# Effects of Manganese (Mn) on the Developing Rat Brain: Oxidative-Stress Related Endpoints

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## Abstract:
We evaluated biochemical endpoints related to oxidative stress in brains of neonatal rats exposed to manganese (Mn). Oral Mn chloride ($MnCl_2$) (0, 25, or 50 mg Mn chloride $kg^{-1}$ body weight per day) was given daily to neonatal rats throughout lactation (i.e. from postnatal day (PND) 1 to 21). As previously reported by [J. Appl. Toxicol. 20 (2000) 179], this treatment paradigm results in increased cerebral cortex (CTY) Mn concentrations in PND 21 rats from both Mn treatment groups. High dose Mn exposure also results in increased cerebellar Mn concentrations. This preliminary study determined whether this exposure paradigm also affects cerebrocortical or cerebellar metallothionein (MT) mRNA levels, glutamine synthetase (GS) activity, GS protein levels, as well as total glutathione (GSH) levels. High dose Mn exposure significantly increased ($P < 0.05$) total cerebrocortical GSH without accompanying changes in any of the other measured parameters. Therefore, it is unlikely that high dose Mn exposure is associated with oxidative stress in this experimental paradigm.
**Keywords:** Manganese; Neurotoxicity; Oxidative stress

## Article:
### *INTRODUCTION*
Manganese (Mn) encephalopathy is characterized by high levels of Mn in the globus pallidus and neuronal loss in the basal ganglia and associated nuclei of the extrapyramidal system (Barbeau, 1984; Nelson et al., 1993; Schochet and Nelson, 1991). Neonatal rats appear to be at an increased risk for Mn-induced neurotoxicity due to their ability to accumulate higher brain Mn levels and develop more pronounced brain pathology than do adults in the face of equivalent or lesser Mn exposures (Chandra and Shukla, 1978; Dorman et al., 2000; Seth et al., 1977; Shukla et al., 1980). Dorman et al. (2000) demonstrated reduced weight gain, increased pulse-elicited acoustic startle response amplitude, and increased striatal dopamine and 3,4-dihydroxyphenylacetic acid levels in neonates given high (50 mg $MnCl_2$ $kg^{-1}$ body weight per day) oral doses of Mn. Factors influencing this increased susceptibility include increased Mn absorption from the gastrointestinal tract (Kostial et al., 1978; Rehnberg et al., 1982), an incompletely formed blood-brain barrier and the virtual absence of biliary Mn excretory mechanisms until weaning (Miller et al., 1975).

One proposed mechanism for Mn-induced neurotoxicity is a cascade of oxidative damage potentiated by the synergism of excess Mn and high concentrations of iron and dopamine in affected brain regions (Aschner, 1997). The brain, in general, is highly susceptible to oxidative damage because it has a high rate of oxidative metabolism, high concentrations of polyunsaturated fatty acids and low to moderate levels of antioxidant enzymes. There is some evidence that the developing rat brain may be more sensitive to oxidative stress. For example, Driver et al. (2000) demonstrated that brain homogenates prepared from neonatal rats incubated in the presence of divalent iron formed higher amounts of reactive oxygen species (ROS) than similarly treated brain homogenates collected from adult rats.

It has been theorized that elevated concentrations of Mn might significantly accelerate the oxidation of dopamine and other catecholamines and concurrently amplify the formation of ROS (Sloot et al., 1996). Donaldson et al. (1980) presented in vitro evidence that divalent Mn increases dopamine autoxidation and thus may induce oxidative damage. It has been demonstrated that divalent Mn ($Mn^{2+}$) catalyzes Fenton-like reactions that generate hydroxyl radical and trigger proteolytic degradation and protein turnover (Wedler, 1993). Ali et al.

(1995) demonstrated dose-related increases in ROS production in rat caudate nucleus after in vivo Mn exposure. Tyree and Archibald (1987) have postulated that trivalent Mn is the neurotoxication of concern. Whether originally entering in the +2, +3, or +4 oxidation state, Mn will, via spontaneous oxidation and dismutation, peroxidative activity, or oxygen radical-mediated oxidation, give rise to $Mn^{3+}$ which in a simple complex, perhaps with catecholamines themselves, will oxidatively destroy dopamine, epinephrine, norepinephrine, and their precursor DOPA in an efficient manner (Archibald and Tyree, 1987).

