatum [Str] and globus pallidus [GP]) has emerged as an outcome of major emphasis in mechanistic studies of manganese neurotoxicity. However, studies have reported seemingly conflicting results on the dopaminergic effects of manganese (see Gwiazda et al., 2006, for review), including decreases, increases, or no change in striatal DA concentrations in manganese-treated animals, possibly reflecting effects of the different exposure regimens on dopaminergic outcomes (Bonilla and Prasad, 1984; Chandra and Shukla, 1981; Eriksson et al., 1987; Gwiazda et al., 2002; Subhash and Padmashree, 1991). Moreover, it has recently been suggested that at lower doses of manganese, other systems such as the GABAergic pathways may be affected (Gwiazda et al., 2002; Zheng et al., 2003). These inconsistent observations may well reflect differences in exposure route, magnitude, duration, etc., between studies, though they also demonstrate the complexity of manganese toxicity and suggest that the factors contributing to its toxicity are not well understood.

A common hypothesis regarding the differential susceptibility of brain regions to manganese is that elevated exposures lead to brain regional differences in the accumulation of manganese and other metals (e.g., iron). It has been reported that rats exposed to very elevated manganese levels via drinking water from an early age displayed increased brain manganese concentrations than did Mn(II), as well as greater effects on measures of the oxidation state of manganese exposure on brain regional uptake and toxicity in intact vertebrate organisms. This need is underscored by several recent cell-based studies showing that Mn(II) and Mn(III) exposures produced different toxic effects on cellular function (Reaney and Smith, 2005). Mn(III) exposure produced significantly greater reductions in intracellular serotonin (5-hydroxytryptophan [5-HT]) concentrations than did Mn(II), as well as greater effects on measures of cellular iron homeostasis, as measured by H-ferritin and TfR protein levels. These data will contribute to a better understanding of the potential relative risks of environmental exposures to manganese in different oxidation states.

**MATERIALS AND METHODS**

**Reagents.** Anti-human mouse TIR antibody was from Zymed Laboratories (Carlsbad, CA), anti-mouse HRP secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), 10% Bis-Tris precast gels was from Invitrogen (Carlsbad, CA), and HPLC-grade solvents (methanol and acetonitrile) were from Fisher Scientific (Fairlawn, NJ). All other reagents were from Sigma Aldrich (St Louis, MO).

**Study design and animals.** This study used a 3 × 2 (Mn dose × oxidation state) factorial design. Thirty-six adult female retired breeder Long Evans rats (~ 8 months old) were obtained from Charles River Laboratories (Wilmington, MA) and randomly assigned to treatment groups following a 1-week acclimation (n = 6/treatment group). Animals were treated with Mn(II) or Mn(III) at nominal doses of 0, 2, or 6 mg Mn/kg body weight/dose via ip injection, delivered three times a week for a duration of 5 weeks (total cumulative doses of 0, 30, or 90 mg Mn/kg body weight). Mn(II)-exposed treatment groups were injected with saline (control, isotonic with Mn(II)-chloride solution) or Mn(II)-chloride solution (32mM), while Mn(III)-exposed groups were injected with 100mM sodium pyrophosphate (control, vehicle) or a Mn(III)-pyrophosphate solution (33mM). Manganese solutions were prepared as previously reported (Reaney et al., 2002). An ip exposure route was used here for several reasons. The internal dose is accurately known, thereby avoiding the uncertainty of dose associated with other exposure routes (oral, inhalation) that would confound our ability to achieve the objectives of the study. The stability and purity of the Mn(III)-pyrophosphate solution have been well characterized (Reaney et al., 2002), allowing the oxidation state delivered to be clearly known. Finally, a large number of rodent studies have evaluated the toxicity of Mn(II)-chloride using an injection exposure route, thereby facilitating inter-study comparison.

Animals were divided into three cohorts of 12 animals, with each cohort balanced by treatment and body weight. Sacrifices occurred 3 days after the last manganese dose for each cohort. Animals were sacrificed by decapitation without anesthesia, and the brain was immediately removed and coronally sectioned on ice to reveal GP, Str, frontal cortex, and Th brain regions. The right hemisphere was collected for elemental determination by PIXE, and the left hemisphere was collected for Western blot and neurotransmitter analysis. Tissues were frozen immediately on dry ice and stored at –70°C until further processing. Concurrently, the liver was removed and stored and whole blood collected in heparin-containing vacutainers. The liver was stored at –70°C and blood at ~20°C.

