Effects of inhaled manganese on biomarkers of oxidative stress in the rat brain

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Abstract:
Manganese (Mn) is a ubiquitous and essential element that can be toxic at high doses. In individuals exposed to high levels of this metal, Mn can accumulate in various brain regions, leading to neurotoxicity. In particular, Mn accumulation in the mid-brain structures, such as the globus pallidus and striatum, can lead to a Parkinson's-like movement disorder known as manganism. While the mechanism of this toxicity is currently unknown, it has been postulated that Mn may be involved in the generation of reactive oxygen species (ROS) through interaction with intracellular molecules, such as superoxide and hydrogen peroxide, produced within mitochondria. Conversely, Mn is a required component of an important antioxidant enzyme, Mn superoxide dismutase (MnSOD), while glutamine synthetase (GS), a Mn-containing astrocyte-specific enzyme, is exquisitely sensitive to oxidative stress. To investigate the possible role of oxidative stress in Mn-induced neurotoxicity, a series of inhalation studies was performed in neonatal and adult male and female rats as well as senescent male rats exposed to various levels of airborne-Mn for periods of time ranging from 14 to 90 days. Oxidative stress was then indirectly assessed by measuring glutathione (GSH), metallothionein (MT), and GS levels in several brain regions. MT and GS mRNA levels and regional brain Mn concentrations were also determined. The collective results of these studies argue against extensive involvement of ROS in Mn neurotoxicity in rats of differing genders and ages. There are, however, instances of changes in individual endpoints consistent with oxidative stress in certain brain tissues.
Keywords: Manganese; Neurotoxicity; Manganism; Oxidative stress; Reactive oxygen species

Article:
1. Introduction
Manganese (Mn) is a naturally occurring element that is widespread in the environment. It is crucial for maintaining the proper function and regulation of many biological processes and is therefore an essential nutrient. Mn is a constituent of many enzymes involved in fat and protein metabolism, and is utilized by various antioxidant enzymes such as Mn superoxide dismutase (MnSOD) and glutamine synthetase (Cotzias, 1958; Lee, 2000; Takeda, 2003; Wedler and Denman, 1984). Additionally, this important element is involved in immune function, regulation of blood sugar, production of cellular energy, reproduction, digestion, bone growth, carbohydrate metabolism, and blood clotting (Aschner, 2000).

Mitochondrial MnSOD uses Mn in scavenging and detoxifying free radicals. Furthermore, MnSOD is critical in preventing or limiting apoptotic and necrotic events resulting from cellular damage caused by reactive oxygen species (ROS) (Halliwell, 2001; Raha and Robinson, 2000). Changes in Mn containing proteins have been observed in many neurodegenerative diseases, including Alzheimer's disease (Frankel et al ., 2000; Markesbery, 1997), amyotrophic lateral sclerosis, and Parkinsonian-like syndrome (Malecki, 2001; Pal et al ., 1999; Zelko et al., 2002), as well neurobehavioral deficits (He et al ., 1994; May, 2000; Normandin et al ., 2002; Wecker et al., 1985).

Mn is common in the environment, and is naturally occurring in air, soil, and water. By virtue of its sulfur-fixing, deoxidizing, and alloying properties, Mn is used extensively in the production of steel and other alloys, accounting for>90% of its global demand (U.S. Geological Survey, 2004). It is also used in other industries,

such as welding and metal working, battery production, glass and ceramics manufacturing, and in formulating the gasoline anti-knock additive methylcyclopentadienyl manganese tricarbonyl (MMT).

In humans, pathologies associated with Mn usually result from exposure to excessive Mn instead of its deficiency. Mn is obtained by mining, and cases of miners being overexposed to Mn have been reported (Pal et al., 1999). Chronic Mn overexposure results in the onset of a neurological phenotype known as manganism. This disease presents with motor symptoms resembling, but distinguishable from, those of Parkinson's disease (Aschner and Aschner, 1991; Lee, 2000; Pal et al., 1999). While airborne M n is considered to be the most relevant route of exposure in occupational settings, increases in blood and brain Mn levels have also been reported in persons with liver disease, which results in impaired clearance of Mn (Burkhard et al., 2003; Rose et al., 1991; Spahr et al., 1996), and those receiving prolonged parenteral nutrition (Iinuma et al., 2003; McKinney et al., 2004; O'Donnell and Radigan, 2003; Suzuki et al., 2003). Additionally, rodent data suggest that iron deficiency may enhance the bioaccumulation of Mn (Erikson et al., 2002a, 2004a; Ellingsen et al., 2003; Kim et al., 2005). Given the prevalence of iron deficiency in numerous parts of the world (ACC/SCN, 1992), certain populations may be at a greater risk to Mn overexposure than others.

