# Manganese-exposed developing rats display motor deficits and striatal oxidative stress that are reversed by Trolox

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## Abstract:

While manganese (Mn) is essential for proper central nervous system (CNS) development, excessive Mn exposure may lead to neurotoxicity. Mn preferentially accumulates in the basal ganglia, and in adults it may cause Parkinson's disease–like disorder. Compared to adults, younger individuals accumulate greater Mn levels in the CNS and are more vulnerable to its toxicity. Moreover, the mechanisms mediating developmental Mn-induced neurotoxicity are not completely understood. The present study investigated the developmental neurotoxicity elicited by Mn exposure (5, 10 and 20 mg/kg; i.p.) from postnatal day 8 to PN27 in rats. Neurochemical analyses were carried out on PN29, with a particular focus on striatal alterations in intracellular signaling pathways (MAPKs, Akt and DARPP-32), oxidative stress generation and cell death. Motor alterations were evaluated later in life at 3, 4 or 5 weeks of age. Mn exposure (20 mg/kg) increased p38MAPK and Akt phosphorylation, but decreased DARPP-32-Thr-34 phosphorylation. Mn (10 and 20 mg/kg) increased caspase activity and F2-isoprostane production (a biological marker of lipid peroxidation). Paralleling the changes in striatal biochemical parameters, Mn (20 mg/kg) also caused motor impairment, evidenced by increased falling latency in the rotarod test, decreased distance traveled and motor speed in the open-field test. Notably, the antioxidant Trolox™ reversed the Mn (20 mg/kg)-dependent augmentation in p38MAPK phosphorylation and reduced the Mn (20 mg/kg)-induced caspase activity and F2-isoprostane production. Trolox™ also reversed the Mn-induced motor coordination deficits. These findings are the first to show that long-term exposure to Mn during a critical period of neurodevelopment causes motor coordination dysfunction with parallel increment in oxidative stress markers, p38MAPK phosphorylation and caspase activity in the striatum. Moreover, we

establish Trolox™ as a potential neuroprotective agent given its efficacy in reversing the Mn-induced neurodevelopmental effects.

**Keywords:** manganese | developing brains | MAPK | DARPP-32 | oxidative stress | behavior | toxicology

**Article:**

## Introduction

Manganese (Mn) is an essential ubiquitous trace element required for normal growth, development and cellular homeostasis (Aschner and Aschner 2005; Aschner et al. 2007). Both in humans and in animals, Mn acts as an essential cofactor of several enzymes required for neuronal and glial cell function, as well as enzymes involved in neurotransmitter synthesis and metabolism (Aschner and Aschner 2005; Aschner et al. 2007). Despite its indispensable role in multiple metabolic functions, excessive Mn exposure may lead to increased metal accumulation in the brain and neurotoxicity in the basal ganglia, particularly globus pallidus, striatum and substantia nigra pars reticulata (Bowman et al. 2011; Dobson et al. 2004; Perl and Olanow 2007; Yamada et al. 1986). Increased brain Mn levels may cause a clinical disorder referred to as manganism, which is characterized by extrapyramidal symptoms resembling idiopathic Parkinson's disease (Menezes-Filho et al. 2009; Roth 2009). Moreover, Mn neurotoxicity has been linked to the development of neurodegenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis (Eschenko et al. 2010) and prion disease (Bowman et al. 2011).

Despite the numerous studies on the neurotoxicity of Mn in adults, little is known about its effects on the developing central nervous system (CNS), which is the most susceptible period to toxic insult (Bondy and Campbell 2005; Grandjean and Landrigan 2006; Rodier 1995; Zheng et al. 2003). Furthermore, several lines of research suggest that developmental exposure to Mn may predispose individuals to later-life neurological disorders (Erikson et al. 2007; Hafeman et al. 2007; Moreno et al. 2009a, b). Notably, newborns retain greater Mn levels than adults (Hafeman et al. 2007), likely due to inefficient excretion, low physiological Fe levels as well as a permeable blood–brain barrier (Roth 2009), or an increased requirement for Mn immediately after birth.

Mn exposure is inherent to environmental and occupational conditions, given its ubiquitous use in water treatment, manufacturing of dry batteries, gasoline additives (as an antiknock agent; methylcyclopentadienyl manganese tricarbonyl, MMT) and fungicides (Burton and Guilarte 2009; Eschenko et al. 2010; Santamaria 2008). Moreover, Mn exposure may also result from administration of parenteral nutrition, where excessive Mn supplementation may predispose primarily children to neurological disorders (Erikson et al. 2007; Hardy 2009; Suzuki et al. 2003).

The late fetal and neonatal periods are characterized by significant changes in brain structure and function as well as cellular and intercellular signaling (Tkac et al. 2003). Substantial acceleration of RNA, DNA and protein synthesis, neuronal migration, growth of glial cells (particularly of astroglia, the main site for glutamate and metal uptake) and myelination of axons continue during the postnatal period (Gottlieb et al. 1977; Rice and Barone 2000; Rodier 1995). The rat brain between postnatal days 7 and 28 (PN7–28) represents an established model for studies on human brain development. From a neurodevelopmental point of view, the rat PN7 corresponds approximately to human gestational week 34, while rat PN28 corresponds to a child between 2 and 3 years of age (Dobbing 1990). Additionally, the preweaning and early postweaning periods coincide with the development of dopaminergic pathways in brain regions such as the prefrontal cortex, nucleus accumbens and striatum, which are critical in regulating behavioral executive functions associated with learning, memory and attention, as well as motor functions (Broaddus and Bennett 1990a, b; Leo et al. 2003; Lozoff and Georgieff 2006; Packard and Knowlton 2002). Given the sensitivity of dopaminergic systems to Mn, early exposure to this metal may result in significant neurological deficits in developing brain structures (Cordova et al. 2012; Kern et al. 2010).

The mechanisms that mediate Mn-induced neurotoxicity are not fully understood, but seem to be associated with mitochondrial dysfunction, leading to cell death via the formation of reactive oxygen species (ROS) and oxidative stress (Gunter et al. 2006; Roth and Garrick 2003; Tamm et al. 2008; Zhang et al. 2008). Higher rates of striatal oxidative stress have been noted in developmentally induced Mn toxicity (Avila et al. 2008; Erikson et al. 2007). Furthermore, an association linking oxidative stress in the striatum with impairment in motor activity has been described (de Oliveira et al. 2007).

Specific signaling pathways coupled with programmed cell death are activated upon in vitro Mn exposure, including protein kinase C, caspases and the mitogen-activated protein kinases, such as c-Jun amino-terminal kinase (JNK), extracellular regulated protein kinase (ERK) and p38MAPK (Cai et al. 2011; Crittenden and Filipov 2011; Hirata et al. 2004; Ito et al. 2006; Jang 2009; Latchoumycandane et al. 2005; Park and Park 2010; Shin et al. 2010). While mostly derived from in vitro studies, recently, we have demonstrated the effects of short-term Mn exposure (PN8–12) on the modulation of ERK1/2, Akt, as well as dopamine and cAMP-regulated phosphoprotein of 32kD (DARPP-32) phosphorylation in the striatum of immature rats (Cordova et al. 2012). Although all these pathways might be involved in the regulation of gene expression, embryogenesis, cell proliferation and migration, cell death/survival and neuroplasticity (Hanada et al. 2004; Svenningsson et al. 2004; Thomas and Huganir 2004), data regarding the modulation of such signaling pathways in response to prolonged Mn exposure during the early postnatal period have yet to be defined.