**Liver and blood manganese concentrations.** Liver (1 g) or blood (1 ml) was aliquoted into 15-ml Teflon digestion vials and processed for manganese analyses by graphite furnace atomic absorption spectrometry using a Perkin Elmer (Boston, MA) 4100ZL Zeeman Spectrometer. Details of sample preparation and analyses have been previously described (Witholt et al., 2000).

**PIXE analysis: brain Mn, Fe, Cu, and Zn.** PIXE spectroscopy is an x-ray fluorescence technique capable of the simultaneous measurement of many elements below a 1-μg/g limit of quantification and micrometer-scale spatial resolution of a sample and thus is well suited for measuring brain regional differences in the accumulation of manganese and other elements in situ (Mauth et al., 1998). To prepare samples for PIXE analysis, 1- to 2-mm coronal slices containing the brain region of interest were mounted on a sectioning chuck at ~20°C. Utilizing a cryostat, successive 20-μm slices were taken until the brain region of interest was revealed. Brain regions were analyzed at the same level for each animal and defined by discrete morphological...
characteristics, using the rat brain atlas of Paxinos and Watson (1986) as a guide. The section chosen (bregma – 1.88 mm) contained all brain regions of interest. At this level the Str is clearly defined by unique tissue striata and clearly bordered by the corpus callosum. The level at which the GP was delineated showed the internal capsule clearly separating the GP from the thalamic nuclei. For a control brain region, the somatosensory cortex was analyzed at the same level. Brain slices of interest were collected onto a nylon foil for PIXE analysis. This sample was then freeze-dried until further analysis (details below). The effacing slice was mounted on a gelatin-coated microscope slide for cresyl violet Nissl staining to make neuronal morphological features more apparent to assure that the proper brain regions were probed during PIXE analyses.

Brain regions of interest (GP, Str, Th, cortex [Cx]) within freeze-dried tissue sections were analyzed for manganese, iron, zinc, and copper using PIXE and scanning transmission ion microscopy (STIM) at the nuclear microprobe facility of Lawrence Livermore National Laboratory (Roberts et al., 1999). PIXE and STIM data were obtained using incident 3 MeV proton microbeams. Regions of interest were delineated on the associated freeze-dried section using a ×40 optical microscope within the microprobe specimen chamber. An absolute encoder-controlled sample stage was used to position the feature of interest in the beam path with a precision better than 10 μm. The beam spot size was maximized to interrogate as much of the feature of interest while avoiding interrogation of surrounding material. Beam spot sizes were square or rectangular and varied between 0.1 × 0.1 mm and 1 × 1 mm. For PIXE analysis, x-rays were detected with an energy-dispersive x-ray detector that subtended a solid angle of ~100 msr to the specimen. The detector was located at an angle of 135° with respect to the incident beam. Charge was collected in a biased Faraday cup located behind the sample. Regions of interest were examined with beam currents of up to 10 nA, for doses of up to 15 μC. X-ray spectra were stored on a computer and analyzed offline.

X-ray spectra were analyzed using the computational iterative PIXE spectrum fitting code PIXE2F to extract characteristic x-ray peak areas (or yields) for elements of interest (Antolak and Bench, 1994). The incident and residual proton energies were used to convert x-ray yields to element concentrations using PIXE2F assuming the composition of the biological material to be C12H10O16N (Bench, 1991; Lefevre et al., 1987, 1991). To verify the system calibration for metal quantitation, thin-film organic matrix standards containing metals at appropriate concentrations were analyzed under the same conditions used for analysis of tissue sections each day the tissues were analyzed. Measured manganese contents from both standards were always within 7% of the known manganese contents. Analysis of the standards revealed that a minimum limit of quantification for manganese in an organic matrix was approximately 1 mg/kg.

### Tissue preparation for neurotransmitters and Western blots

Two-millimeter slices of the left hemisphere of the brain were dissected to collect the region of interest (Str, GP, Th, Cx). Brain regions were weighed, and nine times their weight of an ice-cold 10mM HEPES, pH 7, solution was added in a tube containing a homogenization resin and the sample homogenized for 1 min on ice per the manufacturer’s recommendations (Amersham Biosciences, Piscataway, NJ sample grinding kit). The homogenate was further disrupted with sonication (3 × 1 s pulses at 25 Hz, 3-mm probe), vortexed, and centrifuged at 1000 × g for 10 s. The supernatant was diluted in 2× RIPA buffer for Western blots or in 5× perchlorate buffer for neurotransmitter determination (see below).

