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Prolactin is a peripheral marker of manganese neurotoxicity

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Abstract

Excessive exposure to Mn induces neurotoxicity, referred to as manganism. Exposure assessment relies on Mn blood and urine analyses, both of which show poor correlation to exposure. Accordingly, there is a critical need for better surrogate biomarkers of Mn exposure. The aim of this study was to examine the relationship between Mn exposure and early indicators of neurotoxicity, with particular emphasis on peripheral biomarkers. Male Wistar rats (180–200 g) were injected intraperitoneally with 4 or 8 doses of Mn (10 mg/kg). Mn exposure was evaluated by analysis of Mn levels in brain and blood along with biochemical end-points (see below).

Results—Brain Mn levels were significantly increased both after 4 and 8 doses of Mn compared with controls ($p < 0.001$). Blood levels failed to reflect a dose-dependent increase in brain Mn, with only the 8-dose treated group showing significant differences ($p < 0.001$). Brain glutathione (GSH) levels were significantly decreased in the 8-dose-treated animals ($p < 0.001$). A significant and dose-dependent increase in prolactin levels was found for both treated groups ($p < 0.001$) compared to controls. In addition, a decrease in motor activity was observed in the 8-dose-treated group compared to controls.

Conclusions—1) The present study demonstrates that peripheral blood level is a poor indicator of Mn brain accumulation and exposure; 2) Mn reduces GSH brain levels, likely reflecting oxidative stress; 3) Mn increases blood prolactin levels, indicating changes in the integrity of the dopaminergic system. Taken together these results suggest that peripheral prolactin levels may serve as reliable predictive biomarkers of Mn neurotoxicity.

Keywords

manganese; neurotoxicity; biomarkers; prolactin; behavior

Introduction

Manganese (Mn) is an abundant and essential metal acquired naturally through regular dietary intake (Aschner et al., 2002). Accordingly, Mn levels in organs are usually kept at optimal concentrations (Michalke et al., 2007; Au et al., 2008). Blood Mn levels are maintained by both gastrointestinal (GI) absorption and efficient biliary excretion, the latter representing the main route for Mn elimination from the body (Dorman et al., 2006; Greenberg et al., 1940). In general, humans are exposed to low levels of Mn in water, air and food. Nevertheless, Mn can accumulate in certain brain regions following elevated

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exposures, and Mn-induced neurotoxicity may ensue. Overexposure to Mn can also occur in occupational environments, and cases of Mn neurotoxicity (manganism) have been reported particularly in miners, smelters and workers in the alloy industry where exposures occur predominantly via the inhalation of Mn fumes or Mn-containing dusts (ATSDR, 2000; Aschner et al., 2007). The gasoline additive, methylcyclopentadienyl manganese tricarbonyl (MMT), is another source of airborne Mn (Kaiser, 2003).

The early onset of Mn intoxication is typically subtle, but once established, usually becomes progressive and irreversible, leading to permanent neurological damage (Jiang et al., 2006; Aschner et al., 2007). Thus, early diagnosis is crucial for preventing Mn neurotoxicity in settings of occupational and environmental exposure. Once absorbed, Mn is rapidly distributed throughout the body, concentrating primarily in the bones and in metabolically active organs. However, in all studied mammalian species, including humans, the brain represents the primary target tissue in which Mn persists for the longest duration (Maeda et al., 1997; Lucchini et al., 2000; Crossgrove and Zheng, 2004; Dorman et al., 2006; Kim et al., 2007). T1-weighted magnetic resonance imaging (MRI) corroborates the accumulation of Mn predominantly in the basal ganglia, namely in the *globus pallidus*, *striatum* and *substantia nigra pars reticulata* (Cersosimo et al., 2006; Reaney et al., 2006; Roth, 2006).

The precise mechanisms by which Mn enters the brain are not yet fully understood. As an essential metal, it readily crosses the blood-brain-barrier (BBB) in both the adult and the developing fetus (Aschner and Aschner, 1991). Recently, Aschner et al. (2007) and Au et al. (2008) documented that facilitated diffusion, active transport, divalent metal transport 1 (DMT-1; also known as DCT-1/NRAMP2)-mediated transport and transferrin (Tf)-dependent transport mechanisms are involved in shuttling Mn across the BBB. Mn accumulates in brain regions that are normally rich in iron, most likely due to the fact that the two metals share common transporters (Tf and DMT-1). Studies in Fe-deficient rats have shown Mn accumulation in the *globus pallidus*, hippocampus and *substantia nigra* (ATSDR, 2000), corroborating a close relationship between low Fe levels and increased Mn uptake into the brain.