The biological consequences of developmental Mn exposure may be severe and possibly with late onset, affecting neurogenesis, learning and memory. Furthermore, these early effects may predispose individuals to neurodegenerative disorders that unmask only decades later in life. We

have recently demonstrated that developmental exposure of immature rats to Mn for a short period (5 days; from PN8 to 12) caused marked motor deficits and altered intracellular signaling pathways associated with regulation of proliferation, plasticity and cell death (Cordova et al. 2012) in a manner that might be partially dependent on oxidative stress. The relationship between these signaling pathways and more protracted Mn exposure during the early postnatal period has yet to be defined. Accordingly, the present study was carried out to evaluate alterations in striatal cell signaling pathways, production of cell death, induction of oxidative stress and motor deficits in a model of long-term developmental Mn exposure (20 days, from PN8 to 27). Moreover, the role of a chain-breaking antioxidant (Trolox™) in modulating the effects of Mn on these biochemical and behavioral parameters and its neuroprotective efficacy against Mn-induced neurotoxicity were also evaluated.

## Methods

### Reagents

The primary antibodies anti-ERK1/2, anti-p38MAPK, anti-JNK1/2 and manganese chloride (MnCl2) were obtained from Sigma (St. Louis, MO, USA). The anti-phospho-CREB, anti-CREB, anti-phospho-ERK1/2, anti-phospho-p38MAPK, anti-phospho-JNK1/2/3, anti-Akt, anti-phospho-Akt, anti-DARPP-32, anti-phospho-DARPP-32-Thr-34 and anti-phospho-DARPP-32-Thr-75 antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti-β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acrylamide, bis-acrylamide, β-mercaptoethanol, Hybond™ nitrocellulose, Hyperfilm™ ECL, sodium dodecyl sulfate (SDS), Tris, secondary antibody (anti-rabbit IgG-horseradish peroxidase-conjugated) and ECL™ detection reagents were obtained from GE Healthcare Life Sciences (Piscataway, NJ, USA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™) was obtained from Calbiochem (La Jolla, CA, USA). The N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) was obtained from Biomol (Plymouth Meeting, PA, USA). All other reagents were of the highest analytical grade.

### Animals and treatments

Wistar rats of both genders were obtained from the Federal University of Santa Catarina breeding colony. The animals were maintained in an air-conditioned room (22 ± 1 °C) on a 12-h light/dark cycle with water and food available ad libitum. They were treated, manipulated and euthanized according to the “Principles of Laboratory Animal Care” (NIH publication no. 80-23, revised 1996) and approved by the Committee on the Ethics of Animal Experiments of the

Federal University of Santa Catarina (CEUA/UFSC; www.ceua.ufsc.br; Permit Number: PP00345). All efforts were made to minimize the number of animals used and animal suffering. At birth, the number of pups was randomly culled to eight pups per litter. The treatments began when the pups were 8 days old (PN8). The litters were randomly assigned to each treatment group, and the treatments were carried out at the same time every day (2:00 PM).

For the Mn exposure protocol, four to twelve groups from different litter were used. Each group with eight pups (8 days old) of both sexes was divided into four treatments (control and $MnCl_2$ 5, 10 or 20 mg/kg) with two animals in each treatment group. The pups were treated for 20 consecutive days (8th to 27th postnatal day; PN8–27) with one daily intraperitoneal (i.p.) injection of saline (NaCl, 0.9 %; control) or $MnCl_2$ (5, 10 or 20 mg/kg, diluted in saline), as previously described (Cordova et al. 2004, 2012; Rocha et al. 1995). The dosing regimen was chosen in an attempt to mimic Mn exposures derived from total parenteral nutrition (TPN), which have been associated with significant increases in brain Mn levels both in adults and in children (Ejima et al. 1992; Fell et al. 1996). The highest Mn dose used herein (20 mg/kg) leads to an analogous increase in striatal Mn levels as observed in pediatric patients receiving TPN (Chaki et al. 2000; Ono et al. 1995). Rats were euthanized on the 29th postnatal day (PN29) by decapitation, and the structures of interest were dissected out for neurochemical analyses (Cordova et al. 2004, 2012). Briefly, the brain was carefully removed, the cerebral hemispheres were divided, and the hippocampi were isolated using two fine brushes (Cordova et al. 2012; Rodnight and Leal 1990). This procedure was followed by displacement of the cortex and striatum. The striatum was carefully dissected out and separated from nearby structures with a sharp spatula.

In addition, pups were treated concomitantly with Mn and the antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™) to determine neuroprotective efficacy of this compound against Mn-induced neurotoxicity. The same protocol as previously described was carried out with two pups in each treatment group: saline (0.9 % NaCl; control), Trolox™ 1 mg/kg, $MnCl_2$ 20 mg/kg, $MnCl_2$ 20 mg/kg plus Trolox™ 1 mg/kg (administered 10 min before Mn) (Cordova et al. 2012). The pups' body weights were measured daily from PN8 to 29, and the weight gain (g) is reported as mean ± S.E.M.

Brain metal analyses

Tissue concentrations of Mn and iron (Fe) were analyzed by atomic absorption spectroscopy (Varian AA240™, Varian Inc., Palo Alto, CA, USA) (Fitsanakis et al. 2008). The striatum, hippocampus and cerebral cortex were digested in ultrapure nitric acid (1:10 wt/vol dilution) for 48–72 h in a sand bath (60 °C). One hundred microliters of digested tissue was brought to 1 ml of total volume with 2 % nitric acid and analyzed for Mn and Fe content. The mixture was then centrifuged, and the clear supernatant was used for analysis (100-μl aliquot brought up to a 1 ml

volume with 2 % nitric acid). Bovine liver (10 μg Mn/l) was digested in ultrapure nitric acid and used as an internal standard for analysis. The data are expressed as μg Mn or Fe/g tissue (mean ± S.E.M.).

