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Mechanisms of manganese-induced neurotoxicity in primary neuronal cultures: the role of manganese speciation and cell type

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Short title: Mn toxicity in cortical and cerebellar neurons

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Abstract

Manganese (Mn) is an essential trace element required for the proper functioning of a variety of physiological processes. However, chronic exposures to Mn can cause neurotoxicity in humans, especially when it occurs during critical stages of the central nervous system development. The mechanisms mediating this phenomenon as well as the contribution of Mn speciation and the sensitivity of different types of neuronal cells in such toxicity are poorly understood. This study was aimed to investigate the mechanisms mediating the toxic effects of $MnCl_2$, Mn(II) citrate, Mn(III) citrate and Mn(III) pyrophosphate in primary cultures of neocortical (CTX) and cerebellar granular (CGC) neurons. Cell viability, mitochondrial function and glutathione levels were evaluated after Mn exposure. CGC were significantly more susceptible to Mn-induced toxicity when compared to CTX. Moreover, undifferentiated CGC were more vulnerable to Mn-toxicity than mature neurons. Mitochondrial dysfunction was observed after the exposure to all the tested Mn species. Ascorbate protected CGC against Mn-induced neurotoxicity and this event seemed to be related to the dual role of ascorbate in neurons, acting as antioxidant and metabolic energetic supplier. CTX were protected from Mn-induced toxicity by ascorbate only when co-incubated with lactate. These findings reinforce and extend the notion of the hazardous effects of Mn toward neuronal cells. In addition, the present results indicate that Mn-induced neurotoxicity is influenced by brain cell types, their origins and developmental stages, as well as by the chemical speciation of Mn, thus providing important information about Mn-induced developmental neurotoxicity and its risk assessment.

Keywords: manganese speciation, developmental neurotoxicity, mitochondrial disfunction, cerebellar granule neurons, cortical neurons

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Introduction

Manganese (Mn) constitutes approximately 0.1% of the Earth's crust, but its levels may be locally altered by anthropogenic activities. Mn is released in the air mainly as particulate matter and some of its compounds are soluble in the aquatic environment, where the two main forms are Mn(II) and Mn(IV) (Howe *et al*., 2004). The intermediate specie Mn(III) can be stabilized by ethylenediaminetetraacetic acid (EDTA), pyrophosphate and citrate, which sustain the Mn(II)/Mn(III) redox cycle (Klewicki and Morgan, 1998). Thermodynamic modeling of Mn(II) in serum suggests that it is complexed with albumin (84%), water (6.4%), bicarbonate (5.8%), citrate (2.0%) and other low molecular weight (MW) ligands (1.8%); whereas Mn(III) is bound to transferrin (≅ 100%) (Crossgrove and Zheng, 2004).

Trace amounts of Mn are essential for the proper functioning of a variety of physiological processes, including development, in all living organisms. It integrates many metalloproteins that play vital roles in metabolic processes (e.g. gluconeogenesis; pyruvate carboxylase) and antioxidant defenses (MnSOD and catalase) (Crossgrove and Zheng, 2004). However, several studies indicate that high Mn levels may be toxic to terrestrial and aquatic organisms (for a review, see Howe *et al*., 2004), especially due to its neurotoxic properties (Benedetto et al, 2009).

The Mn concentrations in the tissues of healthy subjects range from 0.1 to 1 µg/g. Of particular importance, the brain has been shown to present the highest retention time, as well as a variable distribution during specific developmental stages (Saric and Lucchini, 2007). This last event probably occurs as result of the differential expression of several metal transport systems within the developmental period (Aschner et al., 2007). In humans, exposures to high Mn levels are associated with a characteristic syndrome termed manganism, which involves both psychiatric symptoms and parkinsonian features (Benedetto et al., 2009). Although occupational exposures and consumption of contaminated well water represent important ways by which humans are exposed to Mn, there is

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increasing evidence of neurotoxicity as result of the oral Mn exposure in infants subjected to parenteral nutrition (Erikson et al., 2007), which has arisen concerns about the developmental neurotoxicity of Mn (Grandjean and Landrigan, 2006). Once in the brain, Mn presents a great affinity for both cortical and subcortical regions, particularly in the basal ganglia. However, experimental studies point to the cerebellum as an important structure that accumulates Mn (Burton et al., 2009; Fitsanakis *et al*., 2011; Sotogaku *et al*., 2000; García et al., 2006a; Yoon et al., 2009).

Several hypotheses to explain the molecular mechanisms mediating Mn-induced neurotoxicity were fostered (for a review, see Quintanar, 2008), but a complete understanding on this theme is still lacking. The hypotheses include (1) mitochondrial dysfunction and impaired energy metabolism, (2) altered levels of neurotransmitters and excitotoxic death, (3) protease activation and apoptotic death and (4) oxidation of cell components by Mn(III). The aforementioned lack of knowledge regarding the molecular mechanisms mediating Mn-induced neurotoxicity is responsible, at least in part, for the absence of effective clinical interventions for Mn poisoning in humans (Aschner et al., 2007) and accepted limits of exposure during the developmental period.

In the aquatic system of the Paranapanema Basin in São Paulo, Brazil, we identified several environmentally relevant Mn fractions (putatively containing Mn(II) citrate, Mn(II) aquo-hydroxyl and Mn(III) pyrophosphate complexes) which pose moderated ecotoxicological risks (Hernández *et al.* 2009). However, there are no comparative data on the potential neurotoxic effects of these Mn species. In fact, the role of chemical speciation in Mn-induced neurotoxicity is still a not well explored subject and certainly deserves additional attention. Further studies on the relationships between Mn neurotoxicity and its speciation are desired.

Because of the previously mentioned aspects concerning Mn-induced neurotoxicity (mainly those related to its chemical speciation), as well as the scarcity of comparative studies on the differential

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susceptibilities of neurons from different brain regions and at different developmental stages, we investigated whether cortical and cerebellar granule neurons have different sensitivity to Mn-induced neurotoxicity and whether Mn chemical speciation is an important determinant of its neurotoxic properties. Primary cultures of cortical neurons (CTX) and cerebellar granule cells (CGC) were used because of the high affinity of Mn for both cerebro-cortical and cerebellar tissues (Fitsanakis et al., 2011). We found that (i) the different Mn species induced mitochondrial disfunction to both cell models; (ii) CGC were significantly more sensitive than CTX to Mn; and (iii) CGC were more vulnerable to Mn during their differentiation period.