Although Mn is a component of several critical enzymes that prevent cellular oxidative stress, such as mitochondrial superoxide dismutase (Aschner et al., 2002; Crossgrove and Zheng, 2004), it can also interact negatively with cellular dopamine, promoting dopamine autoxidation and causing dopaminergic cell death. Hypo- or hyper-activity in dopaminergic neurotransmission leads to a number of disorders, such as Parkinson's disease and schizophrenia (Ben-Jonathan and Hnasko, 2001). Dopamine also acts by binding to D2-receptors on specific pituitary cells (lactotrophs) which are responsible for the secretion of prolactin, thereby inhibiting both the release and the synthesis of this neurohormone (Ellingsen et al., 2003; Takser et al., 2004; Ellingsen et al., 2007). In addition, hypothalamic dopaminergic neurons, which provide dopaminergic efferents to the anterior pituitary gland are themselves regulated by feedback from prolactin through a short-loop feedback mechanism. A variety of other modulators of prolactin secretion (e.g., serotonin, GABA, estrogens and opioids) act at the hypothalamic level by inhibiting or reinforcing the dopaminergic tone (e.g., substance P) (Fitzgerald and Dinan, 2008). Evidence proving that dopamine regulates prolactin release in humans has also been corroborated by observations that drugs which interfere with its release affect circulating prolactin levels (Ben-Jonathan and Hnasko, 2001).

Since Mn stimulates dopamine autoxidation in dopaminergic neurons, it indirectly modulates prolactin secretion, thereby leading to an increase in circulating prolactin levels. Recently, Kim et al. (2009) studied mechanisms that govern changes in rat brain dopamine levels and prolactin production by evaluating the transacting factor of the prolactin gene

(PIT-1). The results of these studies showed a increases in PIT-1 and prolactin, as well as a significant decrease in dopamine levels in rats exposed to Mn. These results are consistent with the role of PIT-1 as a regulator of both dopamine and prolactin levels.

Occupational biomonitoring in populations at risk for Mn exposure has attempted to address the utility of prolactin as a biological index of exposure (Sloot et al., 1996; Smargiassi and Mutti, 1999). Indeed, a strong relationship between Mn exposure and serum prolactin levels has been established in multiple studies (Aschner, 2006; Kim et al., 2007). Mn was positively associated with prolactin in human neonates (Takser et al., 2004) and occupationally exposed men (Mutti et al., 1996; and Smargiassi and Mutti, 1999; Ellingsen et al., 2007). However, negative relationships between Mn and prolactin have also been reported (Roels et al., 1992; Popek et al., 2006). For example, Roels et al. (1992) found that serum prolactin as a predictive measure of effect was unrelated to atmospheric Mn exposure. Such differences in outcomes may be attributable to different exposure times and differences in accumulated brain Mn levels.

In addition to its effect on the dopaminergic system, Mn also inhibits mitochondrial respiration and antioxidant systems. Mn readily induces free radical formation and oxidative stress (Chen and Liao, 2002; Hazzel and Normandin, 2002; Erikson et al. 2004; Marreilha dos Santos et al., 2010) leading to the excessive production of reactive oxygen species (ROS). ROS generation depletes GSH, perturbing the intracellular redox balance as well as the conjugation and excretion of toxic molecules (Meister, 1988, 1991; Duckande et al., 2006; Marreilha dos Santos et al., 2008). *In vitro* studies have shown that the inhibition of GSH synthesis and the consequent impairment of neuronal antioxidant system activity play a detrimental role in oxidative stress-mediated Mn neurotoxicity (Desole et al., 1997; Marreilha dos Santos et al., 2010). Dorman et al. (2000) detected significantly decreased striatal GSH levels following subchronic Mn sulphate (MnSO₄) inhalation. A similar mechanism for Mn neurotoxicity was proposed by Erikson et al. (2004) using lipid peroxides, GSH and metallothionein as biomarkers of free radical production.

The identification and validation of biomarkers is crucial to human toxicology (Smith et al., 2007). To prevent the neurotoxic effects resulting from chronic exposure to Mn, at-risk populations must receive initial and subsequent examinations and necessary follow-up treatment using specific, sensitive and predictive biomarkers. Occupational exposure to Mn has been largely monitored by the determination of Mn levels in blood and urine. Lucchini et al. (1999) observed an association between blood Mn and exposure levels. Apostoli et al. (2000) investigated the suitability of blood and urine Mn levels for exposure assessment, concluding that the two measures could discriminate only between groups of occupationally exposed and control subjects; however, within the exposed groups, there was no association between blood and urinary Mn levels and Mn exposure. Furthermore, blood and urine Mn analyses did not provide information about previous exposures, due to Mn's relatively short blood half-life ($t_{1/2} < 2$ h) (Li et al., 2006). Accordingly, published data question the utility of Mn blood and urine levels as biomarkers of chronic exposure, as they reveal substantial variability in Mn levels in exposed subjects. Since clinical diagnostic medicine relies on effect-related biomarkers (Liu et al., 2008), and the currently available tests (Mn in blood and urine) fail to establish this relationship, attention must be focused on alternative indicators (Smargiassi and Mutti, 1999; Apostoli et al., 2000; Montes et al., 2008).