Western blotting

The brain area of interest (striatum) was dissected and mechanically homogenized in 500 μl of sample buffer (200 mM Tris, 40 mM EDTA, 4 % SDS, pH 6,8), and the homogenates were immediately boiled for 5 min. Sample dilution solution (1:4 vol/vol; 40 % glycerol, 50 mM Tris and minimal bromophenol blue) and β-mercaptoethanol were added to each sample at a final concentration of 5 %. Protein concentrations were estimated by the method described in Peterson (1977) based on a standard curve with bovine serum albumin. The samples (60 μg of total protein/lane) were separated by SDS–PAGE (miniVE Vertical Electrophoresis System™, GE Healthcare Life Sciences, Piscataway, NJ, USA) using 10 % gels (Cordova et al. 2004, 2012). The proteins were transferred to nitrocellulose membrane using a semi-dry blotting apparatus (TE 70 SemiPhor™ Unit, GE Healthcare Life Sciences, Piscataway, NJ, USA) (1.2 mA/cm2; 1.5 h) as described by Bjerrum and Heegaard (1988). The membranes were blocked (1 h) with 5 % skim milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). ERK1/2, JNKs (46 and 54 kDa), p38MAPK, Akt, CREB and DARPP-32 (total and phosphorylated forms) were detected with specific antibodies, diluted in TBS-T (10 mM Tris, 150 mM NaCl, 0,1 % Tween-20, pH 7.5) containing 2.5 % BSA, after overnight incubation. Final primary antibody dilutions were 1:1,000 (anti-phospho-CREB, anti-CREB, anti-Akt, anti-phospho-Akt-Ser-473, anti-DARPP-32, anti-phospho-DARPP-32-Thr-34, anti-phospho-DARPP-32-Thr-75, anti-phospho-JNK1/2/3 and anti-phospho-p38MAPK), 1:2,000 (anti-phospho-ERK1/2 and anti-β-actin), 1:10,000 (anti-JNK1/2 and anti-p38MAPK) or 1:40,000 (anti-ERK1/2). Next, the membranes were incubated for 1 h with anti-rabbit peroxidase-linked secondary antibody (1:4,000) and the reactions developed by chemiluminescence. All steps (blocking and antibody incubations) were followed by three washes (5 min) of the membranes with TBS-T. The optical density (OD) of the bands was quantified with Scion Image™ (Frederick, MD, USA). The phosphorylation level of each protein was determined as a ratio of the OD of the phosphorylated band to OD of the total band. The data are expressed as percentage of the control (considered as 100 %). Values are presented as mean ± S.E.M (Posser et al. 2007).

Quantitation of F2-IsoPs

The F2-isoprostanes (F2-IsoPs) are considered reliable biomarkers of oxidative stress (particularly lipid peroxidation) in both in vitro and in vivo models (Milatovic et al. 2009). Total F2-IsoPs were determined with a stable isotope dilution method with detection by gas chromatography/mass spectrometry and selective ion monitoring as previously described

(Milatovic et al. 2007; Morrow and Roberts 1999). Total F2-IsoPs were measured in the striatum dissected from the animals exposed in vivo to different doses of Mn and stored at −80 °C until analysis. Briefly, the striata were homogenized in Folch solution and lipids extracted from chloroform layer by evaporation (Milatovic and Aschner 2009) and then subjected to chemical saponification using 15 % KOH to hydrolyze bound F2-IsoPs. The homogenates were adjusted to a pH of 3, followed by the addition of 0.1 ng of 15-F2α-IsoP-d4 internal standard. F2-IsoPs were subsequently purified by C18 and silica Sep-Pak extraction and by thin-layer chromatography. They were then analyzed by pentafluorobenzyl ester, a trimethylsilyl ether derivative, via gas chromatography, negative ion chemical ionization-mass spectrometry.

## Caspase activity

Caspase activity was monitored fluorimetrically by the production of fluorescent 7-amino-4-methylcoumarin (AMC) from DEVD-AMC fluorogenic substrate for caspase-3 and related cysteine proteases. Striatal homogenates were prepared (1:5, w/v) in a buffer containing 10 mM HEPES pH 7.4, 42 mM KCl, 5 mM MgCl2, 1 mM phenylmethylsulfonylfluoride, 0.1 mM EDTA, 0.1 mM EGTA, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 % 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) and 1 mM dithiothreitol (DTT) at 4 °C. The reaction was carried out by mixing this homogenate (0.1 mg protein) with a buffer containing 25 mM HEPES pH 7.4, 0.1 % CHAPS, 1 mM EDTA, 10 % sucrose and 3 mM DTT. The reaction was started by the addition of 10 μM caspase-3 fluorogenic substrate DEVD-AMC. Cleavage of the fluorogenic substrate was detected spectrofluorimetrically (Perkin Elmer LS55, Boston, MA, USA) after 2 h of incubation at 37 °C, using excitation and emission wavelengths of 380 and 460 nm, respectively (Zuse et al. 2007). Fluorescence of blank samples containing no fluorogenic substrate was subtracted from these values. Protein content was determined by Lowry's method (Lowry et al. 1951), and the caspase activity was expressed as mean percent of control (100 %) ± S.E.M.

## Behavioral tests

The animals were kept until PN37. All animals were tested at 22, 29 and 36 days of age (3, 4 and 5 weeks old) on a rotarod and on PN37 on an open-field task (Cordova et al. 2012). Animals were habituated to the test room for 1 h prior to the initiation of the behavior tests, which were carried out during the light phase of the cycle (10:00–17:00 h).

## Rotarod analyses

The Rota-Rod system (Insight Equipamentos Científicos, Ribeirão Preto, Brazil) for locomotor assessment measures the time period an animal maintains its balance on a moving cylinder (Aguiar et al. 2009). Animals were first conditioned on a stationary rod for 30 s, and during this time, animals that fell off the cylinder were placed back on the rotarod. Next, the animals were conditioned at a constant speed of 5 rpm for a period of 90 s. Animals that failed the first conditioning were allowed two additional conditioning periods, and those that failed the third conditioning period were omitted from further testing. This assured that all the animals in all the treatment groups attained an analogous baseline (data not shown).

The same basic conditioning methodology was employed in testing treatment and control groups. Thirty minutes after the last conditioning, each animal was placed on the rotarod and its latency for falling determined. The starting speed was 5 rpm, and it was increased by 0.1 revolutions per second.

### Open-field analyses

To evaluate Mn-induced motor changes, the animals were tested in the circular open field (Aguiar et al. 2009). The apparatus consists of acrylic chamber with 50 cm height × 60 cm diameter (Insight Equipamentos Científicos, Ribeirão Preto, Brazil) and is placed in a room with a ceiling-mounted video camera. Each animal was placed in the apparatus for 10 min, and the distance, average speed, number of rearing and grooming were recorded. The data were evaluated with behavioral analysis software, ANY-maze™ (Stoelting, Wood Dale, IL, USA).

### Statistical analyses

Data are expressed as mean ± S.E.M, and statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Duncan's post hoc test when appropriate, except for the rotarod test that does not display a Gaussian distribution. Therefore, for this analysis the nonparametric Kruskal–Wallis test followed by Dunn's post hoc was applied. Analyses were performed with STATISTICA™ 5.1'98 Edition (StatSoft, Tulsa, OK, USA). $p \leq 0.05$ was considered to be statistically significant.

## Results

### Brain metal accumulation

Brain Mn and Fe concentrations were analyzed in rats developmentally exposed to Mn for 20 days. Striatal Mn levels significantly increased, approximately fivefold in the 10 mg Mn/kg ($p < 0.01$) group and seven times in the 20 mg Mn/kg ($p < 0.001$) group (Fig. 1a) relative to controls. Moreover, a significant increase in Mn levels was also observed in the hippocampus ($p < 0.01$ in the 10 mg Mn/kg group; $p < 0.001$ in the 20 mg Mn/kg group; Fig. 1a) and cerebral cortex ($p < 0.01$ in the 10 mg Mn/kg group; $p < 0.001$ in the 20 mg Mn/kg group; Fig. 1a). Fe levels significantly increased (20 and 35 %) only in the striatum of animals treated with 10 or 20 mg Mn/kg, respectively ($p < 0.001$; Fig. 1b). At the termination of the study (PN29), no differences were noted in weight gain (Table 1) for any of the groups when compared with controls.