### Biochemical determinations

For DA determination, four parts of tissue homogenate in 30mM HEPES (pH 7.4) were added to one part of a degassed 5× solution of perchlorate for a final concentration containing 400mM perchlorate, 2mM Na2EDTA, and 2 μM 3,4-dihydroxybenzylamine (DHBA) as internal standard. External standards were diluted with the perchlorate solution in the same proportion as the samples. Supernatant fractions were separated via centrifugation at 16,000 × g for 5 min at 4°C. The sample was injected onto an HPLC (Beckman with Gold Software) fitted with a C18, 3-μm reversed-phase column (100 mm) for separation of DA and metabolites and measured using electrochemical detection (BAS), according to procedures outlined by Freeman et al. (1993). Quantified compounds included DA and its metabolites (3,4-dihydroxyphenylacetic acid and homovanilllic acid) and 5-HT.

Tissue homogenates were also analyzed for gamma amino butyric acid (GABA) and the excitatory amino acids aspartate and glutamate by HPLC-EC after derivatization with o-phthalaldehyde. Derivatization was accomplished by combining a 20-μl volume of the homogenate supernatant obtained during processing for DA measurements (see above) with 208 μl of γ-amino butyric acid-spiked (internal standard BABA) 0.1M borate buffer (pH 9.5) and 32 μl of derivatizing reagent (0.1M borate buffer, 0.05M sodium sulfite, 5% [vol/vol] ethanol, and 0.02M o-phthalaldehyde). External standards were prepared at the time of sample derivatization and derivatized using the same BABA-spiked buffer used with the samples. The derivatized mixtures were analyzed via C18, 3-μm reversed-phase HPLC (150-mm column) with electrochemical detection. Data were expressed relative to wet tissue weight.

Cellular abundance of the proteins TIR, H-ferritin, and tubulin was determined with gel electrophoresis and Western blotting using previously described methods with slight modifications (Kwik-Uribe et al., 2003). Briefly, aliquots of the tissue homogenate in HEPES, pH 7, were further disrupted on ice in an RIPA lysis buffer (phosphatase-buffered saline, containing 1% Igepal-640, 0.5% sodium deoxycholate, 0.1% sodium lauryl sulfate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin). The lysate was then centrifuged at 2000 × g for 10 min at 4°C, an aliquot was taken for protein determination (by the Bradford method), and the remaining supernatant was mixed with 4× NuPAGE sample-loading buffer and stored at –70°C until analysis. Prior to gel loading, all samples were diluted to equal protein concentrations using NuPAGE sample-loading buffer. Lysates in NuPAGE sample-loading buffer were reduced with 10% (vol/vol) 0.75M dithiothreitol and loaded onto NuPAGE 10% Bis-Tris gels (9 μg of protein loaded per lane). Following separation, the proteins were transferred to PVDF membranes and probed with a mouse monoclonal TIR (Zymed Laboratories), H-ferritin (a kind gift from Dr J. Connor, Hershey Medical Center, Pennsylvania State University, Hershey, PA), or rabbit β-tubulin (Santa Cruz Biotechnology). Following immunoblotting with the appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), the protein bands were visualized using an ECL PLUS detection kit (Amersham Pharmacia, Piscataway, NJ). Band intensity was imaged and quantified using a Typhoon scanner equipped with Image Quant software (Amersham Biosciences).

Heparinized whole blood was centrifuged at 1000 × g and the separated plasma fraction removed and stored at ~20°C. Plasma prolactin concentrations were measured by a radioimmunoassay (RIA) with a kit from Diagnostic Systems Laboratories (Webster, TX) with RIA reagents from the National Institute of Diabetes and Digestive and Kidney (NIDDK) Diseases Hormone Distribution Program (Thordarson et al., 2001).

### Statistics

Treatment and pairwise comparisons were performed using two-way analysis of variance and Tukey’s post hoc comparison tests, with manganese exposure and manganese oxidation state as the main factors. The p values less than 0.05 were considered statistically significant for all tests. All analyses were conducted using SYSTAT (SPSS Inc., 10th ed., 2000).

### RESULTS

Rats were exposed to Mn(II)-chloride or Mn(III)-pyrophosphate at cumulative doses of 0, 30, and 90 mg Mn/kg body weight over a 5-week period. These relatively low dose ranges were selected based on previous studies with Mn(II) (Gwiazda et al., 2002; Witholt et al., 2000), showing effects on GABA concentrations in the Str and neuromotor deficits at doses (72 mg Mn/kg body weight) comparable to those used here. These dose ranges are also comparable, though somewhat higher,
to the lowest cumulative dose (10 mg/kg body weight) where adverse neurological effects have been observed in humans occupationally exposed to manganese (Roels et al., 1992). Because we wanted to test the effects of manganese exposure on aged animals, female retired breeders were utilized.