While it is known that excessive Mn can be cytotoxic, the mechanism by which this occurs is still under investigation. It has been hypothesized that cytotoxicity may be due to oxidative stress; specifically, mitochondrial oxidative stress (Dobson et al., 2004; Erikson et al., 2004c; Halliwell, 2001; HaMai and Bondy, 2004; Melov, 2002; Verity, 1999). Mitochondria are critical for numerous essential aspects of cell function, from ATP energy production via the respiratory chain to steroid biosynthesis, heme assembly, pyrimidine biosynthesis, the tricarboxylic acid cycle, and apoptosis (Melov, 2004). However, mitochondria are also the main producers of ROS within the cell (Melov, 2004). The electron transport chain, located on the mitochondrial inner membrane, has a natural "leak" of the highly reactive superoxide radical. Mitochondrial oxidative stress is paradoxically both potentially alleviated and induced by the presence of Mn. This is because the primary endogenous antioxidant of the mitochondrial matrix is MnSOD (Melov, 2004), which removes superoxide by enzymatically converting it to hydrogen peroxide. Hydrogen peroxide can then be converted to oxygen and water by the enzyme catalase. However, hydrogen peroxide can also react through Fenton chemistry with transition metal ions, such as iron, to form the highly reactive hydroxyl radical, which can lead to increased oxidative stress (Ercal et al., 2001; HaMai et al., 2001; Santos et al., 2004). Also, oxidizing conditions within the cell may lead to the oxidation of divalent Mn (Mn^{2+}) to trivalent Mn (Mn^{3+}) , as has been shown in vitro (Archibald and Tyree, 1987). It has been hypothesized that Mn^{3+} is both a pro-oxidant in itself as well as an inhibitor of mitochondrial respiration, leading to increased ROS formation (HaMai et al ., 2001). Significant oxidative stress has severe effects on cell survival pathways, often leading to necrosis or apoptosis (Chong et al., 2005; H inerfeld et al., 2004).

Many previous animal studies of Mn overexposure have utilized oral dosing of M n. However, there has been recent concern regarding overexposure to M n via inhalation. Over the past few years, a series of experiments has been carried out utilizing inhalational Mn exposures sufficient to result in Mn bioaccumulation and allow the examination of several biomarkers of oxidative stress in the brain of the Mn-exposed rats (Dorman et al., 2001, 2004, 2005; Dobson et al ., 2003; Erikson et al., 2004b, 2005, in press). These studies examined neonatal and adult male and female rats as well as senescent male rats exposed to airborne M n for 14–90 days.
In these studies, oxidative stress was indirectly assessed by measuring the levels of two important antioxidants, glutathione (GSH) and metallothionein (MT), as well as glutamine synthetase (GS), an enzyme exquisitely sensitive to oxidative stress. MT and GS mRNA levels and regional brain Mn concentrations were also determined.

Within the brain, GS is exclusively expressed in astrocytes (Martinez-Hernandez et al ., 1977). A Mn-dependent enzyme, GS catalyzes the formation of glutamine from glutamate. The newly formed glutamine is taken up by local glutamatergic or γ-aminobutyric acid-containing (GABA-ergic) neurons where deamination back to glutamate occurs. 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 a sensitive marker for the presence of ROS in the brain (Stadtman, 1992).

The MTs, a class of cysteine-containing intracellular proteins, are highly conserved and widely distributed throughout all cell types. They are important metal binding proteins, with zinc serving as the primary regulator of MT metabolism (Andrews, 1990; Dunn et al ., 1987; Hamer, 1986). 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 (Itano et al ., 1991). Oxidative stress, kainic acid, and 6-hydroxydopamine, a known dopaminergic toxicant and ROS generator, induce MT- I gene expression in the brain (Shiraga et al ., 1993). Likewise, compounds that generate reactive species via redox cycling (e.g., diquat), along with compounds that cause lipid peroxidation (e.g., 3- methylindole) or that deplete cellular antioxidant defense mechanisms (e.g., diamide and dimethyl maleate), increase MT levels in tissue (Bauman et al., 1991).