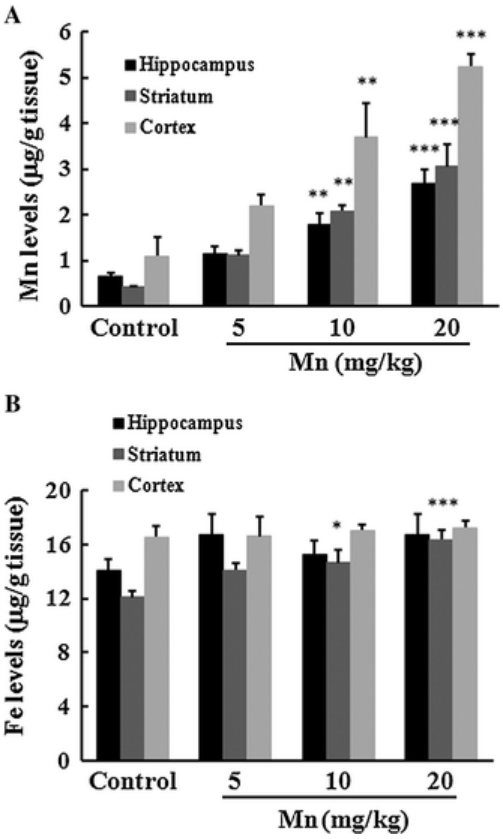


Fig. 1

Effects of Mn exposure on metal accumulation in the hippocampus, striatum and cerebral cortex of immature rats. The panels show the accumulation of Mn (a) and Fe (b). Rat pups were treated with saline (control; NaCl 0.9 %) or MnCl2 at doses of 5, 10 or 20 mg/kg for 20 days (PN8–27), and the brain metal concentrations were analyzed on PN29. Results represent mean ± S.E.M and are expressed in µg metal/g tissue. Statistical analysis was performed by ANOVA followed by Duncan's test. n = 4; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control

**Table 1**

Weight gain in rats developmentally exposed to Mn

| | Body weight PN8 (g) | Body weight PN29 (g) | Weight gain (g) |
|---|---|---|---|
| Control | 13.20 ± 0.78 | 61.88 ± 2.21 | 49.81 ± 1.52 |
| 5 mg Mn/kg | 14.51 ± 0.96 | 65.16 ± 1.91 | 50.65 ± 1.38 |
| 10 mg Mn/kg | 13.39 ± 0.73 | 62.58 ± 1.78 | 49.18 ± 1.21 |
| 20 mg Mn/kg | 13.90 ± 0.70 | 58.54 ± 2.08 | 46.26 ± 1.18 |

Immature rats were treated with saline (control; NaCl 0.9 %) or MnCl2 at doses of 5, 10 or 20 mg/kg for 20 days (PN8–27). The animals' body weights were recorded on PN8 and 29. Results represent mean ± S.E.M and are expressed in grams (g). Statistical analysis was performed by ANOVA; n = 15

Since the striatum is an important target for Mn neurotoxicity (Avila et al. 2008; Bowman et al. 2011; Cordova et al. 2012; Erikson et al. 2007), next we performed neurochemical analyses in this structure.

Effects of Mn exposure on signaling pathways

Phosphorylation and expression of MAPKs, Akt, CREB and DARPP-32 were evaluated on PN29 in the striatum of rats exposed to Mn from PN8 to 27. The phosphorylation of p38MAPK (Fig. 2a) was increased in the 10 (21.25 ± 6.05 %, $p < 0.01$) and 20 mg/kg (33.31 ± 8.14 %, $p < 0.001$) treatment groups. Akt phosphorylation (Fig. 2b) was also significantly increased in the 20 mg/kg group (14.92 ± 2.94 %, $p < 0.001$). Conversely, DARPP-32-Thr-34 phosphorylation (Fig. 2c) was significantly decreased in the 20 mg/kg Mn group (11.05 ± 2.34 %, $p < 0.01$). The phosphorylation and/or expression of ERK1/2, JNK1/2/3 (p54 and p46) and CREB (Fig. 2d) was indistinguishable from controls upon exposures to Mn.