Material and Methods

Materials

Pregnant NMRI mice (16th day of gestation) were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Plastic multi-well culture plates were purchased from CoStar (Corning Science Products, Acton, MA, USA). Fetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco's modified Minimum Essential Medium (DMEM) from Biochrom (Berlin, Germany). Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$; 99.7 %), manganese(III) acetate dihydrate ($(CH_3COO)_3Mn \cdot 2H_2O$; 97 %), sodium citrate tribasic dihydrate (Cit; $(HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O)$, sodium pyrophosphate decahydrate (PPi; $Na_4P_2O_7 \cdot 10H_2O$), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), trypsin, soybean trypsin inhibitor, DNAse, amino acids and poly-D-lysine were obtained from Sigma-Aldrich (Madrid, Spain and São Paulo, Brazil).

Synthesis and characterization of Mn complexes

A $MnCl_2$ stock solution was prepared by dissolving $MnCl_2 \cdot 4H_2O$ in sterile ultra pure water to attain a final concentration of 50 mM. Manganese(II) citrate (Mn(II)Cit) stock solution was prepared by dissolving $MnCl_2 \cdot 4H_2O$ in citrate solution (350 mM) to a final Mn(II) concentration of 50 mM.

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Manganese(III) citrate (Mn(III)Cit) and pyrophosphate (Mn(III)PPi) stock solutions were prepared according to Parker *et al*. (2004) by dissolution of Mn(III) acetate on the respective 373 mM citrate or pyrophosphate solutions to a final Mn concentration of 37.3 mM. Excess ligand (Cit or PPi) guaranteed the stabilization of the metal in the trivalent oxidation state (Klewicki and Morgan, 1998). Mn(II) is stable in aqueous solution at physiological pH. However, Mn(III) is an oxidizing agent that can disproportionate into Mn(II) and Mn(IV) in aqueous solution (Michalke et al., 2007), unless properly chelated. The manganese complexes were synthesized using pure compounds and characterized according to previous reports (Klewicki and Morgan, 1998; Parker et al., 2004; Reaney and Smith, 2005). In this way, UV-VIS and EPR analyses as described below offered solid information about oxidation state of the Mn and the ligands in these complexes. Mn solutions were used immediately after their characterization to guarantee that the exposition process was being executed with the specified Mn species.

Electron Paramagnetic Resonance and electronic analyses

The absence of measurable Mn(II) in the Mn(III) solutions (aqueous and/or in cell culture medium) was confirmed by conventional electron paramagnetic resonance (EPR) using a Varian E-109 Century Series X-band Spectrometer working in the X band (9.5 GHz) at the following experimental conditions: scan range: 1000 gauss, field set: 3365 gauss, Scan Time: 8’, field modulation amplitude: 3.2 gauss, field modulation frequency: 100 kHz, receiver gain: 8×10^4, microwave power: 5 mW, temperature: 323.15 K. The Lande factor for Mn(II) is $g = 2.0054 \pm 0.0005$. These experiments were carried out in 0.4 mm outer diameter quartz tubes, after incubation for 0, 48 and 120 h. None of the Mn complex solutions were deoxygenated for EPR measurement. Electronic spectra were acquired with and UV/visible spectrophotometer and the absorbance of Mn(III)Cit and Mn(III)PPi were registered at 430 nm and 480 nm, respectively (Klewicki and Morgan, 1998).

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Neuronal cell culture and treatment protocols

Primary cultures of cortical neurons were prepared from the cerebral cortices of 16-day-old mice fetuses, whereas those of cerebellar granule cells were prepared from 7-day-old mice pups, as previously reported (Briz et al., 2010; Vendrell et al., 2010). Primary cultures of neocortical neurons are mainly constituted of GABAergic and cholinergic neurons, while primary cultures of cerebellar granule cells are mainly constituted of glutamatergic neurons (Suñol et al., 2008). In both cases, the astrocytic content is maintained low by the addition of antimitotic agents. Animals were handled in compliance with protocols approved by the Generalitat of Catalunya, Spain, in accordance with EU guidelines.

Once adhered to the surface of 96-, 24- or 6-wells microplates, cells were treated with the test solutions (well-defined Mn complexes) either in the first day in vitro (div), when the differentiation process is started (immature neurons) or at 6 div, when the neurons are mature. The treatments lasted 120 h (5 div). The culture medium was not changed until the experiments were performed. All the experiments were carried out in the presence or absence of neuroprotective agents such as ascorbic acid (200 μM) or d,l-Lactate (10 mM; equivalent to 5 mM l-Lactate, *Lac* 5 mM).

Cell viability and mitochondrial activity assays

Cell viability was evaluated with the MTT assay, based on the reduction of 3-[4,5-dimethythiazol-2-il]-2,5-diphenyl-tetrazolium bromide (MTT) to a formazan byproduct via a group of non specific mitochondrial dehydrogenases by viable cells. After 5 days of treatment in vitro, the assay was conducted according to the method described by García *et al*. (2006). Following each exposure period, the cells were washed twice with 0.1 mL of pre-warmed Hanks' solution (37°C) and then incubated for 60 min at 37°C with MTT (0.25 mg/mL). After removal of the MTT solution, 0.2 mL/well of solubilization solution (SDS 5% w/v) was added and the cells were kept overnight at 37°C in darkness. Absorbance was measured at 560 nm in a spectrophotometer plate reader.

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Additionally, neuronal death was evaluated with propidum iodide (PI assay), which is excluded by living cells, but rapidly enters cells with damaged membranes and binds to DNA, rendering them brightly fluorescent, essentially as previously described by Petegnief et al. (2003).

The relative loss of mitochondrial function of the cells exposed to Mn species was determined by using the rhodamine 123 test (Molecular Probes), which is used to measure mitochondrial transmembrane potential (mitochondrial homeostasis). Cells were incubated with rhodamine 123 (final concentration 5 μg/ml) for 30 minutes. After washing, a revealing solution (acetic acid:ethanol, 1:2) was used before fluorometric detection (excitation, 490 nm; emission, 535 nm) (Debbasch et al., 2001).