GSH is a ubiquitous antioxidant formed from glutamate, cysteine, and glycine, leading to the formation of γ-glutamylcysteinylglycine. It constitutes approximately 90% of the intracellular non-protein thiols, and functions in conjugation and elimination of toxic molecules, thereby maintaining cellular redox homeostasis (Meister and Anderson, 1983). The two forms of glutathione, oxidized and reduced (GSSG/2GSH), comprise the most abundant redox coupling mechanism in a cell (Kirlin et al., 1999). Alterations in brain GSH metabolism have been linked to oxidative stress and various neurodegenerative diseases (Gegg et al ., 2003). For example, Sian et al . (1994) demonstrated decreased GSH levels in the substantia nigra of Parkinson's disease patients compared to non-diseased individuals. Correspondingly, GSH levels are significantly and age-dependently lowered in the striatum of Mn-exposed rats (Desole et al ., 1995; Erikson et al., 2004b).

The purpose of this review is to summarize a recent series of studies involving the exposure of rats to inhaled Mn and the measurement of biomarkers for oxidative stress in different brain regions. These studies used rats of both genders and varying ages and employed differing exposure regimens, including post-exposure recovery. Collectively, these studies have provided an in-depth exploration of the impact of airborne- Mn exposure on biochemical markers of oxidative stress in brain regions that accumulate M n to varying degrees. Examination of these studies allows an assessment of whether oxidative stress occurs in the rat brain following Mn inhalation.

2. Materials and methods used in these studies

Detailed information on animal exposures, tissue Mn measurements, and related statistical analyses has been previously published (Dorman et al ., 2001, 2004, 2005). All studies were conducted under Federal guidelines for the care and use of laboratory animals and were approved by the CI IT Centers for Health Research Institutional Animal Care and Use Committee. Several exposure paradigms were used in these studies; acute (14 days; Dorman et al ., 2001), subchronic (90 days; Dorman et al ., 2004), and during gestation and lactation (Dorman et al ., 2005). For the acute exposure, adult male CD rats were exposed 6 h/day, 7 days/week for 14 days (14 exposures) to air (control) or manganese sulfate ($MnSO_4$) at nominal concentrations of 0.03, 0.3, or 3 mg Mn/m^3 . During the subchronic exposure, young adult male and female CD rats and aged (16 months old at the start of the exposure) male rats were exposed 6 h/day, 5 days/week for 90 days (65 exposures) to air or M nSO4 at nominal concentrations of 0.01, 0. 1, or 0.5 mg Mn/m^3, or Mn phosphate (as hureaulite) at 0. 1 mg Mn.

For analysis of pups following exposure during gestation and lactation, male and female rats (parenteral generation) were exposed to air or $MnSO_4$ at nominal concentrations of 0.05, 0.5, or 1.0 mg Mn/m3 starting 28 days prior to breeding. This was continued through breeding and pregnancy for the females (and their gestating pups), and during lactation for the dams and the pups lasting until the pups reached post-natal day (PN D) 18, at which time exposure was halted. Brain tissue was harvested from the pups on either PN D 19 or PN D 45, the latter providing the pups with nearly four weeks of recovery. At the designated time-points in all the above

exposures, animals were euthanized with CO_2. The brain areas of interest were dissected out and weighed, and then placed in high-purity linear polyethylene vials, frozen in liquid nitrogen, and stored at —80 °C until analysis. Tissue manganese concentrations were determined by graphite furnace atomic absorption spectrometry.

Detailed protocols for the biomarker measurements and their statistical analyses can be found in the original publications (Dobson et al ., 2003; Erikson et al ., 2004b, 2005, in press). GS protein levels were analyzed in tissue lysates by western blot analysis using SDS-PAGE electrophoresis. GS and MT mRNA levels were analyzed by northern blot analysis following tissue homogenization and RNA extraction. Total GSH was analyzed by HPLC using an ion-exchange method.

All statistical comparisons were made between groups of Mn-exposed animals and age-matched air-exposed controls. A significance level of $p < 0.05$ was used in all of these studies.

3. Results and discussion

For the purpose of this review, the levels of oxidative stress biomarkers (GS protein and mRNA, MT mRNA, and total GSH) in various brain regions (cerebellum, olfactory bulb, striatum, hippocampus, and hypothalamus) were identified in their original publications (Dobson et al ., 2003; Erikson et al., 2004b, 2005, in press) as either not changing or significantly increasing or decreasing, and presented in tabular format. The only data presented here not included in the original publications are the results for total GSH in the tissues from PND 19 pups, which were provided for this review. In the tissues with concomitant Mn level measurements, significant increases in Mn tissue levels were noted from the original reports (Dorman et al ., 2001, 2004, 2005) and included in the tabular format. Biomarker results and brain Mn increases in regions where Mn levels were measured (cerebellum, olfactory bulb, and striatum) are shown in Table 1. Biomarker results in brain regions without corresponding Mn-level data are shown in Table 2.