The goal of the present study was to determine whether pre-weaning Mn exposure is associated with biochemical changes that might be indicative of oxidative stress in the developing rat brain. The rat is not ideally suited for studies on Mn neurotoxicity, since Mn is ubiquitously distributed in this species, whilst preferentially localized in basal ganglia of primates (Aschner et al., 2001). Nevertheless, since a series of biochemical measurements was conducted in cerebral cortex (CTX) and cerebellar cortex (CB) derived from the animal cohorts previously described by Dorman et al. (2000), we had a unique opportunity to broaden the database on Mn neurotoxicity in this animal model. It should also be noted, that studies in areas most germane to primate Mn-induced neurotoxicity (basal ganglia) could not be performed on this animal cohort, since these areas were unavailable for analyses due to the shortage of tissues. Metallothionein (MT; a protein scavenger of metals) mRNA levels, glutamine synthetase (GS; a key component of glutamate and ammonia detoxification) activity and protein levels, as well as total glutathione (GSH) levels were determined as indicators of oxidative stress (Section 4).

## MATERIALS AND METHODS

### *Animal Treatment*

Primiparous pregnant CD rats (gestational day 13-15) were purchased from Charles River Laboratories (Raleigh, NC). The animals were housed in polycarbonate cages containing cellulose-fiber chip bedding (ALPHA-dri™; Shepard Specialty Papers, Kalama-zoo, MI) which were located in a HEPA-filtered, mass air-displacement room maintained at 20 ± 1 °C and 50 ± 10% relative humidity with a 12-h (07:00/19:00) light—dark cycle. The study was approved by the CIIT Institutional Animal Care and Use Committee (IACUC) and conducted under federal guidelines for the care and use of laboratory animals.

Mn dichloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) was obtained from Sigma Chemical Co. (St. Louis, MO). Mn was dissolved in nanopure water and given to the neonates by mouth using a micropipette for 21 consecutive days at doses of 0, 25 or 50 mg $MnCl_2$ $kg^{-1}$ body weight. The date of parturition was designated as postnatal day 0 (PND 0). On PND 4, litters were randomly reduced to four animals per sex whenever possible. Litters of less than eight pups were not used. Pups were dosed according to the average pup weight for each litter starting on the day after birth (PND 1) until weaning (PND 21). Clinical examinations (Dorman et al., 2000) were performed daily on all the animals before Mn administration. The neonatal rats were killed on PND 21 (with $CO_2$), and samples of cerebellum and CTX were collected and frozen in liquid nitrogen.

### *RNA Isolation and Northern Blot Analysis*

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

For MT, 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 mM Tris pH 8.0 for 1 h at 45 'C. To probe for MT, 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 [alpha-$^{32}$P] dCTP-labeled random primed cDNA probe (approximately 1 X $10^8$ cpm/µg; RadPrime DNA Labeling System, GibcoBRL, Life Technologies, Rockville, MD). Membranes were washed 2-3 times in 2 x 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 0.1 x SSC/0.1%SDS/40 mM Tris buffer and probed for 28s rRNA (Barbu and Dautry, 1989).

### *Protein Isolation and Western Blot Analysis*

Tissue lysates were centrifuged for 10 min at 10,000 x *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 x *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 2% SDS and heated to 65 °C.

Five microliter of 5 x loading buffer (50% glycerol; 10% SDS, 0.25 M Tris pH 6.8) and DTT (final concentration 100 mM) 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 SDS-PAGE with a 5% stacking and 8% resolving acrylamide gels in a 0.1% SDS, 25 mM Tris, 192 mM glycine buffer. Following fractionation, proteins were electrophorectically transferred to a nitrocellulose membrane (Protran, BA83, Schleicher & Schuell, Keene, NH) in 20% methanol, 0.1% SDS, 25 mM Tris and 192 mM glycine for 3 h at 60 V.

Membranes were blocked with 5% low-fat powdered milk in Tris-buffered saline with Tween (TBST, 0.1% Tween, 150 mM NaCl, 20 mM Tris) 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 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).