Cellular manganese accumulation.

Cellular manganese levels were determined by inductively coupled plasma mass spectrometry (ICP-MS), using a PE ELAN DRC II ICP-MS instrument (PerkinElmer Life and Analytical Sciences). Typical daily instrumental parameters are given in Table 1. The cell pellet was lysed via hypo-osmotic shock and sonication in 100 μL of ultra-pure water. An aliquot of 50 μL of the sample was diluted 1:100 in a solution of triton/HNO_3 for Mn analysis (with a method quantification limit of 1 ng Mn / ml sample) and the remaining sample was used for protein determination. The method quantification limit was obtained as 10 x SD (standard deviation) of the 20 consecutive measurements of the blanks multiplied by the dilution factor of samples, in this case 1:100. It corresponds to approximately 1 ng Mn / mg protein.

GSH determination.

Total glutathione (tGSH) levels were estimated by the 5,5’-dithio-bis-2-nitrobenzoic acid (DTNB) recycling method as previously described (Farina et al., 2009). Briefly, cells cultivated in 6-well plates were homogenized in 150 μL of phosphate-EDTA buffer (PE buffer: 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA) and total protein was estimated. The

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homogenate was precipitated with 2% sulfosalicylic acid (w/v) and centrifuged (16,000 × *g*, 5 min at 4 ºC), and the supernatant was used for tGSH determinations. Fifty microliters of the nonprotein supernatant were incubated with assay buffer (PE buffer containing 0.8 mM DTNB and 0.32 U/mL glutathione reductase) in a final reaction volume of 250 µL. The reaction was initiated by the addition of 0.6 mM NADPH. The reaction kinetics of DTNB recycling was monitored at 412 nm for 2 min. The absolute tGSH levels in each sample were calculated based on standards (0 to 25 nmol) and normalized per milligram of protein.

Data analysis

Results are expressed as means ± S.E.M. of three experiments performed in different culture preparations, except when otherwise stated. Each experiment was performed in triplicates. Sigmoidal curves were fitted to concentration–response data. A minimum of six concentration values, all in triplicate, were used to build each curve. Student's t-test, one- and two-way analysis of variance (followed by post-hoc comparison tests) were used for comparison of different experimental conditions. Fitting and statistical analysis were performed using GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA).

Results

The present work aimed to evaluate the neurotoxicity induced by different Mn species as a function of (i) ligand type (water, citrate or pyrophosphate) bound to Mn, (ii) metal oxidation state (Mn(II) or Mn(III)), (iii) neuronal cell type (CGC or CTX) and (iv) neuronal differentiation period (exposure to Mn from 1 div to 6 div or from 6 div to 11 div).

Spectroscopic properties of the Mn complexes

Figure 1A depicts the electronic spectra of Mn(III)Cit and Mn(III)PPi with characteristic peaks at 430 nm and 480 nm, respectively, which confirm the formation of the trivalent Mn complexes

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(Klewicki and Morgan, 1998). Additionally, figure 1B depicts an EPR spectra set of a high-spin Mn(II) state (*d5*, S=5/2), consistent with an EPR signal centered at $g = 2$ with prominent Mn hyperfine structure of six lines equally spaced by 70 gauss. This pattern was not altered in aqueous solution after incubation at 37°C for several hours (Figure 1B (1-3)). However, the EPR signal decreased when the incubation occurred in the presence of cell culture medium (Figure 1B (4-6)). High-spin Mn(III) (*d*4, $S = 2$) systems are "EPR-silent" (Goldberg *et al*. 1997), which can be confirmed both in aqueous and in culture medium (Figure 1B (7-16)).

Cerebellar granule neurons at early differentiation stages are more vulnerable to Mn neurotoxicity.

Primary cultures of cerebellar granule cells were exposed to $MnCl_2$, Mn(II)Cit, Mn(III)Cit and Mn(III)PPi for 5 days; treatments started at day in vitro 1 (div 1 - early differentiation period, with cells starting to produce neurites) or at div 6 (with cells approaching maturity and showing a neurite network connecting cultured cells, which express functional transporters and receptors for neurotransmitters) (Suñol et al., 2008). The MTT assay was used to determine cell viability. Figure 2 shows the LC_{50} values for the different Mn species in cultured cerebellar granule cells exposed for 120 hours at two stages of neural differentiation, indicating that neurons exposed to $MnCl_2$, Mn(II) citrate and Mn(III) pyrophosphate but not Mn(III) citrate during their early developmental stage were more sensitive than differentiated cells. No significant differences were found between Mn species, except for Mn(III)PPi which was the less toxic species in mature neurons ($p < 0.05$, Tukey's test after significant ANOVA). Accordingly, rhodamine 123 test showed that the mitochondrial membrane potential in CGCs exposed from div 1 to div 6 was reduced by all Mn species (Figure 3). EC_{50} values were 16 ± 1 μM, 18 ± 1 μM and 18 ± 1 μM for $MnCl_2$, Mn(II)Cit and Mn(III)Cit, respectively. Finally, the PI assay showed that all Mn species induced a concentration-dependent cytotoxicity (Figure 1, Supplemental Material), determined as nuclei staining with PI after its incorporation through disrupted cytoplasmic membranes. Mn accumulation

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was also determined in CGC exposed during their differentiation period (1-6 div). Figure 4 shows that Mn accumulated in CGCs in a concentration-dependent manner. In addition, comparison between Mn(II)Cit and Mn(III)Cit accumulations showed a higher cell uptake of the Mn(III) species ($p < 0.05$ for 10 μM and $p = 0.08$ for 3 μM).

Cultured cerebellar granule neurons are more vulnerable than cultured neocortical neuron.

Once established that the undifferentiated cell stage was more susceptible to Mn injury, a comparative study was made between CGCs and CTXs at the early developmental stage. Figure 5 shows the concentration-response curves for Mn toxicity in CGCs and in CTX neurons. Table 2 shows the corresponding LC_{50} values, which were significantly different between CGC and CTX for all the Mn species (except for Mn(III)PPi), indicating that CGCs were more vulnerable to Mn-induced toxicity when compared to CTX neurons.