Given these deficiencies in biomonitoring, it is prudent to perform *in vivo* assays to evaluate the relationship between biomarkers of exposure (blood and brain Mn levels) and effect (prolactin, GSH and behavioral assays). Accordingly, the present study was designed to address the consequences of repeated exposures to MnCl₂ and to correlate its neurotoxic

sequalae with reliable and predictive biomarkers of exposure (prolactin), which, in the future, could serve to validate risk in Mn-exposed human populations.

Results

Animal Weight

The rats' weights were recorded on the first day of treatment and after the 4 and 8 doses of Mn, before the sacrifice. As shown in Table 1, on comparing the rats' weights after the 4 and 8 doses of Mn with the respective controls, there was a reduction in the body weight gain in both treated groups but that reduction was only significant for the 8 doses group ($P < 0.001$).

Behavioral assays

Behavioral parameters were studied in control and Mn-treated rats (8 doses) at the beginning of the experiment and 24 h after the last Mn dose, prior to sacrifice. $MnCl_2$ -treated rats showed decreased spontaneous activity compared to controls ($p < 0.05$), both for ambulation and rearing (Fig. 1 and Fig. 2, respectively).

Blood Mn Concentrations

At the end of the experiment (24 h after the last dose), blood was collected, and levels of Mn were measured by means of GFAAS. Both the 4- and 8-dose Mn-treated animals had higher Mn levels in the blood compared with controls. However, the 4-dose-treated group showed no statistical differences compared with the controls. The 8-dose-treated Mn group had significantly higher ($p < 0.001$) blood Mn concentrations compared with the controls as well as the 4-dose-treated group ($p < 0.01$) (Fig. 3).

Brain Mn Concentrations

As shown in Fig. 4, rats receiving either 4 or 8 doses of $MnCl_2$ showed a significant increase in brain Mn concentrations compared with controls ($p < 0.001$). Moreover, brain Mn concentrations in the 8-dose-treated group were significantly increased compared to both the control and the 4-dose-treated groups ($p < 0.01$).

Brain GSH Levels

A decrease in brain GSH levels was observed in both Mn-treated groups; however, the decrease in GSH levels was only statistically significant in the 8-dose-treated group compared to controls ($p < 0.001$) (Fig. 5); levels in the 4-dose-treated group remained indistinguishable from those of the controls.

Serum Prolactin Levels

Prolactin levels were measured in rat serum, and the values were found to be significantly increased in both the 4- and 8-dose-treated Mn groups ($p < 0.001$) compared to controls (Fig. 6). A statistical difference ($p < 0.05$) was also observed between the two Mn-treated groups.

Discussion

The control and prevention of chronic Mn-induced neurotoxicity has particular clinical relevance in occupational, clinical and environmental toxicology as overexposure to this metal can lead to progressive and permanent neurodegenerative damage, resulting in a syndrome analogous to idiopathic Parkinson's disease (Aschner et al., 2002, Crossgrove and Zheng, 2004; Aschner et al., 2007). The need to biomonitor populations at risk has become increasingly important in preventing early and irreversible neurological damage. Thus, it is

imperative that reliable, sensitive and predictive biomarkers are available for identifying populations most susceptible to the debilitating health consequences which can result from Mn exposure. As this objective cannot be met with observational epidemiological studies alone, the present study was designed to correlate Mn exposure with its neurotoxic effects, and, at the same time, to monitor adequate biomarkers of exposure and effect. *In vivo* assays were performed in 2 groups of rats exposed to 4 and 8 doses of MnCl₂ over 8 consecutive days. The effects of Mn exposure were followed by behavioral assessments, as well as the analysis of biomarkers of exposure (Mn concentrations in brain and in blood) and effect (brain GSH and serum prolactin). Changes in behavior were used as indicators of neurotoxic effects.