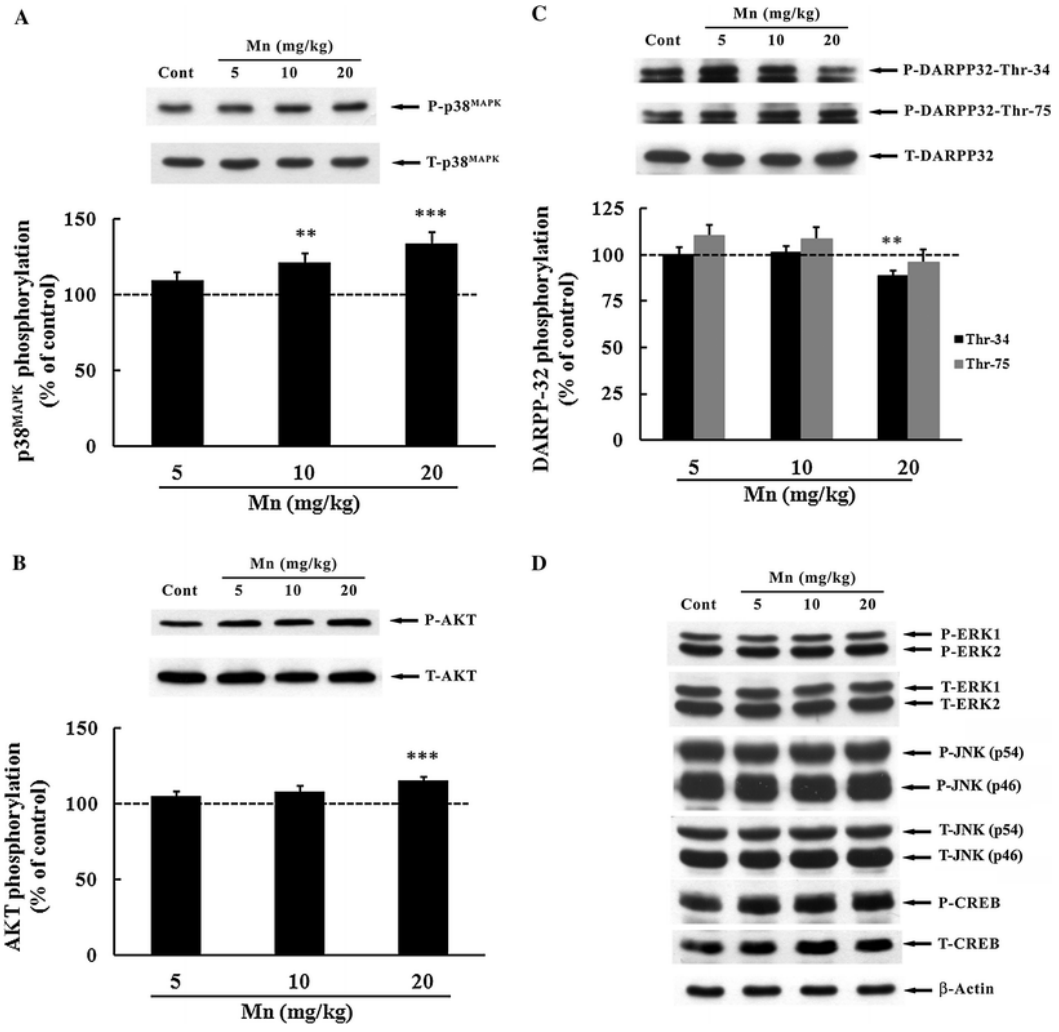


Fig. 2

Effects of in vivo Mn exposure for 20 days on the phosphorylation of MAPK, AKT, CREB and DARPP-32 in the striatum of immature rats. The panels show representative immunoblots and quantification of phosphorylation of p38MAPK (a), AKT (b), DARPP-32-Thr-34 and DARPP-32-Thr-75 (c), ERK1/2, JNKs (p46 and p54), CREB and β-actin (d) in striata of rats treated with saline (control; NaCl 0.9 %) or MnCl2 at doses of 5, 10 or 20 mg/kg/day (PN8–27). The structures were analyzed on PN29. The total and phosphorylated form of each protein was detected with specific antibodies, and the reaction was developed by chemiluminescence. The phosphorylation level of each protein was determined as the ratio of OD of the phosphorylated band to OD of the total band, and the data are expressed as percentage of the control (considered as 100 %). Values are presented as mean ± S.E.M. Statistical analysis was performed by ANOVA followed by Duncan's test. n = 12; ** $p < 0.01$, *** $p < 0.001$ compared to control

Mn exposure may alter signaling pathways associated with cell death and cell survival. Accordingly, next we analyzed the enzymatic activity of caspase-3/7, which plays a key role in

apoptosis. The analysis was carried out with DEVD cleavage fluorescent test in the striata of immature rats exposed to Mn for 20 days. As shown in Fig. 3, Mn significantly increased ($p < 0.001$) caspase activity both in the 10 mg/kg (30.10 ± 6.23 %) and in the 20 mg/kg (29.17 ± 6.51 %) treatment groups compared with the controls.

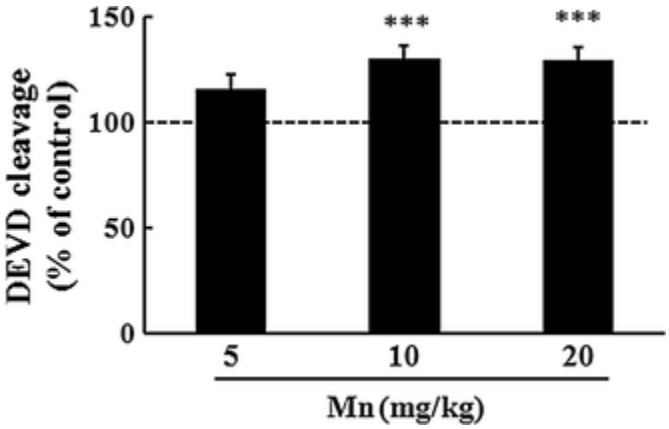


Fig. 3

Caspase activity measured by the DEVD cleavage test in the striata of immature rats exposed in vivo to Mn for 20 days (PN8–27). The panel shows the DEVD cleavage test from rats treated with saline (control; NaCl 0.9 %) or MnCl2 at doses of 5, 10 or 20 mg/kg/day (PN8–27) and the structures analyzed at PN29. Results represent mean ± S.E.M and are expressed as percentage of control (100 %). Statistical analysis was performed by ANOVA followed by Duncan's test. n = 8; *** $p < 0.001$ compared to control

Oxidative stress in Mn neurotoxicity

Recently, we demonstrated increased brain F2-IsoPs generation in a short-term model of Mn exposure (Cordova et al. 2012). Here, we also evaluated F2-IsoPs generation in rat striata upon a more protracted exposure to this metal. Consistent with our previous results, exposure to Mn at all doses led to a significant increase ($p < 0.001$; Fig. 4) in F2-IsoPs generation in the striata of developing rats.

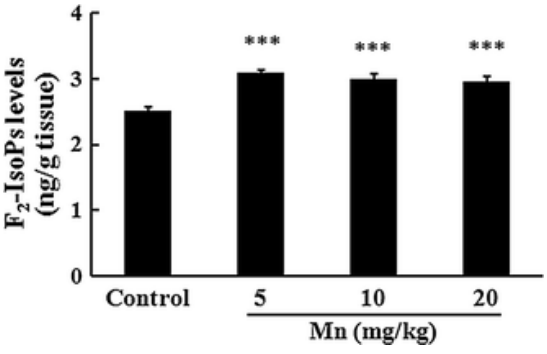

Fig. 4

F2-IsoPs production in the striata of immature rats exposed in vivo to Mn for 20 days (PN8–27). The panel shows F2-IsoPs production in rats treated with saline (control; NaCl 0.9 %) or MnCl2 at doses of 5, 10 or 20 mg/kg/day (PN8–27). Striata were analyzed on PN29. Results represent mean ± S.E.M and are expressed as ng F2-IsoPs/g tissue. Statistical analysis was performed by ANOVA followed by Duncan's test. n = 4; *** $p < 0.001$ compared to control

Effects of Mn exposure on motor activity

As shown in Fig. 5, overall performance on the rotarod test was indistinguishable between controls and rats treated with 5 or 10 mg Mn/kg. However, animals treated with 20 mg Mn/kg showed a significant decrease ($p < 0.05$) in the overall latency for falling off the rotarod versus controls.

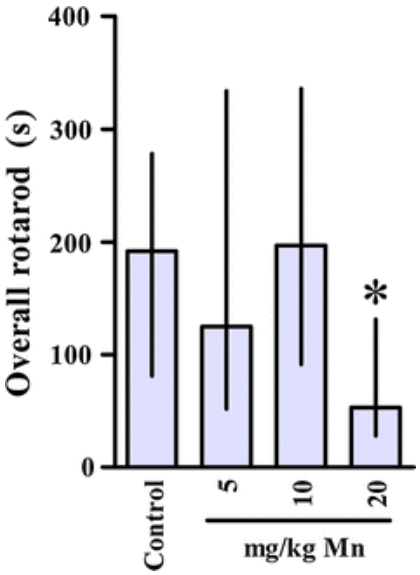


Fig. 5

Motor coordination in rats exposed in vivo to Mn. Control and treated rats were tested on 22, 29 and 36 days of age (3, 4 and 5 weeks old) on the rotarod task. The graphic shows the overall latency for falling in rats treated (PN8–27) with saline (control; NaCl 0.9 %) or MnCl2 at doses of 5, 10 or 20 mg/kg/day. Results represent median ± interquartile range and are expressed in seconds (s) to latency for falling derived from twelve independent experiments. Statistical analysis was performed by Kruskal–Wallis followed by Dunn's post hoc test. * $p < 0.05$ compared to control

Protective effects of Trolox™ on Mn-induced neurotoxicity

In order to gain a better understanding on the role of oxidative stress in mediating Mn-induced neurotoxicity, we co-treated animals with the chain-breaking antioxidant Trolox™ (1 mg/kg) and Mn (20 mg/kg). As shown in Fig. 6a, Trolox™ fully reversed the effect of Mn on p38MAPK phosphorylation, to the levels indistinguishable from controls ($p < 0.01$). However, Trolox™ failed to block the Mn-induced increase in Akt phosphorylation (Fig. 6b). Interestingly, Trolox™ per se also increased Akt phosphorylation (35.55 ± 10.92 %, $p < 0.05$; Fig. 6b). Furthermore, Trolox™ reversed the Mn-induced increase in caspase activity to the levels indistinguishable from controls ($p < 0.05$ relative to Mn alone, Fig. 7) and decreased Mn-mediated F2-IsoPs production ($p < 0.001$ relative to Mn alone; Fig. 8). No changes in the rats' weight were noted upon Trolox™ treatment (Table 2). Finally, as shown in Fig. 9, co-treatment with Mn and Trolox™ caused a significant ($p < 0.05$) improvement in motor coordination compared to Mn-treated animals.