Oxidative stress has been proposed as an important mechanism mediating Mn toxicity and decreased glutathione (GSH) levels have been reported as a consequence of Mn exposure (Erikson et al., 2006). Therefore, we investigated whether different GSH levels in both cell types could be related to their different vulnerabilities to Mn toxicity. Total GSH (tGSH) levels in mature control CGC and CTX cells were 113 ± 17 and 216 ± 29 nmol GSH/mg protein, respectively ($p < 0.01$; n = 8-10). In order to look for early signs of Mn on GSH antioxidant defences, we exposed the cells to low concentrations of Mn that did not induce significant cytotoxicity. Exposure for 5 days in vitro did not decrease GSH levels in both cell types (Figure 6). Instead, a trend for increasing tGSH levels was observed in CTX neurons for 10 – 40 μM $MnCl_2$ ($p = 0.09$), although this increase did not reach statistical significance.

Protection against Mn neurotoxicity by ascorbic acid and lactate

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Ascorbic acid (200 μM) protected the differentiating CGC neurons (1-6 div) from both $MnCl_2$- and Mn(III)Cit-induced neurotoxicity (Figures 7A and 7B, respectively). However, ascorbic acid did not protect CTX when Mn concentrations were close to the EC_{50} (Figure 8A). Other antioxidants, such as probucol (3 μM), trolox (10 μM) or propyl gallate (10 μM), also failed to protect cultured CTX neurons against Mn-induced toxicity (data not shown). Lactic acid also presented a significant protective effect against Mn-induced neurotoxicity in CGCs (Figures 7C and 7D). Unlike ascorbic acid, lactate was effective in protecting CTX neurons against $MnCl_2$-induced neurotoxicity (Figure 8B). In addition, the combination of ascorbic acid and lactate significantly protected CTX neurons against Mn-induced toxicity (Figure 8C).

Discussion

The electronic spectra of the Mn(III) species (Figure 1A) display characteristic maximum absorptions (λ_{max}) at 430 nm and 480 nm for Mn(III)Cit and Mn(III)PPi, respectively (Klewicki and Morgan, 1998), which are attributed to metal-centered *d-d* transitions. Divalent Mn species (aqueous Mn(II) and Mn(II)Cit; spectra not shown) are high spin d^5 species whose *d-d* transitions are spin-forbidden, which translate in very low molar absorptivities and therefore virtual absence of absorbance in the visible spectrum (Shriver and Atkins, 2003). Further characterization of the complexes was performed by EPR spectroscopy (Figure 1B). High-spin Mn(II) species (d^5, S = 5/2) give rise to spectra centered at g = 2 with characteristic manganese hyperfine structure of six equally spaced lines at 70 gauss at room temperature (Schiemann et al., 2003). This pattern is not altered in aqueous solution after incubation at 37°C for several hours (spectra 1-3). However, incubation with cell culture medium induced decreased EPR signals (spectra 4-6). This observation may be rationalized in terms of Mn(II) coordination to high molecular weight ligands (e.g. albumin), which causes broadening of the spectra due to faster relaxation processes (Schiemann et al., 2003). Manganese EPR-silent species include Mn(III) (d^4, S = 2) (Goldberg *et al*. 1997) and are observed in Figure 1b (spectra 7-16). In this case, conventional EPR-allowed ΔMs = ±1 transitions

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are not observed at normal X-band due to a combination of large zero-field splitting values and unfavorable relaxation effects (Mabbs and Collison, 1992).

We found that cultured cerebellar granule neurons (CGC) were more vulnerable to Mn-induced toxicity than cultured cortical neurons (CTX). A decrescent order of cell vulnerability to Mn can be suggested based on the LC_{50} values of $MnCl_2$ in different cell types: cerebellar granule neurons (LC_{50}: 25 – 50 µM; this work) > cortical neurons (LC_{50}: 100 – 180 µM; this work; Lee et al., 2009; Tamm et al., 2008) > hippocampal and mesencephalic dopaminergic neurons (LC_{50}: 250 – 500 µM; Cai et al., 2011; Stanwood et al., 2009; Tamm et al., 2008) > astrocytes (Lee et al., 2009). The selective vulnerability of certain brain areas / neural cell types can be accounted for by different cell antioxidant defenses, neurotransmitter phenotype and / or intracellular Mn accumulation. In this sense, we found significantly higher levels of the endogenous antioxidant GSH in CTX when compared to CGC which could be responsible, at least in part, for the higher resistance of CTX to Mn. This is believed because oxidative stress mediates Mn-induced toxicity (Benedetto et al., 2009) and glutathione, an important antioxidant molecule with crucial functions in the CNS, presents decreased levels in the brain of rodents exposed to Mn (Erikson et al., 2006). Another important aspect that likely modulates Mn-toxicity is the cellular ability of accumulating and/or exporting this metal, which has been reported to be responsible for differential susceptibilities of different encephalic structures (or even different cell types) to manganese-induced toxicity (Aschner et al., 2007). In this regard, cell accumulation of Mn is driven by several transport mechanisms, among them the divalent metal transporter (DMT1) (Aschner et al., 2007). Immunohistochemistry for DMT1 antibody reveals higher expression of DMT1 in the cerebellum than in the cortex of the rat (Burdo et al., 2001), which is in line with the high accumulation of Mn in the cerebellum of rats treated with Mn (Fitsanakis et al., 2011). Of particular importance, it is noteworthy that impairments in the cerebellar function can induce loss of both cognitive and motor capacity (Hoppenbrouwers et al., 2008), which have been diagnosed in humans exposed to Mn during early life stages (Grandjean and Landrigan, 2006; Menezes-Filho et al., 2011). We also

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demonstrated that, independently of the chemical speciation, Mn was more neurotoxic to undifferentiated than to differentiated cells. This observation, which corroborates the results of Tamm et al. (2008) with stem cells exposed to $MnCl_2$, reinforces the idea that the developing systems/cells are more susceptible to the hazardous effects played by potential toxicants, including Mn. Likewise, rats perinatally exposed to Mn had increased expression of DMT1 in the brain, the cerebellum being one of the most affected areas as well as one of the areas with significant accumulation of Mn (Garcia et al., 2006a).