A significant and progressive decrease in the rats' weights was noted in response to Mn treatments (Table 1). These results are consistent with other published data (Torrente et al., 2005; Finkelstein et al., 2007). Motor activity measured at the end of the experiment (after 8 doses) revealed a significant decrease ($P < 0.05$) in spontaneous activity for both ambulation and rearing in Mn-treated animals compared to controls (Fig. 1 and Fig. 2, respectively). The observed decrease in spontaneous activity in the open field due to MnCl₂ treatment is consistent with earlier observations (Roels et al., 1997; Zheng et al., 2003; Ávila et al., 2010). This Mn-induced decrease in motor activity most likely reflects the vulnerability of the dopaminergic system (Verity, 1999; Hazell and Normandin, 2002).

Brain and blood Mn levels were selected as biomarkers of exposure. Mn has a short blood half-life ($t_{1/2} < 2$ h) (Maynard and Cotzias, 1955) and following exposure, it is quickly eliminated from the circulation. Currently, there are no predictive, reliable or consistent biomarkers that exhibit a correlation between Mn exposure and its neurotoxic effects. Recently, Apostoli et al. (2000) investigated the suitability of blood and urinary Mn levels for exposure assessment, concluding that while both blood and urine Mn can discriminate between exposed and unexposed workers, the variability of these indicators was exceedingly and unacceptably high (with only 13% of the variance explained), thereby failing to provide adequate and accurate usage for the biological monitoring of at-risk populations.

As shown in Fig. 3, blood Mn levels in both the Mn-treated groups were higher than those of the controls. However, the blood Mn level was only significantly higher in the 8-dose-treated group. These results corroborate those reported by others, suggesting that blood Mn concentration is a poor biomarker for Mn exposure (Garcia-Aranda et al., 1983), especially since even a 4-dose exposure, in which Mn blood levels were indistinguishable from controls, led to significant increases in brain Mn (Fig. 4) and serum prolactin concentrations (Fig. 6). Additionally, the Mn concentration in the brain was shown to be dose-dependently higher in the 4- and 8-dose Mn-treated groups (~3 and ~5 fold, respectively) compared to controls (Fig. 4).

Based on previous proposed mechanisms of Mn neurotoxicity (Erikson et al., 2008; Kim et al., 2009), we also analyzed two other biomarkers of effect (brain GSH and serum prolactin levels). Our results (Fig. 5) show a significant decrease in GSH levels in rats exposed to 8 doses of MnCl₂ and an insignificant decrease in the 4-dose Mn-treated group. A decrease in GSH has already been observed in other studies with rats exposed to Mn (7.5, 15.0, 30.0 mg/kg body weight) (Yin et al., 2008), thus corroborating the presence of oxidative stress. In a recent *in vitro* study performed in our lab, we also confirmed a decrease in GSH levels in rat brain endothelial cells (RBE4 cells) exposed to several concentrations of MnCl₂ (Marreilha dos Santos et al., 2008).

It is well known that Mn produces ROS with the depletion of antioxidant defense mechanisms (Stredrick et al., 2004). Nevertheless, alterations in antioxidant defenses do not

represent the only known consequences of Mn-induced toxicity. In fact, Mn stimulates dopamine autoxidation in the dopaminergic neurons and modulates prolactin secretion, leading to an increase in circulating prolactin levels. Given that prolactin levels are regulated by dopamine, Mn-induced dopamine depletion has been posited to increase prolactin release and prolactin serum levels. This hypothesis was confirmed by our data (Fig. 6), which show increased levels of serum prolactin in male rats exposed to either 4 or 8 doses of Mn. Prolactin levels in both groups were significantly higher than in controls, as well as different from each other, demonstrating a dose-dependent effect (Fig. 6).

We are cognizant that the studies were carried out in males and the possibility exists that the result would differ were they conducted in females, reflecting gender-specific prolactin levels. Although the tuberoinfundibular dopaminergic system can be considered as the final common pathway of all neural mechanisms controlling prolactin secretion, other neurotransmitters, neuropeptides and hormones modulate the sensitivity of lactotrope cells to dopaminergic tonic inhibition (Gudelsky, 1981). Among the various neuromodulators, estrogens play an important role, since they increase the amount of prolactin excreted. It has been previously shown that estrogen exerts a variety of actions on hypophyseal function, including a direct stimulatory effect on the pituitary to increase prolactin synthesis and/or release (Nicoll and Meites, 1962; Chen et al., 1970). The increasing blood concentrations of estrogen during late pregnancy and during some stages of estrous cycle appear responsible for the elevated levels of prolactin in females. According with Ferroni et al. (1992), in female workers exposed to low perchloroethylene concentrations in dry-cleaning shops, a shift in the distribution of prolactin levels rather than an increased prevalence of abnormally high values were observed, as compared to controls. While most of the workers exposed to metals are men, for women exposed to Mn the need for proper female control group should be considered. Furthermore, baseline serum prolactin levels for women should be measured. Finally, consideration should be directed at the interaction between prolactin levels during pregnancy and risk to Mn.