Manganese Concentrations in Blood and Liver

Mn(III) exposures produced significantly higher blood manganese levels than comparable exposures to Mn(II) (Fig. 1a). This is reflected by the significant effect of manganese dose ($F_{2,29} = 567, p < 0.001$) and oxidation state ($F_{1,29} = 29.9, p < 0.001$) on blood manganese levels, and the significant dose × oxidation state interaction ($F_{2,29} = 7.40, p = 0.002$).

The highest Mn(III) exposure produced greater manganese concentrations in the liver than the corresponding Mn(II) exposure, as reflected by the overall dose ($F_{2,29} = 9.94, p = 0.001$) and oxidation state effect ($F_{1,29} = 2.81, p = 0.104$; interaction $F_{2,29} = 5.57, p = 0.009$) on total manganese concentrations in the liver (Fig. 1b). The above interaction reflects the ~56% increase versus control in liver manganese concentrations of rats exposed to the highest dose of Mn(III). There was only a ~5% nonsignificant increase in liver concentrations in Mn(II)-treated rats.

Manganese Concentrations in the Brain

Brain manganese levels increased significantly in all brain regions with increasing manganese dose and oxidation state, though within a dose or oxidation state there was no measurable difference among the four different brain regions measured (Str, GP, Th, and Cx; $p_{\text{Brain Region}} > 0.1$) (Figs. 2a and 2b, Table 1). Other metals (Fe, Cu, Zn) did not measurably change with manganese exposure (dose or oxidation state), and the relative concentrations of these elements did not vary among different brain regions (Table 1).

There was a clear effect of increasing manganese dose and oxidation state on increasing manganese concentrations in all four brain regions measured (GP, Str, Th, Cx), with Mn(III) exposure producing higher brain manganese concentrations than Mn(II) exposures (GP Mn dose $F_{2,30} = 112, p < 0.001$, Mn ox $F_{1,30} = 13.9, p = 0.001$, interaction $p > 0.05$; Str Mn dose $F_{2,30} = 113, p < 0.001$, Mn ox $F_{1,30} = 10.3, p = 0.003$, interaction $F_{2,30} = 4.23, p = 0.024$; Th Mn dose $F_{2,30} = 114, p < 0.001$, Mn ox $F_{1,30} = 11.9, p = 0.002$, interaction $F_{2,30} = 6.81, p = 0.004$; Cx Mn dose $F_{2,30} = 49.3, p < 0.001$, Mn ox $F_{1,30} = 5.12, p = 0.031$, interaction $> 0.05$). Because there were no measurable differences between brain regions in the accumulation of manganese, the brain regional manganese values were treated as replicates, values were averaged together for each individual animal, and the data were statistically reanalyzed. This reanalysis further showed an effect of both dose ($F_{2,30} = 207, p < 0.001$) and oxidation state ($F_{1,30} = 18.5, p < 0.001$; interaction $F_{2,30} = 10.8, p < 0.001$) on increasing brain manganese concentrations (Fig. 2c).

Comparing the brain manganese concentrations (averaged across brain regions) for each dose within oxidation state to the blood manganese concentrations within animals showed a notable curvilinear relationship (Fig. 3). The best-fit curves for Mn(II)- and Mn(III)-exposed animals were produced using Michaelis-Menton–type equations of the general form: $y = \frac{B_{\text{max}} \times x}{K_d + x}$, where $B_{\text{max}} = 4.4$ (95% CI = 3.9–4.8) and 5.3 (95% CI = 4.6–6.0) for Mn(II)- and Mn(III)-exposed animals, respectively. The $B_{\text{max}}$ term reflects the asymptote of the best-fit curve, which in this case represents the saturation brain manganese concentration for each oxidation state. The $B_{\text{max}}$ terms for the Mn(II)- versus Mn(III)-exposed animals were marginally not significantly different ($F_{1,31} = 3.96, p = 0.055$), suggesting that Mn(III) exposures produce higher brain manganese concentrations at saturation than do Mn(II) exposures. The $K_d_{\text{Mn(II)}} = 22.4$ and $K_d_{\text{Mn(III)}} = 37.7$ were not statistically different ($F_{1,31} = 1.42, p = 0.242$).