### *Glutamine Synthetase Activity*

An aliquot of the tissue lysate (150 µg) was added to a fresh microcentrifuge tube and the volume was brought up to 400 µl with lysis buffer. A 2x reaction buffer was prepared fresh to provide a final assay concentration of 120 mM L-glutamine, 10 mM sodium arsenate, 50 mM imidazole and 30 mM hydroxylamine, pH 6.5. Immediately prior to use, $MnCl_2$ (30 µM final concentration) and ATP (200 µM final concentration) were added to the 2x reaction buffer. The 2 x reaction buffer (400 µl) was added to the 400 µl of tissue lysate. The microcentrifuge tubes were gently vortexed and incubated at 37 °C for 60 min. Standards of L-glutamic acid γ-monohydroxamate (γ-glutamyl hydroxamate) were prepared in imidazole lysis buffer and incubated in parallel with the experi-mental samples. Following incubation, 200 µl of $FeCl_3$ buffer ($FeCl_3 \cdot 7H_2O$ 15% w/v trichloroacetic acid 25% v/v, hydrochloric acid 2.5 N) was added and proteins were allowed to precipitate for 60 min at 4 °C, after which time the samples were centrifuged for 10 min at 10,000 x *g*. The absorbance of the supernatant was measured at 490 nm with a molecular devices $V_{max}$ kinetic microplate reader (Sunnyvale, CA).

### *GSH Level Determination*

Tissue samples (50-100 mg) were homogenized in 1 ml of 10% (v/v) percholoric acid containing 1 mM bathophenanthroline disulfonic (BPDS) acid and L-γ-glutamyl-L-glutamate. The mixture was vortexed and centrifuged and an aliquot was removed for HPLC analysis (Fariss and Reed, 1987) 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 approximately 50 pmol, which equated to approximately 0.4 nmol/mg protein (Lash and Tokarz, 1990; Lash and Woods, 1991).

### *Statistical Analysis*

All experiments were conducted using five to seven treated neonates per Mn treatment group. To determine statistical significance, one-way analysis of variance (ANOVA) was used and when the overall significance resulted in rejection of the null hypothesis ($P < 0.05$), the source of the variance was determined with the Student—Newman—Keuls post-test. All analyses were performed using GraphPad InStat version 3.02 for Windows (GraphPad Software, San Diego, CA). Unless otherwise noted, all data presented represent mean ± S.E.M. values.

## RESULTS

### *GS Protein Expression and Activity*

Mn treatment did not significantly alter either GS protein expression or the levels of GS enzyme activity in either the CTX or the CB (Table 1).

Table 1
GS protein levels and activity[a]

| Endpoint | Cortex | Cerebellum |
|---|---|---|
| GS protein levels | | |
| Control | 100.00 ± 1.75 | 100.00 ± 3.60 |
| Low Mn dose | 93.55 ± 2.47 | 95.35 ± 7.57 |
| High Mn dose | 88.97 ± 6.42 | 95.30 ± 8.09 |
| GS activity | | |
| Control | 100.00 ± 7.84 | 100.00 ± 7.82 |
| Low Mn dose | 121.18 ± 9.13 | 98.92 ± 8.49 |
| High Mn dose | 121.23 ± 16.33 | 97.32 ± 6.70 |

[a] Values represent percentage of control mean ± S.E.M. ($n = 5$ and 9 for the CTX and cerebellum, respectively). Samples were derived from different litters. *$P < 0.05$ vs. respective control.

### *GSH Levels*

Mn treatment of neonatal rats did not significantly alter the levels of total GSH in the CB (Table 2). However, a statistically significant increase ($P < 0.05$) in total GSH levels was noted in the CTX of pups treated with the high Mn dose when compared to control rats (Table 2).

Table 2
Total GSH levels in CTX and cerebellum from Mn-exposed neonatal rats[a]

| Endpoint and treatment group | Cortex | Cerebellum |
|---|---|---|
| Total GSH | | |
| Control | 100.00 ± 7.60 | 100.00 ± 9.74 |
| (nmol $mg^{-1}$ protein) | 0.571 ± 0.04 | 0.814 ± 0.08 |
| Low Mn dose | 80.56 ± 4.21 | 87.95 ± 6.64 |
| (nmol $mg^{-1}$ protein) | 0.460 ± 0.02 | 0.716 ± 0.05 |
| High Mn dose | 122.19 ± 5.84* | 122.72 ± 9.61 |
| (nmol $mg^{-1}$ protein) | 0.698 ± 0.03 | 0.999 ± 0.08 |

[a] Values represent percentage of control mean ± S.E.M. ($n = 5$ and 9 for the CTX and cerebellum, respectively). Samples were derived from different litters.
* $P < 0.05$ vs. respective control.