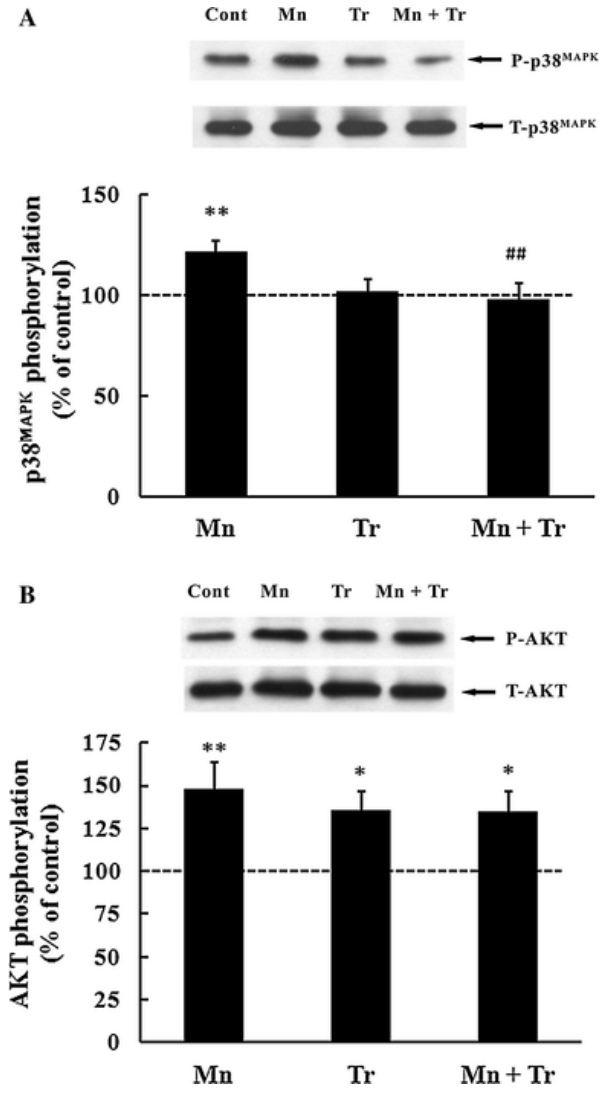

Fig. 6

Effects of in vivo exposure to Mn and/or Trolox™ for 20 days (PN8–27) on phosphorylation of p38MAPK (a) and Akt (b) in the striatum of immature rats. The panels show representative immunoblots and quantification of p38MAPK and Akt phosphorylation from rats treated with saline (control; NaCl 0.9 %), MnCl2 20 mg/kg (Mn), Trolox™ 1 mg/kg (Tr) or MnCl2 20 mg/kg plus Trolox™ 1 mg/kg (Mn + Tr) for 20 days (PN8–27). The structures were analyzed on PN29. The total and phosphorylated form of each protein was detected with specific antibodies, and the reaction was developed by chemiluminescence. The phosphorylation level of each protein was determined as a ratio of OD of the phosphorylated band to OD of the total band, and the data are expressed as percentage of the control (considered as 100 %), and the values are presented as mean ± S.E.M. Statistical analysis was performed by ANOVA followed by Duncan's test. n = 12; * $p < 0.05$, ** $p < 0.01$, compared to control; ## $p < 0.01$ compared to 20 mg Mn/kg group

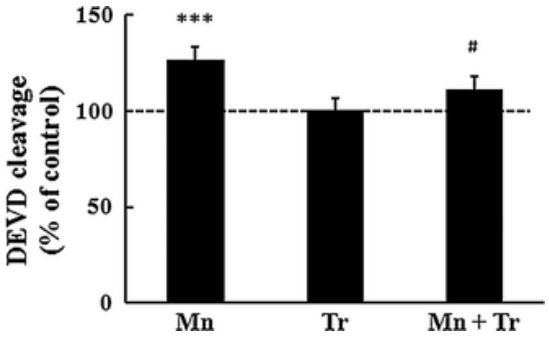


Fig. 7

Effects of Trolox™ on caspase activity measured by DEVD cleavage test in striata from immature rats exposed in vivo to Mn for 20 days (PN8–27). The panel shows the DEVD cleavage test from rats treated with saline (control; NaCl 0.9 %), MnCl2 20 mg/kg (Mn), Trolox™ 1 mg/kg (Tr) or MnCl2 20 mg/kg plus Trolox™ 1 mg/kg (Mn + Tr) for 20 days (PN8–27). The structures were analyzed on PN29. Results represent mean ± S.E.M and are expressed as percentage of control (100 %). Statistical analysis was performed by ANOVA followed by Duncan's test. n = 8; *** $p < 0.001$ compared to control; # $p < 0.05$ compared to 20 mg Mn/kg group.

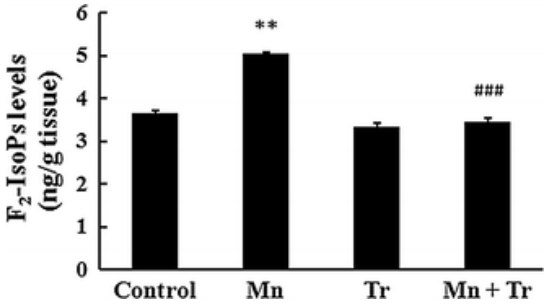


Fig. 8

F2-IsoPs production in striata from immature rats exposed in vivo to Mn and/or Trolox™ for 20 days (PN8–27). The panel shows F2-IsoPs production from rats treated with saline (control; NaCl 0.9 %), MnCl2 20 mg/kg (Mn), Trolox™ 1 mg/kg (Tr) or MnCl2 20 mg/kg plus Trolox™ 1 mg/kg (Mn + Tr) for 20 days (PN8–27). The structures were analyzed on PN29. Results represent mean ± S.E.M and are expressed as ng F2-IsoPs/g tissue. Statistical analysis was performed by ANOVA followed by Duncan's test. n = 4; ** $p < 0.01$ compared to control; ### $p < 0.001$ compared to 20 mg Mn/kg group

**Table 2**

Effects of Mn and/or Trolox™ on body weight gain in immature rats exposed in vivo

| | **Body weight PN8 (g)** | **Body weight PN29 (g)** | **Weight gain (g)** |
|---|---|---|---|
| Control | 13.79 ± 0.68 | 65.74 ± 2.91 | 51.94 ± 2.37 |
| Mn | 13.19 ± 0.70 | 60.08 ± 2.59 | 46.89 ± 2.03 |
| Tr | 14.08 ± 1.21 | 64.71 ± 3.85 | 50.64 ± 2.85 |
| Mn + Tr | 14.02 ± 0.72 | 64.22 ± 2.68 | 50.20 ± 2.13 |