An important attribute of these studies is the use of inhalation exposure to Mn. Concerns exist regarding airborne exposure to Mn, especially in occupational settings. The concentrations used in these studies (0.01–3 mg Mn/m^3) were chosen to ensure tissue Mn bioaccumulation. Thus they are quite high when compared to environmental exposures, and often exceed the occupational limits as well. The American Conference of Governmental and Industrial Hygienists' threshold limit value-time-weighted average (ACGI H TLVTWA) for Mn is 0.2 mg/m^3, which is a concentration to which most workers can be exposed without adverse effects (ACGIH, 1999). The current inhalation reference concentration (RfC) for Mn, which is relevant to environmental exposures and is set by the U.S. Environmental Protection Agency (USEPA), is 0.05 μg Mn/m^3 (USEPA, 1993). An RfC is an estimate of an exposure level that is likely to be without an appreciable risk of deleterious effects for a lifetime. Thus the Mn concentrations used in these studies ranged from 200 to 60,000 times higher than the Mn RfC. The study designs were often successful in achieving Mn bioaccumulation in several brain regions, including the striatum, a target-tissue for Mn neurotoxicity.

The predominant Mn species used in these inhalation exposures is $MnSO_4$, which is relatively water-soluble and therefore more readily transferred from lung tissue into the blood. This subsequently led to higher brain concentrations in preliminary studies than inhalation of Mn phosphate (Dorman et al ., 2001). Mn phosphate, which is less water-soluble and less readily delivered to the brain (Dorman et al ., 2001), was used for comparison to assess the relationship of solubility to brain Mn bioaccumulation in the 90 days studies (Dorman et al., 2004).

Table 1

Changes in glutamine synthetase (GS) protein, GS mRNA, metallothionein (MT) mRNA, and total glutathione (GSH) concentrations in the cerebellum, olfactory bulb, and striatum of rats following acute (14 days) or subchronic (90 days) Mn exposure, or on post-natal days 19 or 45 after exposure through gestation and lactation until PND 18

		Cerebellum				Olfactory Bulb				Striatum			
		GS Protein	GS mRNA	MT mRNA	Total GSH	GS Protein	GS mRNA	MT mRNA	Total GSH	GS Protein	GS mRNA	MT mRNA	Total GSH
14 Day[a]	**mg Mn/m^3**												
Young Males	0.03	–	↑	–	–	–	nd	nd	–	–	nd	nd	–
	0.3	–	–	–	–	↑	nd	nd	–	–	nd	nd	–
	3	↓	↑	–	–	↑	nd	nd	–	–	nd	nd	–
90 Day[b]													
Young Males	0.01	–	↓	↓	–	–	↓	↓	↓	–	–	–	–
	0.1	–	↓	↓	–	–	↓	↓	↓	–	–	–	–
	0.5	–	↓	↓	–	–	–	–	↓	–	–	–	–
	0.1P	–	↓	↓	–	–	–	–	↓	–	–	–	–
Young Females	0.01	–	–	–	–	–	–	–	–	–	–	–	↓
	0.1	–	–	–	–	–	–	–	↑	–	–	–	↓
	0.5	–	–	–	–	↑	–	–	↑	–	–	–	↓
	0.1P	–	–	–	–	–	–	–	–	–	–	–	↓
Aged Males	0.01	–	–	–	–	–	–	–	–	–	–	–	↓
	0.1	–	–	–	–	–	–	–	–	–	–	–	↓
	0.5	–	–	–	↓	–	–	–	–	–	–	–	↓
	0.1P	–	–	–	↓	–	–	↓	–	–	–	–	–
Pups[c]													
PND19	0.05	↓	–	–	–	–	–	–	–	–	↓	–	–
	0.5	↓	–	–	–	–	–	–	–	–	↓	↓	↓
	1	↓	–	–	–	–	–	–	–	–	↓	↓	↓

	Gender:	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
PND45	0.05	–	–	–	–	–	–	↓	–	–	–	–	↓	–	–	–	↓	–	–	–	↓	–	↓	–	–
	0.5	–	–	–	–	–	–	↓	–	–	–	–	↓	–	–	–	↓	–	–	–	↓	–	↓	–	–
	1	–	–	–	–	–	–	↓	–	–	–	↑	–	–	–	–	↓	–	–	–	↓	–	↓	–	–