Under *in vivo* conditions, Mn(II) has been reported to be more toxic than Mn(III) (EPA, 1975). Our in vitro results show that Mn(II) and Mn(III) species presented similar toxicities to cerebellar granule neurons (CGC; containing ~ 95 % of glutamatergic neurons). Conversely, Mn(III)PPi displayed higher cellular toxicity than the other Mn species towards cultured cortical neurons (CTX; containing a mixture of GABAergic, glutamatergic and cholinergic neurons). Likewise, in PC12 cells (dopaminergic neurons), Mn(III)PPi was more toxic than Mn(II) ($MnCl_2$) (Reaney and Smith, 2005). Thus, the different chemical species of Mn may induce different degrees of toxicity depending on the targeted neuronal cell. However, even though different neuronal cell types have different susceptibilities to the different Mn species, it is not possible to state, thus far, that a given Mn species will display specific toxicity toward a particular neurotransmitter system. Because of the different behavior of Mn(III)PPi regarding toxicity in mature CGC and in CTX with respect to the other Mn species, further studies are needed to elucidate whether different mechanisms of toxicity and/or different Mn accumulation are underlying factors. On the other hand, with a particular emphasis in the citrate-containing complexes, we found higher accumulation of Mn(III)citrate in comparison to Mn(II)citrate, which is in agreement with the results shown by Reaney and Smith (2005).

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MTT is a tetrazolium salt actively transported into cells and reduced to formazan byproduct in a reaction catalyzed by a large group of non-specific mitochondrial dehydrogenases (Ying et al., 2001), including NADH dehydrogenase, malate dehydrogenase, and succinic acid dehydrogenase. Thus, the decrease of MTT reduction by the cells after Mn exposure represents a good indicator of the loss of mitochondrial enzyme activity and a signal of early progression for cell death as well. As undifferentiated CGCs were highly susceptible to Mn citotoxicity in the MTT assay, additional experiments were carried out to better understand the mechanisms related to such phenomenon. In agreement with the MTT assay, we found that all Mn species induced a significant reduction of mitochondrial homeostasis as determined by the rhodamine 123 test, traditionally used to measure mitochondrial transmembrane potential (Figure 3). The effects of Mn toward mitochondria from cultured cells have also been reported in primary cultures of astrocytes (Yin et al., 2008). Of particular importance, Yin and collaborators showed that Mn induced apoptosis in cultured astrocytes via mitochondrial-dependent pathways (Yin et al., 2008). Based on these evidences and on our data with different Mn species, it is reasonable to suggest that mitochondria might represent an important primary organelle mediating Mn-induced neurotoxicity.

The tripeptide glutathione (GSH, γ-glutamylcysteinylglycine) may function either as an intracellular metal chelator, a metal detoxifying agent or a radical scavenger (Hamai and Bondy, 2004). Of particular importance, some studies have shown decreased GSH levels after Mn exposure (Erikson et al., 2006). We found no significant effects of Mn on GSH levels in both cell models, although there was a trend for increasing GSH levels at lower Mn concentrations, as well as a trend for decreasing GSH levels at Mn concentration higher than LC_{50} ($p = 0.09$). In order to substantiate the hypothesis of oxidative stress in Mn-induced neurotoxicity, several radical scavengers were tested in an attempt to find potential neuroprotective effects. However, only ascorbate, a well-known antioxidant required by all mammalian cells (Karaczyn et al., 2006), was effective in protecting cultured neurons against Mn toxicity, although with some differences between both cells models.

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We found that ascorbic acid was much less effective against Mn-induced neurotoxicity in CTX neurons than in CGCs (Figures 7A, 7B and 8A). This could be associated, at least in part, to a disruption in the cell and mitochondrial coupling antioxidant system ascorbate:GSH (Li et al, 2001). The ascorbate:GSH ratio in neurons is 4:1 (Rice et al., 2000), and we found that CGC have less GSH that CTX, consequently CGC must be more vulnerable than CTX. Ascorbate protection was more effective in CGC probably because these cells might uptake and retain slightly more ascorbate than CTX (Caprile et al., 2009), although both models have similar ascorbate basal levels (Rice et al., 2002). Another possible explanation for the higher protection afforded by ascorbate in CGC when compared to CTX could be the protective effects of ascorbate against excitotoxic events (May et al., 2006). This hypothesis is based on the fact that CGC cultures present approximately 95% of glutamatergic neurons. In agreement to this, glutamate receptor antagonists have been reported to protect against Mn-induced neurotoxicity under *in vivo* conditions (Xu et al., 2010).

The recovery of cell viability with d,l-lactate (*Lac,* 5 mM) treatment (Figures 7C, 7D and 8B) agrees with the hypothesis of energy depletion as an important mechanism mediating Mn neurotoxicity (Quintanar, 2008). Lactate is the end-product of the glycolysis and is preferentially used by neurons as major oxidative substrate. This implies that Mn affects the cell lactate metabolism in these cell models, especially the glutamatergic neurons (CGC), as suggested by Zwingmann et al. (2003). Our results agree with those reporting that lactate (as well as pyruvate) plays a more important role as energy fuel for glutamatergic neurons than for GABAergic neurons (Schousboe, et al., 2007).

In view of these results and especially the low efficiency of the antioxidants in CTX (Figures 8A and 8B), we tested the co-administration of lactate and ascorbate in this model, searching for increased neuroprotection. Indeed, this approach led to full protection against cellular Mn toxicity (Figure 8C), probably by a mechanism coordinated by these molecules. For example, it has been

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reported that the maintenance of GSH levels and the redox homeostasis is a primary function of the pentose phosphate pathway (Vaughn and Deshmukh, 2008). By this way ascorbate and dehydroascorbate can be metabolized at a high rate to lactate or glucose in cells unable to produce ascorbate (Banhegyi *et al.*, 1997), such as neurons. Additionally, extracellular ascorbic acid can inhibit glucose transport and stimulate lactate transport in neuronal cells (Castro et al., 2009) as well as the antioxidant system ascorbate:GSH which led to the maintenance of ascorbate and lactate levels.