The best correlations for the exposure effects of Mn were noted for brain Mn content and prolactin serum levels (Fig. 7), as well as prolactin and neurobehavioral assays suggesting that prolactin may serve as a sensitive biomarker of cumulative exposure to Mn and as a predictive biomarker of neurotoxic effects (Table 2). To confirm this data, further research is currently being conducted, specifically directed at correlating serum prolactin levels with neurobehavioral changes in rats repetitively and simultaneously exposed to Mn and other metals, thus mimicking various occupational exposures.

Materials and Methods

Chemicals

Manganese chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 99.99%: Sigma-Aldrich), nitric acid (HNO_3 , 65% suprapure: Merck), hydrogen peroxide (H_2O_2 , 30%: Aldrich), sterile saline solution.

Animals

Experiments were performed according to the Guidelines for Animal Experimentation as set forth by the NIH. Male Wistar rats (Charles River Laboratories @, Barcelona, Spain) weighing 150–171g were used for the experiments. Animals were housed in an isolated room and adapted to controlled environmental conditions for 10 days prior to experimentation. Room temperature was maintained at 24°C, with a relative humidity control of 50–70% and a 12-h light/dark cycle. Animals had free access to tap water and to a

daily dose of food (Letica1 Ref. IPM-R20). General husbandry conditions were checked daily and the rats' weights recorded.

Mn Exposure

Rats were randomly assigned to 3 separate groups. Two groups of six rats received a daily i.p. injection of Mn. One Mn-exposed group received a total of 4 doses of MnCl₂ (10 mg/Kg), and the other Mn-exposed group received 8 doses of MnCl₂ (10mg/kg). Eight untreated rats were used as controls (control animals received daily injections of sterile saline during 4 or 8 consecutive days). The animals were sacrificed 24 h after the last dose was administered.

Behavioral Assays

Spontaneous Locomotor Activity—Motor activity was assessed in the control and Mn-treated groups receiving a total of 8 doses of Mn. Motor activity was first assessed at the beginning of the experiment and after the last treatment, just prior to sacrifice. The order of testing was randomized, and the observer was blinded to the animals' treatments.

Two behavioral parameters were studied: ambulation (number of crossings within 5 min in an open field) and rearing (number of times within 5 min where both forelegs were elevated from the floor in the same open field). The floor of the open field measured 60 cm × 90 cm and was divided into six equal squares. The floor was surrounded by an opaque wall 30 cm high. The rats were tested individually, and after each session, the open field was swiped with alcohol. Because a large number of environmental conditions can affect motor activity (e.g., sound level, cage design, lighting, temperature, humidity, and various odors), recordings were carried out in an isolated room at a fixed hour of the day with controlled light and sound, thus eliminating any confounding variables. At the beginning of each session, the animals were placed at the center of the open field, and the behavior of each rat was observed for 5 min (Ladefoged et al., 1994).

Tissue Collection and Analytical Procedures

Before sacrifice (24 h after the last dose), all rats were anesthetized with pentobarbital (20 mg/kg, i.p.), and blood was collected by cardiac puncture. Immediately after sacrifice, the brain was dissected out, weighed and stored at -80°C.

Several parameters were determined by analytical procedures (see below). Mn was measured in the brain and blood by atomic absorption spectrometry; brain Fe levels were measured by atomic absorption spectrometry; GSH was analyzed by high performance liquid chromatography (HPLC); and prolactin levels were determined by an enzyme immunoassay method.

Analytical Methods

Determination of Mn in biological samples

Microwave-Assisted Acid Digestion—Brain and blood samples were digested with an oxidizing acid mixture of 4:1 (v/v) 65% suprapure nitric acid: hydrogen peroxide, prior to graphite furnace atomic absorption spectrometry (GFAAS) analysis. Eighty mg of brain homogenate were weighed, and 2.9 mL of the oxidizing mixture were added to a cup. The cups were sealed, placed inside the respective bombs (Parr Microwave Acid Digestion Bombs ®) and subjected to microwave irradiation (30", 900 W). Solutions were kept at 4°C until analysis.

Graphite Furnace Atomic Absorption Spectrometry—Mn concentrations were determined by GFAAS with a Perkin Elmer AAnalyst™ 700 atomic absorption spectrometer equipped with an HGA Graphite Furnace, a programmable sample dispenser (AS 800 Auto Sampler, and WinLab 32 for AA software). Mg (NO₃)₂·6 H₂O (0.84 mol/L) was used as a chemical modifier. Each measurement consisted of two injections into the graphite furnace. Results were expressed as micrograms of Mn per g of brain tissue and micrograms of Mn per milliliter of blood. Calibration curves were obtained with standard solutions of 5, 10, 15 and 25 µg/L of MnCl₂. The limit of detection was 0.05 µg Mn/L.