↓: Decreased when compared with age and gender-matched air-exposed controls ($p < 0.05$); ↑: increased when compared with age and gender-matched air-exposed controls ($p < 0.05$); –: no statistical difference from age and gender-matched air-exposed controls; nd: not determined, shading indicates significant tissue Mn increase when compared with age and gender-matched air-exposed controls ($p < 0.05$). Exposures are Mn sulfate in mg Mn/m^3 except 0.1P, which is Mn phosphate (hureaulite) at 0.1 mg Mn/m^3. [a]Dobson et al. (2003) and Dorman et al. (2001); [b]Erikson et al. (2004b) and Dorman et al. (2004); [c]Erikson et al. (2005, in press) and Dorman et al. (2005).

Table 2
Changes in GS protein, GS mRNA, MT mRNA, and total GSH concentrations in the hippocampus and hypothalamus of rats following acute (14 days) or subchronic (90 days) Mn exposure, or on post-natal days 19 or 45 after exposure through gestation and lactation until PND 18

	Hippocampus				Hypothalamus			
	GS protein	GS mRNA	MT mRNA	Total GSH	GS protein	GS mRNA	MT mRNA	Total GSH
14 days[a] (mg Mn/m^3)								
Young males								
0.03	–	–	–	–	↑	–	–	–
0.3	–	–	–	–	↑	–	↑	–
3	–	–	–	–	↑	–	–	↓
90 days[b]								
Young males								
0.01	–	–	–	–	–	–	–	–
0.1	–	–	–	–	–	–	–	–
0.5	–	↓	↓	–	–	–	–	–
0.1P	↑	–	–	–	↓	–	–	–
Young females								
0.01	–	–	–	–	–	–	–	–
0.1	↓	–	–	–	–	–	–	–
0.5	–	–	–	–	–	–	↓	–
0.1P	–	–	–	–	–	↑	–	–
Aged males								
0.01	↓	–	–	–	–	↓	–	–
0.1	↓	–	↓	–	↓	–	–	–
0.5	–	↓	↓	–	–	–	–	–
0.1P	–	–	↓	–	–	–	–	↓
Pups[c]								
PND 19								
0.05	–	–	–	–	–	–	–	–
0.5	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	–	–

	Gender															
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
PND 45																
0.05	–	–	–	–	↓	–	–	–	–	–	–	–	↓	–	–	–
0.5	–	–	–	–	↓	–	–	–	–	–	–	–	↓	–	–	–
1	–	–	–	–	–	–	–	–	–	–	↑	–	–	–	–	–

↓: Decreased when compared with age and gender-matched air-exposed controls ($p < 0.05$); ↑: increased when compared with age and gender-matched air-exposed controls ($p < 0.05$); –: no statistical difference from age and gender-matched air-exposed controls, exposures are Mn sulfate in mg Mn/m^3 except 0.1P, which is Mn phosphate (hureaulite) at 0.1 mg Mn/m^3.

[a] Dobson et al. (2003).
[b] Erikson et al. (2004b).
[c] Erikson et al. (2005, in press).

In cases of chronic clinical manganese toxicity in humans (Cotzias, 1958), there can be relatively poor correlation between tissue manganese concentrations and clinical signs since the injury to the brain tissue may be present well after an exposure has occurred and tissue manganese concentrations (including brain) have returned to more normal levels. The current studies explored whether oxidative stress likely plays a role in manganese neurotoxicity by employing exposure times from 14 to 90 days. Brain manganese concentrations were highest after the end of the exposures and declined thereafter. We therefore hypothesize that if manganese exposure triggers oxidative stress, then associations between manganese exposure, brain manganese concentration, and markers for oxidative stress would occur simultaneously in the pathogenesis of the disease syndrome and should be apparent in these less than chronic studies.

The brain regions under examination in these studies are known to accumulate Mn to varying degrees following inhalation (Dorman et al., 2004). In these studies, the olfactory bulb accumulated Mn most readily, primarily due to the contribution of direct olfactory transport of Mn to the bulb, in addition to systemic delivery (Dorman et al., 2002). The contribution of olfactory transport to brain levels in humans is unknown, but is hypothesized

to be less than in rodents due to large anatomical differences between rodents and humans that favor delivery of inhaled xenobiotics to the rodent olfactory system (Aschner et al., 2005; Dorman et al., 2002). The striatum, known to accumulate Mn in overexposed humans and primates, tended to accumulate Mn in the rodent model, particularly at higher doses and longer durations, although not to the extent of the olfactory bulb (Dorman et al., 2004). The cerebellum is not traditionally regarded as a target tissue for Mn accumulation, although it occasionally exhibited an increase in Mn content over control tissue following high does for long durations (Dorman et al., 2004). The levels of Mn in the hippocampus and hypothalamus were not investigated in these studies, although these brain regions are also not traditionally regarded as being involved in the development of manganism.