Brain regional GABA, DA, and 5-HT Concentrations

GABA levels increased significantly with manganese exposure in the GP and tended to increase in the Str (Fig. 4a, Table 2). Manganese exposure increased GABA levels in the GP
by ~15–30% over controls (dose $F_{2,29} = 3.69, p = 0.037$; ox $F_{1,29} = 0.047, p = 0.83$; interaction $p > 0.05$). There were similar, though marginally nonsignificant, GABA increases of 15–45% in the Str (15–45%) (dose $F_{2,29} = 2.92, p = 0.07$; ox $F_{1,29} = 5.92, p = 0.021$; interaction $p > 0.05$) but not the Th (dose $F_{2,29} = 0.308, p = 0.740$; ox $F_{1,29} = 1.09, p = 0.310$; interaction $p > 0.05$) (Table 2). It is worth noting that differences in the effect of oxidation state were apparent if the data were grouped by non–manganese exposed versus manganese exposed (30 and 90 mg/kg combined) within oxidation state. Mn(III) exposure led to a significant increase of ~41% ($p = 0.010$) in Str GABA levels, while Mn(II) exposure resulted in no measurable change (~4%, $p > 0.1$) compared to control.

Brain DA concentrations in the GP were altered by manganese dose and oxidation state, though this was most evident by the significant difference between the Mn(II) versus Mn(III) effect at the highest exposure dose (dose $F_{2,29} = 0.304$, $p = 0.741$; ox $F_{1,29} = 1.42, p = 0.243$; interaction $F_{2,29} = 4.52, p = 0.020$) (Table 2, Fig. 4b). There was no manganese effect on DA levels in the Str (dose $F_{2,29} = 0.375, p = 0.690$; ox $F_{1,29} = 0.052, p = 0.820$; interaction $p > 0.05$) (Table 2). The significant treatment interaction in the GP reflects the significantly different effects of the highest Mn(II) and Mn(III) exposures on DA concentrations, with a trending decrease in DA with Mn(II) exposure and significantly higher DA in Mn(III)-exposed animals (Fig. 4b). DA and 5-HT levels in the Th were below the limit of quantification for the method.

Brain 5-HT concentrations (Table 2) were not measurably altered by manganese exposure, though levels trended up with Mn(III) exposure: GP Mn dose $F_{2,29} = 0.40, p = 0.67$; Mn ox $F_{1,29} = 2.18, p = 0.15$; interaction $p > 0.05$; Str Mn dose $F_{2,29} = 2.65$. Interestingly, similar to GABA levels in the Str, there was a significant effect of manganese oxidation state on Str 5-HT concentrations when the data were grouped by manganese exposed (30 and 90 mg/kg combined) versus not manganese exposed within oxidation state. Mn(III) exposure led to a significant increase of ~31% ($p = 0.028$) in Str 5-HT levels, while Mn(II) exposure resulted in no change (~9%, $p > 0.1$).

TfR and H-ferritin Protein

TfR protein abundance trended toward an increase with increasing manganese dose in all brain regions measured (GP, Str, Th), though the manganese dose effect was significant only in the GP (GP Mn dose $F_{2,29} = 5.29, p = 0.011$, ox $F_{1,29} = 0.534, p = 0.471$, interaction $p > 0.05$; Str dose $F_{2,29} = 2.56$, $p = 0.090$, ox $F_{1,29} = 0.074, p = 0.787$, interaction $p > 0.05$; Th dose $F_{2,29} = 1.37, p = 0.27$, ox $F_{1,29} = 0.001, p = 0.97$,
### TABLE 1

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<td></td>
<td>Th (99)</td>
<td>100 ± 7.4</td>
<td>103 ± 8.0</td>
<td>100 ± 9.0</td>
<td>100 ± 6.5</td>
<td>95.4 ± 7.3</td>
<td>89.2 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>Cx (97)</td>
<td>100 ± 5.9</td>
<td>104 ± 3.7</td>
<td>108 ± 6.7</td>
<td>100 ± 4.3</td>
<td>94.1 ± 4.5</td>
<td>91.5 ± 5.3</td>
</tr>
</tbody>
</table>

*Values in parentheses are average trace metal concentrations in brain region of control animals in units of micrograms per gram dry weight (ppm).

interaction $p > 0.05$ (Fig. 5a). Overall, however, brain TfR levels were significantly increased with manganese exposure when the changes, relative to controls, were averaged across brain regions for each individual animal (Mn dose $F_{2,29} = 6.92, p = 0.003$, Mn ox $F_{1,29} = 0.258$, $p = 0.615$, interaction $p > 0.05$) (Fig. 5b). For these latter analyses, each brain region was treated as a replicate measure and all brain regions averaged within an animal; average brain values for each animal were averaged within treatment group.