Immature rats were treated with saline (control; NaCl 0.9 %), MnCl2 20 mg/kg (Mn), Trolox™ 1 mg/kg (Tr) or MnCl2 20 mg/kg plus Trolox™ 1 mg/kg (Mn + Tr) for 20 days (PN8–27). The animals' body weights were recorded on PN8 and PN29. Results represent mean ± S.E.M and are expressed in grams (g). Statistical analysis was performed by ANOVA; n = 8

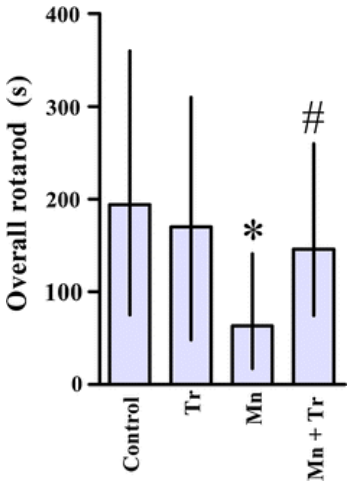

Fig. 9

Effects of Trolox™ on motor coordination of immature rats exposed to Mn. Rats were treated for 20 days (PN8–27) with saline (control; NaCl 0.9 %), MnCl2 20 mg/kg (Mn), Trolox™ 1 mg/kg (Tr) or MnCl2 20 mg/kg plus Trolox™ 1 mg/kg (Mn + Tr) and tested on 22, 29 and 36 days of age (3, 4 and 5 weeks old) on the rotarod task. The graphic shows the overall latency for falling, and results represent mean ± interquartile range and are expressed as seconds (s) to latency for falling derived from twelve independent experiments. Statistical analysis was performed by Kruskal–Wallis followed by Dunn's post hoc test. * $p < 0.05$ compared to control., # $p < 0.05$ compared to 20 mg Mn/kg group

In the open-field test, animals treated with the highest Mn dose (20 mg/kg) showed a significant ($p < 0.05$) reduction in the distance traveled (Fig. 10a) and speed (Fig. 10b) versus controls. Co-treatment with Trolox™ did not block the Mn-induced deficits (Fig. 10). Mn exposure did not alter grooming (Fig. 10c) or rearing (Fig. 10d) behaviors.

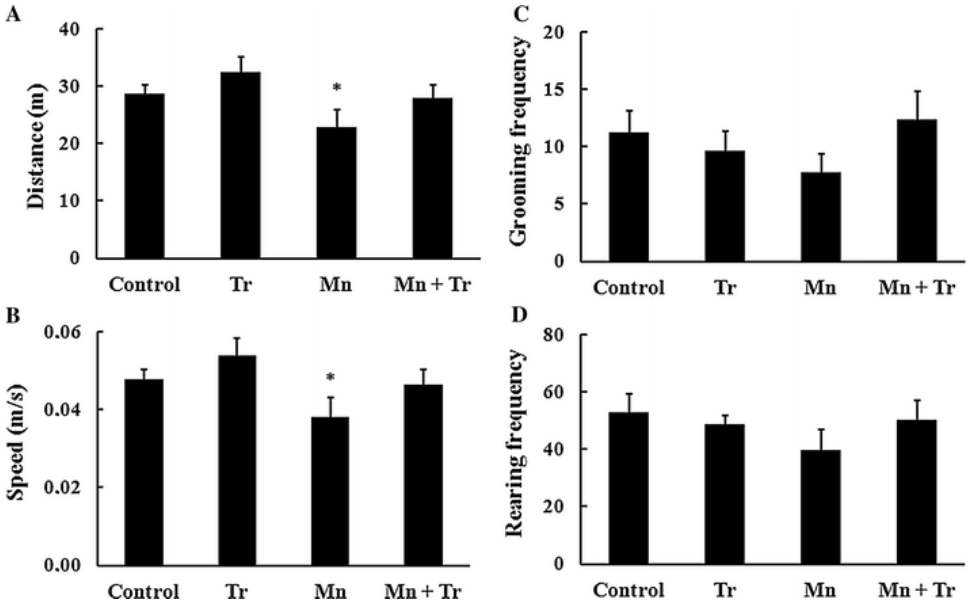


Fig. 10

Evaluation of motor changes induced by Mn and/or Trolox™ exposure of immature rats in the circular open-field task. Rats were treated with saline (control; NaCl 0.9 %), MnCl2 20 mg/kg (Mn), Trolox™ 1 mg/kg (Tr) or MnCl2 20 mg/kg plus Trolox™ 1 mg/kg (Mn + Tr) for 20 days (PN8–27). The panels show the distance (m) (a), speed (m/s) (b), grooming frequency (c) and rearing frequency (d). Results represent mean ± S.E.M. Statistical analysis was performed by ANOVA followed by Duncan's test. n = 12; *$p < 0.05$ compared to control

Discussion

Mn is an essential metal for living organisms, but excessive levels may cause pathological processes characterized by irreversible CNS damage (Lazrishvili et al. 2009). The occupational exposure of adults to Mn represents a toxicological concern given its widespread use in manufacturing of dry batteries, gasoline additives and fungicides (Burton and Guilarte 2009; Eschenko et al. 2010; Santamaria 2008). In newborns and children, both breast milk (Ljung et al. 2009; Yoon et al. 2011) and TPN (Fell et al. 1996) contribute to increased Mn body burden; however, from a toxicological viewpoint, TPN likely represents a more critical concern for exposure (Aschner and Aschner 2005). The exposure regimen used herein was chosen in an attempt to mimic Mn exposures in infants receiving TPN feeds. Our results established a sevenfold increase in striatal Mn levels in exposed rats (Fig. 1a). It is noteworthy that TPN in pediatric patients has also been shown to cause a sevenfold increase in blood and striatal Mn levels (Chaki et al. 2000; Ono et al. 1995). Accordingly, the present exposure protocol leads to striatal Mn levels that are likely inherent to TPN-fed children, particularly those with simultaneous liver failure disease (Fell et al. 1996).

Given the dearth of information on Mn neurotoxicity in the developing brain, the present study addressed the potential for this metal to generate oxidative stress and alter intracellular signaling pathways and behavior in developing rats. Recently, we showed increased oxidative stress and modulation of ERK1/2, Akt and DARPP-32 in PN14 rats upon in vivo exposure to Mn (Cordova et al. 2012). However, given the importance of the second to fourth postnatal weeks in maintaining optimal neurodevelopment, here we tested the effects of protracted Mn exposure (PN8–29) to allow for better understanding of the molecular and behavioral effects of this metal on rat brain development.