In conclusion, here we report that *in vitro*, irrespective of the Mn species, undifferentiated CGCs were more susceptible to Mn toxicity than mature neurons. We also found that CGCs were more vulnerable than CTX neurons against Mn-induced cell toxicity. Incubation studies with ascorbic acid and lactate suggest that cellular Mn toxicity was mediated by mitochondrial energy metabolism impairment and oxidative stress, with the potential occurrence of lactate and ascorbate metabolism disruptions. Our results reinforce and extend the notion of the hazardous effects of Mn toward neuronal cells. In addition, the present findings indicate that Mn-induced neurotoxicity is influenced by brain cell types, their origins and developmental stages, as well as by the chemical speciation of Mn. The data point to Mn as a mitochondrial disrupting agent and suggest that cerebellar impairment, especially in early life periods, might underlie Mn-induced developmental neurotoxicity.

Supplementary Data

Mn-induced cytotoxicity was assessed by means of the incorporation of propidium iodide through damaged cell membrane. Both MnCl2, Mn(II)Cit and Mn(III)Cit induced a concentration-dependent cell death in primary cultures of cerebellar granule cells exposed to Mn species from div 1 to div 6.

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Legends

Figure 1: a) Electronics spectra for trivalent species and ligands. b) EPR spectra from $MnCl_2$ (2 mM) in aqueous solution (1, 2 and 3 after 0 h, 48 h and 120 h at 37ºC) and in culture medium (4, 5 and 6 after 0 h, 48 h and 120 h at 37ºC); Mn(III)PPi (1 mM) in water solution (7, 9 and 11 after 2 h, 48 h and 120 h at 37ºC) and in culture medium (8 and 10 after 0 h and 48 h at 37ºC); Mn(III)Cit (1 mM) in water solution (12, 14 and 16 after 0 h, 48 h and 120 h at 37ºC) and in culture medium (13 and 15 after 2 h and 48 h at 37ºC) .

Figure 2: Bar diagram of LC_{50} values for the cytotoxic effects of manganese species after exposure for 120 hours at differentiating (from div 1 to div 6; white bars) and differentiated (from div 6 to div 11 div; black bars) stages of cultured cerebellar granule neurons. Values represent mean ± S.E.M. Two-way ANOVA indicated significant differences among both neuronal differentiation stages ($F_{1,34} = 56.8$; $p < 0.0001$). * $p< 0.05$ and ** $p < 0.001$ vs corresponding Mn complexes exposure at 1 – 6 div. + $p < 0.05$ vs exposure to $MnCl_2$.

Figure 3: Mitochondrial activity (rhodamine 123 test) of cerebellar granule cells after 120h (from div 1 to div 6) of treatment with $MnCl_2$ (gray bars), Mn(II)Cit (white bars) and Mn(III)Cit (black bars) Values are means ± S.E.M of 3 independent experiments. * $p < 0.01$ vs control after a significant one-way ANOVA.

Figure 4: Intracellular manganese concentrations. Cerebellar granule cells were exposed to $MnCl_2$ (gray bars), Mn(II)Cit (white bars) or Mn(III)Cit (black bars) for 120 h (from div 1 to div 6). Values are means ± SEM of 3 independent experiments. *$p < 0.05$), ** $p < 0.01$ vs non-exposed cells, and # $p < 0.05$ between Mn(II)Cit and MN(III)Cit (Bonferroni's post test after significant two-way ANOVA).

Figure 5: Concentration-response curves for the effects of Manganese species on cell viability in primary cultures of cerebellar granule neurons (■) and of cortical neurons (▲). Cells were exposed for 120 h (from div 1 to div 6). Values are means ± SEM of at least 3 independent experiments, each performed in triplicate.

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Figure 6: Effect of $MnCl_2$ on intracellular GSH levels in primary cultures of cerebellar granule neurons (A) and of cortical neurons (B). Cells were exposed for 120 h (from div 1 to div 6). Values are means ± SEM of at 4 - 6 independent experiments, each performed in triplicate.

Figure 7: Protection by ascorbic acid (A and B) and lactate (C and D) of manganese-induced neurotoxicity in cerebellar granule cells. Cells were exposed to $MnCl_2$ (A and C) or Mn(III)Cit (B and D) for 120 h (from div 1 to div 6), in the absence (black bars) or in the presence (white bars) of 200 μM ascorbic acid (A and B) or 5 mM lactate (C and D). Values are means ± SEM of at least 3 independent experiments, each performed in triplicate. Two-way ANOVA showed significant differences for manganese concentrations and the absence/presence of ascorbic acid or lactate. * $P < 0.05$ and ** $P < 0.01$ with respect to corresponding controls; + $P < 0.05$ and ++ $P < 0.01$ with respect to corresponding manganese concentration (one-way ANOVA followed by Dunnet's test).

Figure 8: Effects of ascorbic acid (A), lactate (B) and ascorbic plus lactate (C) in manganese-induced neurotoxicity in Cortical Neurons. Cells were exposed to $MnCl_2$ or Mn(III) citrate for 120 h (from div 1 to div 6), in the absence (black bars) or in the presence (white bars) of ascorbic acid, lactate or a combination of both. Values are means ± SEM of at least 3 independent experiments, each performed in triplicate. Two-way ANOVA showed significant differences for manganese species and the absence/presence of ascorbic acid or lactate. * $P < 0.05$ and ** $P < 0.01$ with respect to corresponding controls; + $P < 0.05$ with respect to corresponding manganese concentration (one-way ANOVA followed by Dunnet's test).

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Table 1.- ICP-MS operating conditions

PerkinElmer Elan DRC II	
Spray chamber	Cyclonic
Nebulizer	Meinhard®
RF power (W)	1100
Ar nebulizer gas flow (L min−1)	0.7–0.9 (optimized daily)
Measures	
Scan mode	Peak hopping
Resolution (amu)	0.7
Replicate time (s)	1
Dwell time (s)	50
Sweeps/reading	20
Integration time (ms)	1000
Replicates	2
Isotope	^{55}Mn

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Table 2.- LC_{50} (μM) values for the cytotoxicity of Mn species in primary cultures of cerebellar granule neurons and of cortical neurons.