Table 3
MT mRNA levels in the CTX and CB of Mn-treated pups[a]

| | Cortex | Cerebellum |
|---|---|---|
| Control | 100.00 ± 19.67 | 100.00 ± 5.49 |
| Low Mn dose | 90.87 ± 44.09 | 133.07 ± 40.84 |
| High Mn dose | 57.03 ± 29.13 | 154.49 ± 22.75 |

[a] Values represent percentage of control mean ± S.E.M. ($n = 5$ and 8 for the low Mn dose for the CTX and cerebellum, respectively, $n = 5$ and 6 for the high Mn dose for the cerebral cortex and cerebellum, respectively). Samples were derived from different litters.

### *MT mRNA Levels*

Northern blot analyses revealed that treatment of neonatal rats with either the low or high Mn dose did not alter the levels of MT mRNA in either the CTX or the CB (Table 3).

## DISCUSSION

The exposure to Mn in our experiments is approximately 75-150-fold higher than from consumption of rat's milk (Keen et al., 1981) and will result in an approximately two-fold increase in brain Mn concentrations in weanling animals (Dorman et al., 2000). As reported by Dorman et al. (2000), weanling rats given either 25 or 50 mg $kg^{-1}$ body weight per day $MnCl_2$ from PND 1 to 21 had significantly increased cortical Mn concentrations when compared with control animals given water. Increased cerebellar Mn levels were also observed in neonates exposed to the high Mn dose (50 mg $MnCl_2$ $kg^{-1}$ body weight per day).

Mn has been shown to enhance the autoxidation and/ or turnover of intracellular catecholamines, resulting in increased production of ROS and other cytotoxic metabolites, concomitant with depletion of antioxidant defense mechanisms (Donaldson, 1987; Graham, 1984; Liccione and Maines, 1988; Parenti et al., 1988). Injection of Mn produced changes in monoamine concentrations in different brain regions of rats dosed with either $MnCl_2$ ($Mn^{+2}$) or MnOAc ($Mn^{+3}$) (Parenti et al., 1988). ROS were measured by a molecular probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), and both divalent and trivalent Mn led to ROS generation; however, $Mn^{+3}$ was shown to be an order of magnitude more potent than $Mn^{+2}$ (Ali et al., 1995). Consistent with Mn-induced oxidative stress, Desole et al. (1995) reported that allopurinol antagonized Mn-induced oxidative stress by inhibiting dopamine-induced oxidative stress in the brainstem of 3-month-old male rats dosed with Mn ($MnCl_2$). The same authors (Desole et al., 1994) have also established that the response of striatal cellular defense mechanisms (e.g. increase in ascorbic acid (AA) oxidation, decrease in GSH levels) highly correlated with changes in markers of dopaminergic system activity and uric acid levels, providing evidence for Mn-induced oxidative stress via xanthine oxidase.

Brenneman et al. (1999) failed to demonstrate elevated striatal 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentrations in young rats orally exposed to 0, 25, or 50 mg $kg^{-1}$ per day of $MnCl_2$ from PND 1 to 49 despite observing elevated Mn levels in mitochondria isolated from the striatum. Their findings suggest that even relatively high dose oral Mn exposure does not significantly alter 8-OHdG levels in either mitochondrial or nuclear DNA. A failure to observe increased 8-OHdG levels following Mn exposure does not eliminate ROS formation in the pathogenesis of Mn-induced neurotoxicity. As suggested by Halliwell (2000) 8-OHdG may be only a minor product of oxidative DNA damage. Indeed, 8-OHdG may be difficult to measure because of the ease with which it is formed artifactually during isolation, hydrolysis and analysis of DNA. Accordingly, direct measures of mRNA, protein expression and activity of proteins that are known to be "markers" for oxidative stress (such as GS; Levine et al., 1981; Liaw et al., 1993; Nakamura and Stadtman, 1984) and redox status (total, reduced and oxidized GSH; MT) are likely to provide more meaningful assessment of oxidative stress.