GS protein is highly sensitive to oxidative stress because it is rapidly deactivated by oxidation and subsequently removed by protein turnover (Stadtman, 1992). Thus, under conditions of oxidative stress, it could be expected that the level of GS protein would decrease. This in turn may lead to an increase in GS mRNA, as homeostatic mechanisms would likely respond to maintain GS protein at an optimal level. GS protein did decrease in a few cases, particularly in the cerebellum of PND 19 pups following exposure through gestation and lactation to PND 18. There was not, however, any compensatory change in GS mRNA, and GS protein changes were not evident at PND 45. In the only case in which GS protein decreased and GS mRNA increased (in the cerebellum of young males treated for 14 days with $_{MnSO4}$ at 3.0 mg Mn/m^3), tissue Mn was not significantly elevated. It is unknown why GS protein increased in several cases, such as in the hypothalamus of the young males treated for 14 days with all doses of MnSO4. One could speculate this response represents an over-compensation for GS protein oxidation, although this has not been previously demonstrated. Alternatively, the increases could be unrelated to oxidative stress, perhaps reflecting increased accumulation of Mn and a subsequent increase in the expression of the Mn containing GS enzyme.

Importantly, GS protein levels in the striatum, a target tissue for Mn-induced neurotoxicity, were unaffected in any paradigm, even in cases when striatal Mn was significantly elevated. GS mRNA decreased in the striatum of PND 19 pups and in the female pups at PND 45 at all doses. The toxicological significance of decreased GS mRNA, especially without corresponding GS protein changes, is not currently understood. In the olfactory bulb, which showed preferential accumulation of Mn, only GS protein increases were evident, and in only three of the fourteen cases in which olfactory bulb Mn was elevated. In several cases, GS protein levels were decreased in the hippocampus of young females (0. 1 mg Mn/m^3 MnSO4) and old males (0.0 1 and 0. 1 mg Mn/m^3) and hypothalamus of young males (0. 1 mg Mn/m^3 Mn phosphate) and aged males (0. 1 mg Mn/m^3 MnSO4) during the 90-day study. However, these changes were neither dose-dependent nor accompanied by any GS mRNA changes.

MT is a metal-binding and antioxidant protein induced in the brain in response to oxidative stress (Andrews, 1990; Dunn et al., 1987; Hamer, 1986; Shiraga et al., 1993). Thus, an expected result of oxidative stress would be an increase in MT mRNA, in an attempt to respond to the oxidative challenge. No increase in MT mRNA was observed in any of the brain regions examined across all the studies with one exception. Increased MT mRNA was detected in the hypothalamus of young males treated with $_{MnSO4}$ at the mid-dose level (0.3 mg Mn/m^3) for 14 days. There were, however, a number of incidences where MT mRNA decreased. The most notable decreases occurred in the cerebellum of young males at all doses (despite significant Mn accumulation only at the 0.5 mg Mn/m^3 MnSO4 dose) and the hippocampus of aged males (all doses except the lowest MnSO4 dose) during the 90-day study, and in the striatum of female pups at all doses on PND 45 (after Mn levels had returned to normal). Otherwise, decreases in MT mRNA do not appear to be dose-dependent. It is unclear as to what decreases in MT mRNA signify, as it is currently unknown if and how oxidative stress might play a role in decreasing MT mRNA. This effect has been shown in vitro as well, as primary-cultured astrocytes exposed to 300 µM $_{MnSO4}$ displayed a significant decrease in MT mRNA expression when compared to control cells (Erikson et al., 2002b). It is possible that increased Mn may have altered the metabolism of zinc or copper, two known modulators of MT expression (Dunn et al., 1987), but this is currently speculative.