Mn Species	**Cerebellar granule neurons**	**Cortical neurons**
$MnCl_2$	27 ± 2 (13)	180 ± 90 (3) *
Mn(II) citrate	47 ± 11 (4)	314 ± 182 (3) *
Mn(III) citrate	36 ± 4 (11)	261 ± 41 (3) *
Mn(III) pyrophosphate	48 ± 11 (3)	69 ± 17 (4)

Cultured cells were exposed during 120 hours beginning at day in vitro 1. Values represent: mean ± S.E.M (number of experiments in parenthesis)

*P < 0.05, **p < 0.01, ***p < 0.001 vs cerebellar granule neurons (Bonferroni test) after significant 2-way ANOVA (p < 0.0001).

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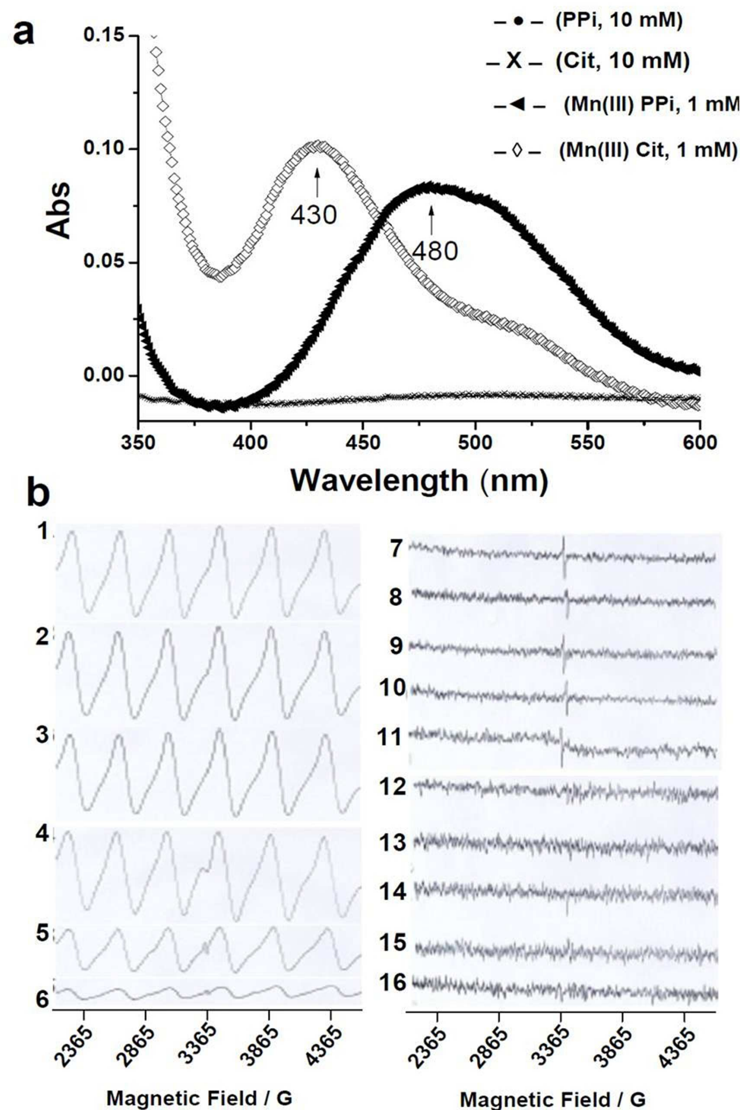

Figure 1: a) Electronics spectra for trivalent species and ligands. b) EPR spectra from MnCl2 (2 mM) in aqueous solution (1, 2 and 3 after 0 h, 48 h and 120 h at 37ºC) and in culture medium (4, 5 and 6 after 0 h, 48 h and 120 h at 37ºC); Mn(III)PPi (1 mM) in water solution (7, 9 and 11 after 2 h, 48 h and 120 h at 37ºC) and in culture medium (8 and 10 after 0 h and 48 h at 37ºC); Mn(III)Cit (1 mM) in water solution (12, 14 and 16 after 0 h, 48 h and 120 h at 37ºC) and in culture medium (13 and 15 after 2 h and 48 h at 37ºC) .
147x235mm (300 x 300 DPI)

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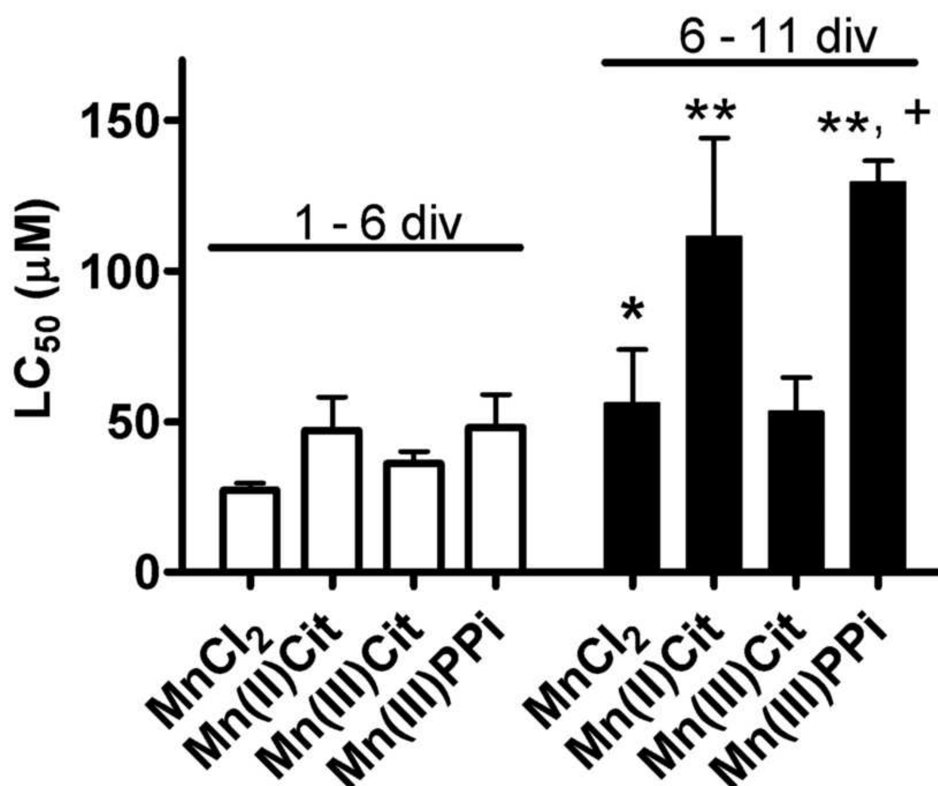