Brain GSH Analysis

Sample Preparation—Brain tissue was homogenized, and samples were weighed (approximately 20 mg). PBS solution was added (10 times the weight in volume), and samples were macerated. Samples were then sonicated (30 cycles; Conditions: Cycle 0.5, Amplitude 100) and centrifuged for 10 minutes at 10,000 rpm. The supernatant was collected in 1 ml Eppendorf tubes, and samples were analyzed by HPLC. Samples were handled on ice, and only non-metallic utensils were used.

HPLC analyses—GSH levels in brain tissue were measured by HPLC. The equipment was supplied with a Supelcosil LC-18-S reverse phase column, a Waters 2475 fluorimeter detector and a Waters 2695 separation module. The data were analyzed with Water Empower II Software.

Determination of Prolactin in Serum

Serum prolactin was quantified by an enzyme immunoassay (Citomed ®). Plates were read in an Anthos Zenyth 3100 microplate detector. The limit of quantification was 0.6 ng/mL.

Statistical Analysis

All analyses were conducted with Microsoft Office Excel® 2003 and SPSS Statistics® version 16.0, 2007. All results were analyzed for normal distribution with the Kolmogorov-Smirnov test. Variables were compared by tests for homogeneity of variance (Levene's test) and one-way analysis of variance (ANOVA). For multiple comparisons, ANOVA complemented with *post hoc* Tukey's HSD (all pairwise), and the Dunnett's test (pairwise versus control) was performed. Data are presented as mean values ± standard error of the mean (S.E.M.). The limit for statistical significance was set at $p < 0.05$ (significance level of 95%).

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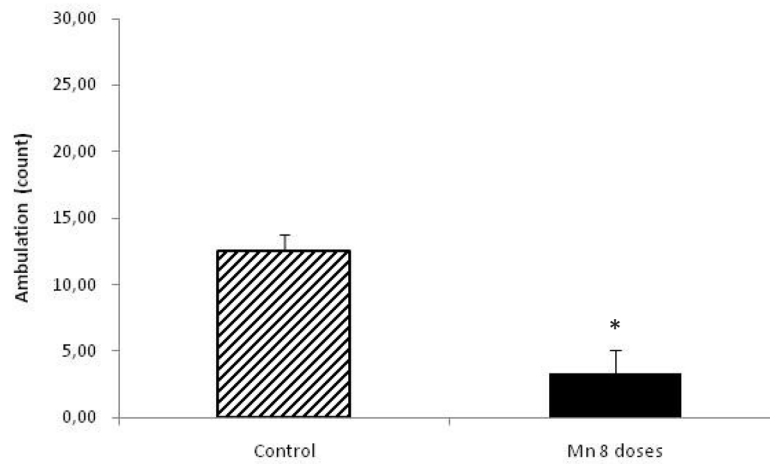


Figure 1. Ambulation in rats repeatedly exposed to 8 doses of MnCl_2 (10 mg/kg) vs. controls. Each bar represents the mean \pm S.E.M. for $n=6$ (* $p<0.05$).

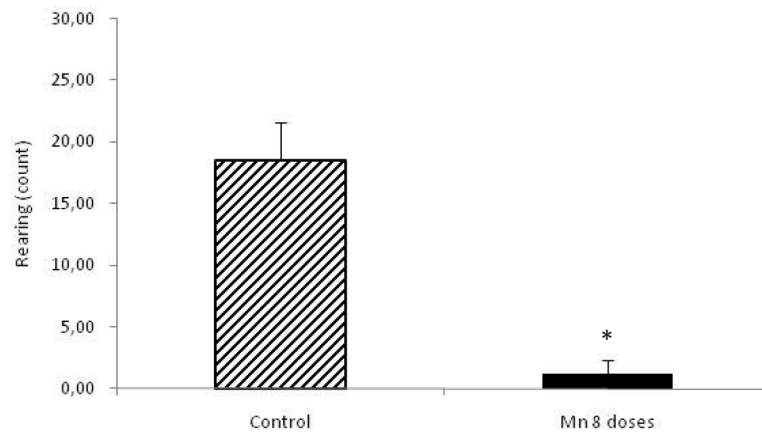


Figure 2. Rearing in rats repeatedly exposed to 8 doses of MnCl₂ (10 mg/kg) vs. controls. Each bar represents the mean \pm S.E.M. for n=6 (*p<0.05).