GSH is a ubiquitous intracellular antioxidant linked with oxidative stress and various neurodegenerative diseases (Gegg et al., 2003). It would be expected that an oxidative stressor would lead to decreases in GSH in tissue. In fact, such decreases have been shown both experimentally (Desole et al., 1995) and in patients with neurodegenerative diseases (Sian et al., 1994). Intriguingly, total GSH did indeed decline in the olfactory bulb of young males at all doses of Mn in the 90-day study, concurrent with significant Mn accumulation. Conversely, total GSH increased in the olfactory bulb of young females in the same study in the two doses that produced significant Mn accumulation (MnSO4 at $\geq$ 0. 1 mg Mn/m^3) . Total GSH also decreased in the striatum of aged males at 90 days at all three MnSO4 doses, but Mn accumulation was only significant in the highest MnSO4 dose (0.5 mg Mn/m^3) . Total GSH decreased in the striatum of young females at all doses of M nSO4 and Mn phosphate, but none of these tissues had significant Mn accumulation. Total GSH also decreased in the cerebellum of male pups (all doses) and the olfactory bulb of female pups (all doses) at PN D 45, at a time when tissue Mn levels were normal, but following a significant Mn elevation at PN D 19.

While there are a few changes in individual endpoints in discrete brain regions that suggest the occurrence of oxidative stress, there are no consistent changes across endpoints in any particular tissue, across gender and age, to verify that Mn induced oxidative stress is occurring in these studies. When viewed in isolation, there are some changes in individual endpoints consistent with oxidative stress in certain tissues.

In particular, total GSH in the striatum was significantly lowered in most of the female and aged male rats at 90 days and in the PN D 19 pups. However, significant striatal Mn accumulation, while present in the PN D 19 pups, was absent in the females and present only in the highest dose in the aged males. Furthermore, these changes in GSH were not accompanied by changes in any other endpoints in the manner expected if oxidative stress were involved. In aggregate, these studies do not provide compelling evidence for oxidative stress occurring following high dose Mn inhalation in rats. There are, however, several instances of changes which might indicate the existence of specific oxidative reactions in possible sensitive subpopulations. However, even in these groups, the changes are not consistent across endpoints.

Even though there are changes in various oxidative stress biomarkers inherent in the studies reviewed here, they often do not change in the direction expected if oxidative stress were involved, and/or they do not show a consistent dose-dependence with Mn treatment. These changes could be unrelated to Mn exposure (i .e. biological variability), or related to Mn exposure but unrelated to oxidative stress. A few may be statistical artifacts given the number of tests done in this series of experiments (e.g., Tables 1 and 2 present data for 488 biomarker tests with 85 positive results at a significance level of $p < 0.05$). Also, there remains the possibility that some changes may be due to oxidative stress but in an unpredicted direction (i.e. overcompensation). Lastly, oxidative stress is observed in a broad range of neurotoxic and pathologic conditions and often is not a primary mechanism of injury. Thus, evidence for oxidative stress involvement may only be correlative and not indicative of causation.

When considering the possible involvement of oxidative stress in these studies, it is important to consider the inherent limitations of the experimental endpoints used. All of these biomarkers are indirect measurements of oxidative stress, which rely on a biochemical response to the oxidant rather than a measure of oxidant activity per se. GS protein levels were measured in these experiments, but not GS protein activity, which may be a more sensitive indicator of oxidative stress (Stadtman, 1992). Thus the protein being measured could have been inactivated by oxidative stress but still available for measurement in these assays, although this is unlikely as the deactivated form of GS is rapidly degraded (Stadtman, 1992). Measurements of mRNA alone are sometimes misleading, as changes in protein levels can occur without changes in mRNA levels and vice versa when a protein is translationally regulated. Therefore, it is not expressly known if MT protein levels fluctuated in these experiments. However, since only a single incidence of an increase in MT mRNA was found across all of these studies, it is unlikely that toxicologically significant increases in MT protein levels occurred following Mn inhalation.

Measurements of total GSH do not indicate the reduced/ oxidized ratio of the intracellular GSH pool. It is possible for GSH to be oxidized (to GSSG) in response to oxidative stress without large changes in total GSH. Only the study with PND 19 pups (Erikson et al., in press) reported GSH, GSSG, and the GSH/GSSG ratio. In that study, the GSH/GSSG ratio was reduced in the hippocampus (at 0.5 and 1 mg Mn/m3) and olfactory bulb (0.05, 0.5, and 1 mg Mn/m3) of PND 19 pups when total GSH levels did not significantly change, while changes in striatal GSH/GSSG (at 0.5 and 1 mg Mn/m3) were reflected in total GSH at the same doses.