Consistent with earlier observations (Normandin et al. 2002; Roth 2009; Yamada et al. 1986), we noted increased Mn levels in the striatum of rats dosed with 10 and 20 mg Mn/kg (Fig. 1a). However, a significant increase in Mn levels was also noted in the hippocampus and cerebral cortex, corroborating previous studies showing the propensity of Mn to accumulate throughout the CNS, both in young and in adult animals (Dorman et al. 2000; Morello et al. 2008, 2009a; Normandin et al. 2002; Schneider et al. 2009). In addition, we observed a discrete increase in Fe levels in the striatum of PN29 animals treated with 10 and 20 mg Mn/kg (Fig. 1b), but not in animals treated with a lower dose (5 mg Mn/kg). Notably, increased Fe levels in the absence of significant changes in the level of other metals were previously observed in the striatum of juvenile mice exposed to Mn (Erikson et al. 2007; Hafeman et al. 2007; Moreno et al. 2009a, b). Therefore, it is possible that Mn exerts its effects on the immature striatum, at least in part, secondary to altered Fe metabolism. It is noteworthy that the increment in Fe levels approached 20–35 %, while Mn levels increased 400–650 % at 10 and 20 mg Mn/kg, respectively. Moreover, significant elevation in F2-IsoPs concentration was observed with all the Mn doses, including the 5 mg Mn/kg in which the striatal Fe level remained indistinguishable from controls (Fig. 1a). In contrast, other studies reported decreased brain Fe levels in animals exposed to excessive Mn (Fitsanakis et al. 2010; Garcia et al. 2006; Guilarte and Chen 2007; Guilarte et al.

2006; Hansen et al. 2009) paralleled by increased transferrin (Tf) plasma levels, and divalent metal 1 transporter (DMT-1) and Tf receptor (TfR) expression (Garcia et al. 2006). While these changes may possibly explain the increased levels of Fe observed in our study, additional studies will be required to determine expression and/or activity of these transporters in the treatment protocol described herein.

According to our previous study (Cordova et al. 2012), Mn exposure led to increased striatal oxidative stress, characterized by significant elevation in F2-IsoPs concentrations (Fig. 4). F2-IsoPs are prostaglandin-like molecules produced by the free radical-mediated peroxidation of arachidonic acid (Milatovic and Aschner 2009), which can be efficiently reduced by antioxidant treatment (Milatovic et al. 2011). Several studies have shown oxidative stress to be a major mechanism for Mn-induced neurotoxicity in both in vitro and in vivo models (Benedetto et al. 2009; Gunter et al. 2006; Milatovic et al. 2009; Roth and Garrick 2003; Tamm et al. 2008; Zhang et al. 2008). Mn-induced ROS generation (Avila et al. 2008; Park and Park 2010; Roth 2009) is triggered by dysfunctional mitochondria, resulting in both apoptotic and necrotic cell death (Roth and Garrick 2003; Tamm et al. 2008; Yin et al. 2008), triggering the opening of the permeability transition pore (Roth 2009; Zhang et al. 2004). Consistent with these observations, we show that Mn induced an increase in striatal caspase activity (Fig. 3) and that Trolox™ attenuated the Mn (20 mg/kg)-induced increase in F2-IsoPs generation (Fig. 8) and restored caspase activity to control levels (Fig. 7).

In addition to oxidative stress generation and activation of caspases, many classic intracellular signaling pathways associated with programmed cell death are altered by Mn treatment in vitro. Among them are the activation of MAPKs and Akt (Gonzalez et al. 2008; Hirata et al. 2004; Ito et al. 2006; Li et al. 2010; Park and Park 2010; Prabhakaran et al. 2008; Yin et al. 2008). Herein, we observed p38MAPK and Akt activation in the striatum of immature rats exposed for 20 days to Mn (Fig. 2a, b). Interestingly, co-treatment with Trolox™ led to a significant reduction in p38MAPK phosphorylation to the levels indistinguishable from controls. This effect suggests that Mn-mediated p38MAPK activation might be occurring via a ROS-dependent mechanism (Fig. 6a). Conversely, the activation of Akt by Mn (20 mg/kg; Fig. 2b) appeared independent of oxidative stress, since Trolox™ was ineffective in reversing the Mn-dependent Akt activation (Fig. 6b). It is noteworthy that Trolox™ itself also increased Akt phosphorylation, consistent with the previous studies (Choi et al. 2012; Sun et al. 2012). Although our results point to the ability of Mn to activate the Akt pathway (present study; Cordova et al. 2012; McDougall et al. 2011), the understanding of the mechanism and the toxicological significance of this effect remain elusive. Accordingly, it is possible that ROS production-independent mechanisms, such as neurotransmitter imbalances, might be involved in the Akt activation in response to Mn. Nonetheless, additional studies are necessary to address this possibility.

DARPP-32 phosphorylation was also altered in response to 20-day Mn exposure (Fig. 2c). DARPP-32 is highly expressed in striatal medium spiny neurons, and it is implicated in modulation of motor responses in the striatum (Polissidis et al. 2010) by integrating

dopaminergic and glutamatergic pathways. In this way, D1 receptor activation by dopamine stimulates DARPP-32 phosphorylation at Thr-34 (via cAMP/PKA) and thereby converts DARPP-32 into a potent inhibitor of protein phosphatase 1 (PP1). Conversely, DARPP-32 is also phosphorylated at Thr-75 in response to glutamate (via Cdk5 activation), converting DARPP-32 to an inhibitor of PKA (Svenningsson et al. 2004). Recently, we demonstrated the modulation of DARPP-32 by Mn in a short-term exposure protocol (Cordova et al. 2012), noting increased phosphorylation of DARPP-32 at Thr-34 in PN14 rats treated with 5 and 10 mg Mn/kg. Herein, we noted a slight decrease in the phosphorylation of DARPP-32-Thr-34 and no changes at Thr-75 in PN29 rats exposed to 20 mg Mn/kg (Fig. 2c). These differences might potentially reflect changes in striatal dopaminergic signaling, which alter the availability of dopamine and/or dopamine receptors (Guilarte et al. 2008).

In addition to the cell signaling alterations, we also noted significant behavioral changes in developing rats exposed to Mn (20 mg/kg) for 20 days, characterized by a significant decline in motor coordination (Fig. 5). Co-treatment with Mn and Trolox™ significantly improved the animals' performance in the rotarod task (Fig. 9). Mn (20 mg/kg) also decreased the distance and speed traveled in the open-field task (Fig. 10), but in this case Trolox™ failed to mitigate the Mn-induced effects. Altered movement has been previously shown to be associated with increased oxidative stress, mainly in the striatum (de Oliveira et al. 2007). Furthermore, an in vivo study conducted in nonhuman primates showed a direct correlation between the levels of Mn in the caudate nucleus, putamen and globus pallidus, and the severity of the behavioral deficits (Schneider et al. 2009). Thus, the significant accumulation of Mn in the striatum, the generation of oxidative stress, apoptotic cell death and disturbed cell signaling in response to Mn exposure might directly or indirectly trigger these behavioral deficits. However, this possibility needs to be confirmed in future studies.

Mn has a relatively long half-life in the CNS, consistent with a slow elimination rate (Morello et al. 2008). Exposure of developing individuals to Mn levels that exceed the homeostatic capacity may result in an overload condition, with increased risk for neurodegenerative diseases, such as Parkinson's disease at later life stages (Lucchini and Zimmerman 2009). Our study shows that developmental exposure to Mn induced oxidative stress, perturbed cell signaling pathways and triggered behavioral dysfunction. Moreover, we showed that Trolox™, a potent antioxidant, significantly attenuated the Mn-induced ROS generation. Therefore, future studies could be profitably directed to determine the therapeutic efficacy of this agent in reducing Mn-induced neurodevelopmental effects.

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Conflict of interest

None of the authors has any known or perceived conflict of interest with the contents of this manuscript.

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