There was a trending increase in H-ferritin protein levels in the GP and Str with increasing manganese dose. Insufficient sample volume was available for measurement of H-ferritin in the Th. There was an effect of manganese dose to increase H-ferritin in the GP (Fig. 6) and an effect of manganese oxidation state in the Str, but no effects were observed on the average H-ferritin levels across these brain regions (GP dose $F_{2,29} = 5.05, p = 0.013$, ox $F_{1,29} = 0.797, p = 0.379$; interaction $p > 0.05$; Str dose $F_{2,29} = 1.29, p = 0.291$, ox $F_{1,29} = 7.40, p = 0.011$, interaction $F_{2,29} = 3.68, p = 0.038$; average of GP and Str within animal, Mn dose $F_{2,29} = 2.99, p = 0.067$, ox $F_{1,29} = 2.14, p = 0.155$, interaction $p > 0.05$). β-Tubulin levels did not change in any of the brain regions or treatment groups measured (GP Mn dose $F_{2,29} = 0.186, p = 0.831$, ox $F_{1,29} = 0.017$, $p = 0.897$; Th Mn dose $F_{2,29} = 1.235, p = 0.305$, ox $F_{1,29} = 0.409, p = 0.528$; Str Mn dose $F_{2,29} = 0.615, p = 0.547$, ox $F_{1,29} = 0.532, p = 0.472$).

### Plasma Prolactin Concentrations

Plasma prolactin levels significantly decreased with increasing manganese dose but not manganese oxidation state (Mn dose $F_{2,29} = 3.9, p = 0.032$; Mn ox $F_{1,29} = 0.003$, $p = 0.958$; interaction $p > 0.05$). When data across oxidation state treatment were averaged and reanalyzed by manganese dose,
there remained a significant manganese dose effect to decrease plasma prolactin levels ($p = 0.022$) (Fig. 7).

**DISCUSSION**

This study shows that the oxidation state of manganese exposure is important in modulating manganese retention and accumulation. Mn(III) exposures as Mn(III)-pyrophosphate resulted in greater accumulation/retention of manganese in the blood, liver, and brain compared to equimolar exposures to Mn(II)-chloride. The higher brain manganese levels produced by the Mn(III) exposures were associated with increases in brain GABA concentrations, TIR levels, and H-ferritin levels, particularly in the GP. These data substantiate the need to better understand the relative effects of manganese exposures in different oxidation states and how these exposures impact specific brain regions.

The higher levels of tissue manganese accumulation/retention with Mn(III) exposures (Figs. 1 and 2) evidence different toxicodynamics for the Mn(II) versus Mn(III) exposures. If it is assumed that similar amounts of the ip administered Mn(II) and Mn(III) entered the circulation for a given dose, these results would then suggest that there is significantly less efficient clearance of manganese from the Mn(III) exposures. These observations are consistent with previous animal model studies which showed that Mn(II) exposures in the form of Mn(II)-chloride or Mn-protein complexes (e.g., globulins) are more efficiently cleared from the blood by the liver than are Mn(III) exposures as the manganic-transferrin complex (Chua and Morgan, 1997; Gibbons et al., 1976). We hypothesize that the higher liver concentrations observed with Mn(III) exposures (Fig. 1b) could be due to possible sequestering of manganese in the abundant ferritin stores of the liver and hence less

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**TABLE 2**

Mean GABA, DA, and 5-HT Concentrations in the GP, Str, and Th of Control and Mn(II)- and Mn(III)-Exposed Rats.