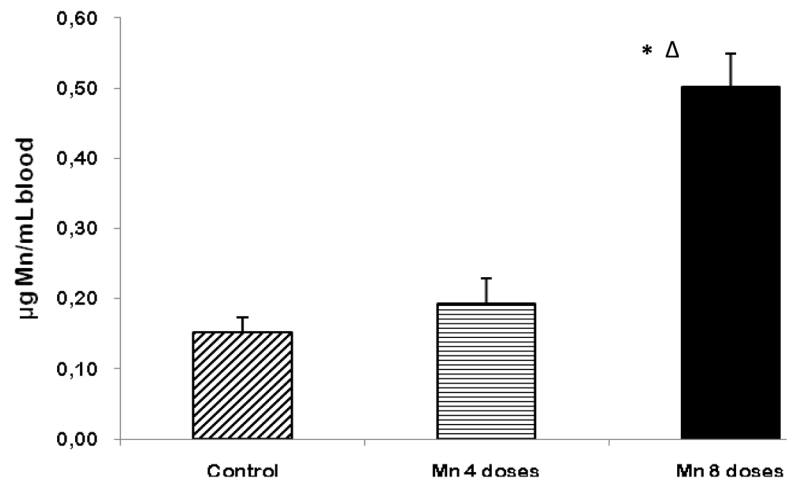


Figure 3. Mn levels in blood of rats repeatedly exposed to 4 and 8 doses of MnCl_2 (10 mg/kg). Bars represent the mean \pm S.E.M. for $n=6$ (* $p < 0.001$, vs. control; $\Delta p < 0.01$, vs. 4-dose Mn-treated group).

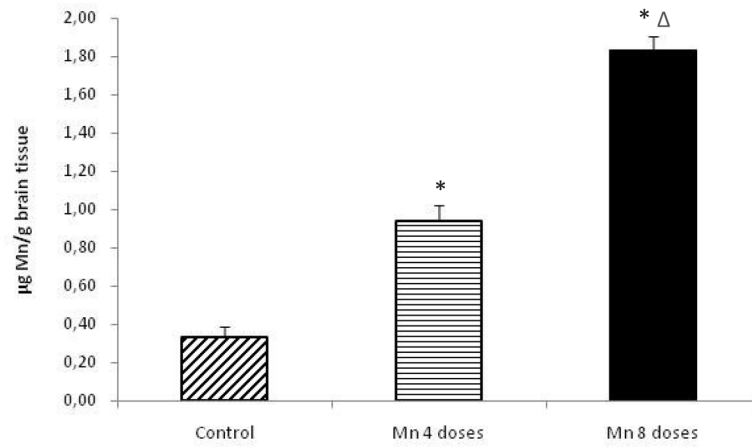


Figure 4. Mn levels in brain tissue of rats repeatedly exposed to 4 and 8 doses of MnCl_2 (10 mg/kg). Bars represent the mean \pm S.E.M. for $n=6$ (* $p<0.001$, vs. control; $\Delta p<0.01$, vs. 4-dose Mn-treated group).

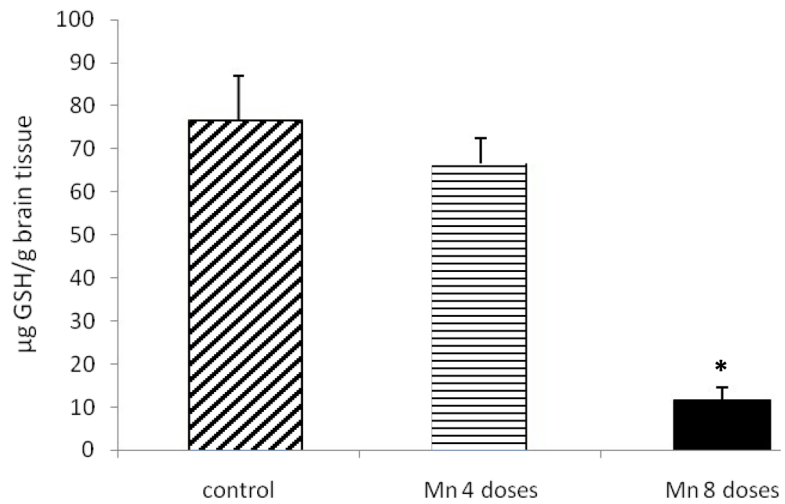


Figure 5. GSH levels in brain tissue of rats repeatedly exposed to 4 and 8 doses of MnCl_2 (10 mg/kg). Bars represent the mean \pm S.E.M. for $n=6$ (* $p<0.001$, vs. control).