Brenneman et al. (1999) reported Mn-induced elevations in ROS levels in the PND 49 rat cerebellum, but not striatum. In the present study we only observed an absolute increase in the total amount of GSH in the cortex of PND 21 rats given Mn throughout lactation. The dose of Mn used resulted in significantly increased cerebellar and cortical Mn levels in neonates exposed to the high Mn dose (50 mg $MnCl_2$ $kg^{-1}$ body weight per day) (Dorman et al., 2000). The tripeptide GSH (γ-glutamylcysteinylglycine) is the major anti-oxidant in mammalian cells constituting nearly 90% of the intracellular non-protein thiols (Anderson and Meister, 1983). It is important in maintaining the intracellular redox status of protein thiols, for protection against endogenous and exogenous sources of oxidative stress, and for the conjugation and excretion of toxic molecules (Meister, 1988, 1991; Stohs and Bagchi, 1995). It is unclear at this point whether this modest increase in GSH reflects oxidative stress, but given that we failed to note a significant change in levels of oxidized GSH in the cortex (data not shown), these preliminary data do not substantiate a pro-oxidant effect by Mn.

Within the CNS, GS is localized almost exclusively in astrocytes (Martinez-Hernandez et al., 1977). Glutamine produced by astrocytes via the action of GS is released from astrocytes and taken up by neighboring glutamatergic or GABAergic neurons as precursors for neurotransmitter synthesis as part of the glutamate—glutamine cycle (Benjamin and Quasstel, 1975; Schousboe et al., 1992; Sonnewald et al., 1997). In addition to providing glutamine to neurons, other glutamate-derived metabolites, such as the tricarboxylic acid (TCA) cycle precursors lactate, malate and citrate are utilized by neurons for energy production (Sonnewald et al., 1991). Thus, inhibition of GS activity may result in decreased neuronal energy levels, decreased synthesis of glutamate and GABA, and the inability to detoxify ammonia within the CNS. Decreases in GS activity might be mediated by multiple mechanisms. The GS macromolecule is susceptible to oxidative modification resulting in decreased synthetic activity (Levine et al., 1981; Liaw et al., 1993; Nakamura and Stadtman, 1984). In addition to direct inactivation by ROS, the oxidized GS protein under-goes rapid degradation by intracellular proteases leading to decreases in both activity and protein levels. The present study failed to establish a statistically significant effect of Mn on GS protein levels and GS activity in cortex or cerebellum at either low or high dose. Given the exquisite susceptibility of GS to oxidative modification, the absence of decreased synthetic activity upon Mn exposure likely reflects on the absence of Mn-induced oxidative stress.

The MTs, a class of cysteine-containing intracellular proteins, are characterized by a molecular mass of 6-7 kDa. These highly conserved proteins are expressed in all mammals with a wide organ and cellular distribution and they represent an important metal binding protein (Andrews, 1990; Dunn et al., 1987; Hamer, 1986). MT's antioxidant properties may depend on its metal speciation, namely, the ability of oxygen free radicals to release zinc from MT thiolate clusters (Hainaut and Milner, 1993). Circumstantial evidence supports the hypothesis that increased levels of MTs could potentially neutralize ROS in peripheral tissues and the CNS. Data in support of the antioxidant function of MT, derived from in situ hybridization studies, reveal that bacterial endotoxin induces MT gene expression in the brain (Itano et al., 1991). Transcriptional induction of the MT-I gene in the CNS is also increased by oxidative stress, kainic acid and 6-hydroxydopamine, which generates free radicals and is known to be toxic to dopaminergic neurons (Shiraga et al., 1993). Hypoxia produces a long lasting protection against kainic acid-induced injury, such that when kainic acid is injected into the CNS one week later, both seizures and neurodegenerative changes in the hippocampus and pyriform cortex are markedly reduced (Dalton et al., 1995). Collectively, these studies suggest a singularly important role for constitutive levels of MT as an antioxidant. In the present study we noted increased MT mRNA production in the cerebellum from PND 21 rats in the high Mn dose group; however, this increase was statistically insignificant, possibly due to the high variability in the blots and a definitive answer to this issue will have to await further studies.

In summary, the present study determined whether oxidative stress plays a role in Mn-induced neurotoxicity in preweanling rats. Our preliminary results indicate a modest, yet significant change in the level of total GSH in rats exposed to high Mn dose versus controls. The change was limited to the cortex and it was absent from the cerebellum. The other measures of oxidative stress (MT mRNA; GS activity and protein levels) were unperturbed in both regions (cerebellum or cortex) and dose regimens (high and low Mn) compared with control rats. Given that the cortex does not represent a target area for Mn toxicity in primates, it cannot be

unequivocally stated at this point that our data support the ability of Mn to induce oxidative stress in neonatal rats. Studies in our laboratory are presently being carried out to address this issue, focusing on Mn-induced oxidative stress in basal ganglia, representing preferential sites for Mn accumulation and toxicity in humans.

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