<table>
<thead>
<tr>
<th>Neurotransmitter</th>
<th>Brain region (control conc.)*</th>
<th>Mn(II), 0 mg/kg</th>
<th>Mn(II), 30 mg/kg</th>
<th>Mn(II), 90 mg/kg</th>
<th>Mn(II), 0 mg/kg</th>
<th>Mn(II), 30 mg/kg</th>
<th>Mn(II), 90 mg/kg</th>
<th>Mn(III), 0 mg/kg</th>
<th>Mn(II), 30 mg/kg</th>
<th>Mn(II), 90 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>GP (3.16)</td>
<td>100 ± 7.5</td>
<td>100 ± 6.0</td>
<td>118 ± 9.4</td>
<td>100 ± 14</td>
<td>95 ± 10</td>
<td>129 ± 15</td>
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<tr>
<td></td>
<td>Str (2.71)</td>
<td>100 ± 7.7</td>
<td>115 ± 17</td>
<td>94 ± 9.8</td>
<td>100 ± 5.8</td>
<td>145 ± 18</td>
<td>137 ± 9.4</td>
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</tr>
<tr>
<td></td>
<td>Th (1.50)</td>
<td>100 ± 8.3</td>
<td>125 ± 18</td>
<td>111 ± 14</td>
<td>100 ± 19</td>
<td>96 ± 12</td>
<td>105 ± 10</td>
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<tr>
<td></td>
<td>GP (12.6)</td>
<td>100 ± 23</td>
<td>106 ± 25</td>
<td>59 ± 8.8</td>
<td>100 ± 26</td>
<td>75 ± 20</td>
<td>158 ± 30</td>
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<tr>
<td></td>
<td>Str (33.8)</td>
<td>100 ± 6.3</td>
<td>99 ± 16</td>
<td>105 ± 17</td>
<td>100 ± 10</td>
<td>98 ± 8.0</td>
<td>113 ± 15</td>
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<td>Th (&lt; DL)</td>
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</tr>
<tr>
<td>DA</td>
<td>GP (12.6)</td>
<td>100 ± 9.0</td>
<td>98 ± 8.9</td>
<td>99 ± 9.2</td>
<td>100 ± 18</td>
<td>126 ± 18</td>
<td>119 ± 16</td>
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<td></td>
<td>Str (2.74)</td>
<td>100 ± 4.0</td>
<td>118 ± 19</td>
<td>101 ± 12</td>
<td>100 ± 3.5</td>
<td>137 ± 12</td>
<td>125 ± 11</td>
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<td>Th (&lt; DL)</td>
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<td>5-HT</td>
<td>GP (1.23)</td>
<td>100 ± 7.5</td>
<td>100 ± 6.0</td>
<td>118 ± 9.4</td>
<td>100 ± 14</td>
<td>95 ± 10</td>
<td>129 ± 15</td>
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<td></td>
<td>Str (2.74)</td>
<td>100 ± 7.7</td>
<td>115 ± 17</td>
<td>94 ± 9.8</td>
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<td>145 ± 18</td>
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<tr>
<td></td>
<td>Str (33.8)</td>
<td>100 ± 6.3</td>
<td>99 ± 16</td>
<td>105 ± 17</td>
<td>100 ± 10</td>
<td>98 ± 8.0</td>
<td>113 ± 15</td>
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<td></td>
<td>Th (&lt; DL)</td>
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<td>&lt; DL</td>
<td>&lt; DL</td>
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</tbody>
</table>

*Values in parentheses are average neurotransmitter concentrations in control brain regions of control animals in units of picomoles of neurotransmitter per gram of wet tissue weight.

< DL = below the analytical detection limit for the method.
efficient clearance of manganese when exposures occur as Mn(III).

The higher brain manganese concentrations with Mn(III) exposures, compared to those produced from exposures to Mn(II), appear to be due predominantly but not solely to the higher concentrations of manganese in blood. The best-fit curvilinear associations between blood and brain manganese concentrations imply that for significantly elevated blood manganese levels, Mn(III) exposures result in higher brain manganese concentrations than comparable exposures to Mn(II) (Fig. 3). If so, this suggests that the availability of blood manganese to the brain or the retention of manganese in the brain may differ depending in part on the oxidation state of manganese exposure. This suggestion is consistent with studies indicating that manganese normally exists in both Mn(II) and Mn(III) species within the blood (Hancock et al., 1973; Harris and Chen, 1994; Herak et al., 1982; Scheuhammer and Cherian, 1985) and with studies showing that Mn(II) and Mn(III) utilize different transport mechanisms for cellular uptake and transport across the blood-brain barrier; DMT-1 is known to transport Mn(II) species, and TfR-mediated endocytosis transports Mn(III)-transferrin species (Aschner and Gannon, 1993; Murphy et al., 1991; Zheng et al., 2003).
There was a clear effect of manganese dose and oxidation state on a number of brain outcomes, including levels of neurotransmitter and proteins involved in cellular iron metabolism (Table 2, Figs. 4–6). The oxidation state of manganese exposure had a significant impact on GABA in the Str and GP, as well as H-ferritin levels in the Str. However, these effects must be qualified by the fact that the Mn(III) exposures produced higher brain manganese levels—making it difficult to distinguish brain effects arising from manganese concentration versus oxidation state of exposure. A notable exception to this qualification is the effect of the highest Mn(III) exposure to significantly increase DA levels by ~60% in the GP, compared to an ~40% decrease caused by comparable Mn(II) exposures, relative to their respective controls (Fig. 4b). The mechanistic basis for this latter observation remains unclear, though a recent review of manganese effects on striatal DA content in rodent models has shown highly variable effects, from no change, increase, or decreases in striatal DA (Gwiazda et al., 2006), though none of these studies compared the effects of soluble Mn(II) versus soluble Mn(III) exposures.