Lastly, the endpoints assessed in these studies do not provide direct evidence that Mn-induced neurotoxicity occurred in the exposed animals. However, brain Mn levels seen in the Mn-exposed rats in these studies are consistent with other studies in which evidence of neurotoxicity was reported (Dorman et al., 2000). Despite these limitations, the fact that there are fewer biomarker changes across endpoints than would be expected if significant oxidative stress were involved in Mn neurotoxicity argues against the widespread involvement of ROS in these studies.

The literature contains accounts that both support and reject the notion that oxidative stress is involved in Mn-induced neurotoxicity. Much of the evidence supporting a role for oxidative stress in the toxicity comes from in vitro studies. Rat pheochromocytoma (PC12) cells treated with Mn exhibited a decrease in GSH content, catalase activity, and mitochondrial activity (Seth et al., 2002). GSH depletion potentiated Mn-induced dopamine depletion in PC12 cells, while the antioxidant ascorbate antagonized this effect (Desole et al., 1997). Chen and Liao (2002) reported that in vitro Mn treatment of astrocytes time-dependently shifted the intracellular redox potential towards the oxidized state. Pretreatment of astrocytes with antioxidants blocked Mn-induced mitochondrial permeability transition (MPT) (Rao and Norenberg, 2004), a key step in the cell death pathway. Mouse catacholaminergic (CATH.a) cells were protected from Mn-induced cellular toxicity by GSH and its precursor, N-acetyl cysteine (NAC) (Stredrick et al., 2004).

Alternatively, Sziraki et al . (1998) reported protection of nigrostriatal neurons by Mn from iron-induced oxidative stress independent of MnSOD activity, again demonstrating a potential duality in the effects of Mn. Using X-ray absorption near-edge structure (XANES), Gunter et al. (2004) found no evidence for the presence of the potential oxidizer Mn 3+ in brain, liver, and heart mitochondria, even after incubation under conditions promoting ROS production, suggesting intramitochondrial Mn exists primarily in the less-reactive Mn 2+ state. Further investigation by XAN ES failed to show evidence for stabilization or accumulation of any Mn 3+ complex through oxidation of M n 2+ by ROS in PC 12 cells and PC 12 cells induced by nerve growth factor to display a phenotype more similar to neurons (Gunter et al ., 2005), or in neuroteratocarcinoma (NT2) cells and primary cultures of rat astrocytes under a variety of conditions (Gunter et al., in press).

Evidence both in support of and inconsistent with oxidative stress involvement in Mn neurotoxicity in vivo exists as well. One group reported a decrease in GSH levels in the striatum of aged rats exposed to M n (Desole et al., 1995), and that GSH depletion potentiated Mn-induced ascorbate oxidation in the striatum and brainstem of young rats (Desole et al., 1997).

Hazell et al . (2006) recently reported that rats treated intraperitoneally with high doses of Mn displayed pathological changes in astrocyte morphology known as Alzheimer type II astrocytosis, and co-treatment with the antioxidant and GSH precursor NA C blocked the development of the pathology. This data indicates that oxidative stress is involved in the phenotypic change. However, Brenneman et al . (1999) reported that oral exposure to high doses of Mn in developing CD rats resulted in increased brain and mitochondrial M n levels, increased motor activity, and decreased body weight, but found no evidence of increased striatal ROS or whole-brain 8-OHdG levels, a marker for oxidative DNA damage. A recent review of studies involving oral and inhaled Mn exposure (using neonatal and adult rats respectively) revealed only a few cases providing evidence in support of the oxidative stress hypothesis (Erikson et al., 2004c). Another recent review, considering a broader range of studies, found evidence both for and against oxidative stress, but concluded ―the preponderance of evidence indicates that oxidative stress and mitochondria play major roles‖ in Mn-induced neurotoxicity (Dobson et al., 2004).

4. Conclusions

The current report argues against extensive involvement of ROS in Mn neurotoxicity in rats of differing genders and ages. There are, however, instances of changes in individual endpoints consistent with oxidative stress in certain tissues. The literature contains mixed accounts regarding the hypothesis that oxidative stress is involved in Mn-induced neurotoxicity. Evidence both in support of and inconsistent with oxidative stress involvement in the toxicity can be found in studies performed in vitro and in vivo. This report, in conjunction with the mixed literature in the field, assures that the potential role of ROS in Mn neurotoxicity will continue to be debated. Currently, further work is underway to test the hypothesis that Mn exposure induces oxidative stress in the nonhuman primate brain following subchronic $MnSO_4$ inhalation. These data should prove useful in inferring the mechanism of Mn neurotoxicity in nonhuman primates and ultimately in humans.

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