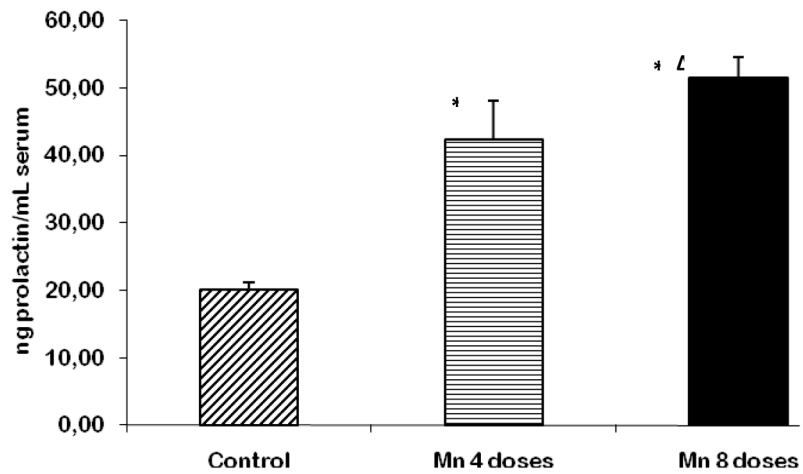


Figure 6. Prolactin levels in the serum of rats repeatedly exposed to 4 and 8 doses of MnCl_2 (10 mg/kg). Bars represent the mean \pm S.E.M. for $n=6$ (* $p<0.001$ vs. control; Δ $p<0.05$, vs. 4-dose Mn-treated group).

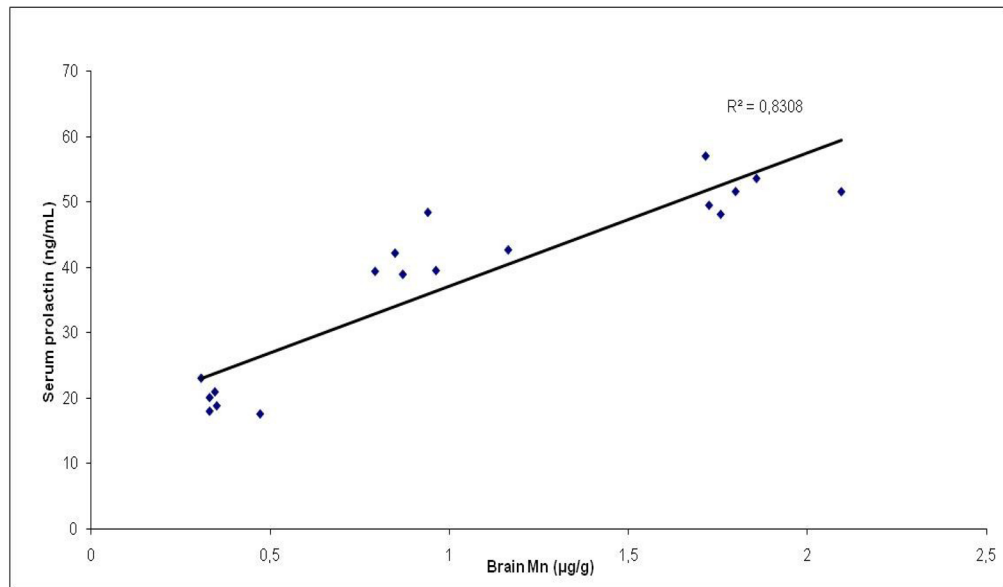


Figure 7. Correlation between Mn levels in the brain and prolactin levels in the serum of rats repeatedly exposed to 4 and 8 doses of $MnCl_2$ (10 mg/kg; n=6).

Table 1

Growth rate measured in % of body weight gain (mean \pm SD), between the first and the last dose, of rats repeatedly exposed to 4 and 8 doses of MnCl₂ (10 mg/kg).

	growth rate %
Control 4 doses (saline)	10,0 \pm 4,4
Mn 4 doses	4,7 \pm 2,1
Control 8 doses (saline)	22,3 \pm 3,7
Mn 8 doses	3,8 \pm 1,1 *

* Significant different from respective control (p<0.001).

Table 2

Correlation between biomarkers/behavioral parameters with serum prolactin and brain Mn in rats exposed to 4 and 8 doses of Mn Cl₂ (10 mg/Kg).

Correlation	R²
Brain Mn/Serum prolactin	0,8306
Blood Mn/Serum prolactin	0,4870
Brain GSH/Brain Mn	0,7850
Brain GSH/Serum prolactin	0,4108
Blood Mn/Brain Mn	0,7524
Rearing/Prolactin	0,8623
Ambulation/Prolactin	0,8715