Previous studies have shown that both dopaminergic and GABAergic systems of the basal ganglia are affected by manganese (Autissier et al., 1982; Bonilla, 1978; Chandra and Shukla, 1981; Eriksson et al., 1987; Gianutsos and Murray, 1982; Gwiazda et al., 2002; Lipe et al., 1999; Neff et al., 1969). While reported manganese effects on brain DA levels are quite variable, possibly due to experimental differences between studies (Gwiazda et al., 2006), rodent studies by large are consistent in reporting increases in striatal GABA levels following manganese exposure (few studies have evaluated GABA levels in the GP) (Bonilla, 1978; Bonilla and Prasad, 1984; Gianutsos and Murray, 1982; Gwiazda et al., 2002; Lipe et al., 1999). These GABAergic effects are consistent with histological and MRI evidence from monkeys and human studies indicating that the GABA-rich GP is among the most sensitive brain regions affected by manganese. Our data suggest that the apparent increased susceptibility of the GP and Str may be due to their inherent sensitivity to manganese since there were no measurable differences in accumulated manganese, iron, copper, or zinc across the GP, Str, Th, and Cx brain regions measured here.

Increases in TIR protein and H-ferritin expression with increasing manganese dose were apparent in the Str and GP, though a statistically significant dose response occurred only in the GP. The concurrent increase in TIR and H-ferritin protein levels evidence disruption of iron regulation since normally these proteins respond in opposite directions to iron deficiency or overload. An increase in TIR protein levels or mRNA resulting from exposure to Mn(II) (Kwik-Uribe et al., 2003; Zheng and Zhao, 2001; Zheng et al., 1999) and Mn(III) (Reaney and Smith, 2005) has been previously observed. There are clear toxicological implications for these outcomes since disruption of cellular iron metabolism due to elevated manganese exposure has been implicated in increased oxidative stress and cellular dysfunction, including association with neurodegenerative diseases such as Parkinson’s disease (Dexter et al., 1991; Gorell et al., 1997; Riederer et al., 1989; Sofic et al., 1988).

Serum prolactin levels have been evaluated as a marker for manganese exposure in the occupational setting by several studies (Ellingsen et al., 2003; Mutti et al., 1996). The rationale for the use of serum prolactin as a surrogate of manganese exposure is based on the assumption that increases in systemic manganese results in a reduction of brain regional DA leading to a lack of inhibition of prolactin synthesis and secretion in the pituitary gland. Here we found that despite the lack of robust changes in DA concentrations in the brain regions analyzed (GP, Str, Th), serum prolactin concentrations decreased with increasing manganese exposure. Previous animal studies have reported varied effects of manganese exposure on serum prolactin levels, including no effect (Merritt and Brown, 1984) or increased serum prolactin levels (Merritt and Brown, 1984). While the basis for the apparent differences in the effect of manganese on serum prolactin is not clear, the fact that significant alterations in serum prolactin levels exist suggests that altered serum prolactin may be an important toxicological outcome of manganese exposure.

In summary, the cumulative dose range of manganese used here (30–90 mg Mn/kg body weight) is at the lower range of exposures used in studies reporting adverse effects in rodents (Bonilla and Prasad, 1984; Chandra and Shukla, 1981; Eriksson et al., 1987; Gwiazda et al., 2002; Subhash and Padmashree, 1991). Exposures to Mn(III) produced higher blood and brain manganese concentrations than equimolar exposures to Mn(II), though there were no measurable differences in the manganese accumulation across the four brain regions examined here. Similarly, there were no differences in the brain region levels of iron, copper, and zinc due to manganese treatment. However, there were effects of manganese dose and oxidation state on a number of brain outcomes. These data substantiate the heightened susceptibility of the GP and Str brain regions to manganese, and they indicate that the oxidation state of manganese exposure may be an important determinant of tissue toxicodynamics and subsequent neurotoxicity.

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