Figure 2: Bar diagram of LC50 values for the cytotoxic effects of manganese species after exposure for 120 hours at differentiating (from div 1 to div 6; white bars) and differentiated (from div 6 to div 11 div; black bars) stages of cultured cerebellar granule neurons. Values represent mean ± S.E.M. Two-way ANOVA indicated significant differences among both neuronal differentiation stages ($F_{1,34} = 56.8$; $p < 0.0001$). * $p < 0.05$ and ** $p < 0.001$ vs corresponding Mn complexes exposure at 1 – 6 div. + $p < 0.05$ vs exposure to $MnCl_2$.
73x60mm (300 x 300 DPI)

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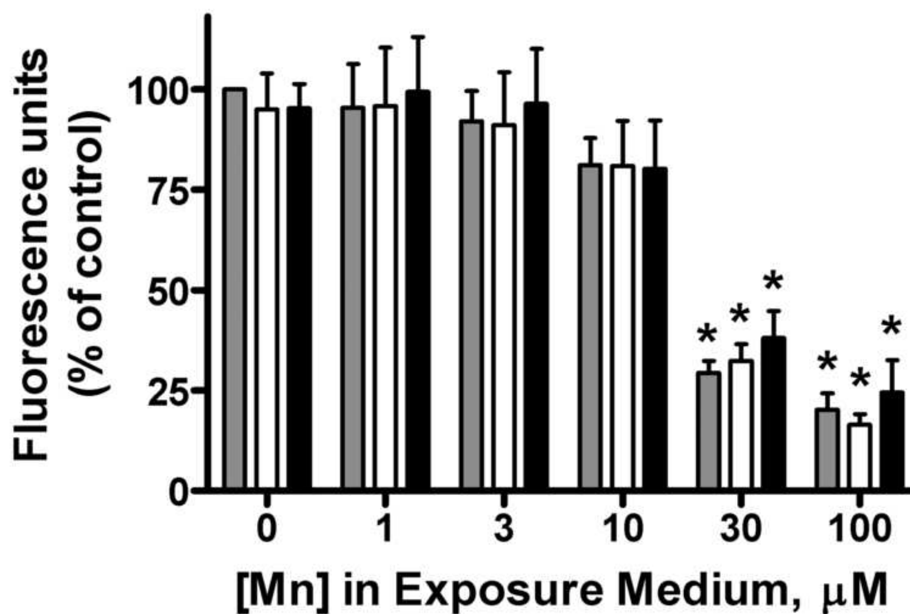

Figure 3: Mitochondrial activity (rhodamine 123 test) of cerebellar granule cells after 120h (from div 1 to div 6) of treatment with MnCl2 (gray bars), Mn(II)Cit (white bars) and Mn(III)Cit (black bars) Values are means ± S.E.M of 3 independent experiments. * $p < 0.01$ vs control after a significant one-way ANOVA.
69x47mm (300 x 300 DPI)

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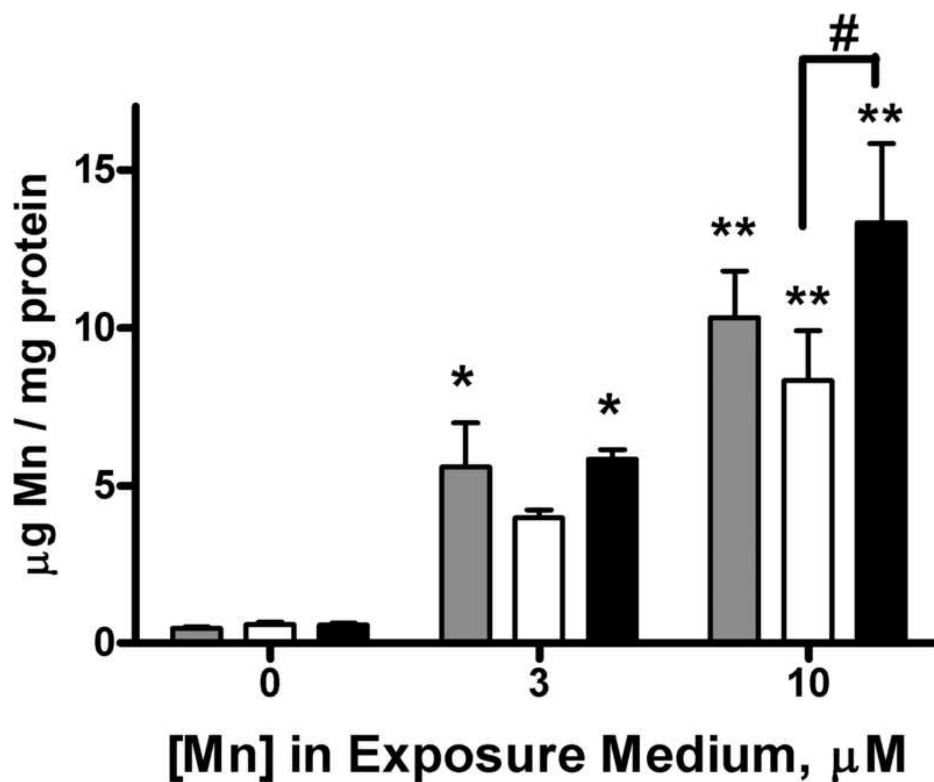

Figure 4: Intracellular manganese concentrations. Cerebellar granule cells were exposed to MnCl2 (gray bars), Mn(II)Cit (white bars) or Mn(III)Cit (black bars) for 120 h (from div 1 to div 6). Values are means ± SEM of 3 independent experiments. *p < 0.05), ** p < 0.01 vs non-exposed cells, and # p < 0.05 between Mn(II)Cit and MN(III)Cit (Bonferroni's post test after significant two-way ANOVA).
72x59mm (300 x 300 DPI)

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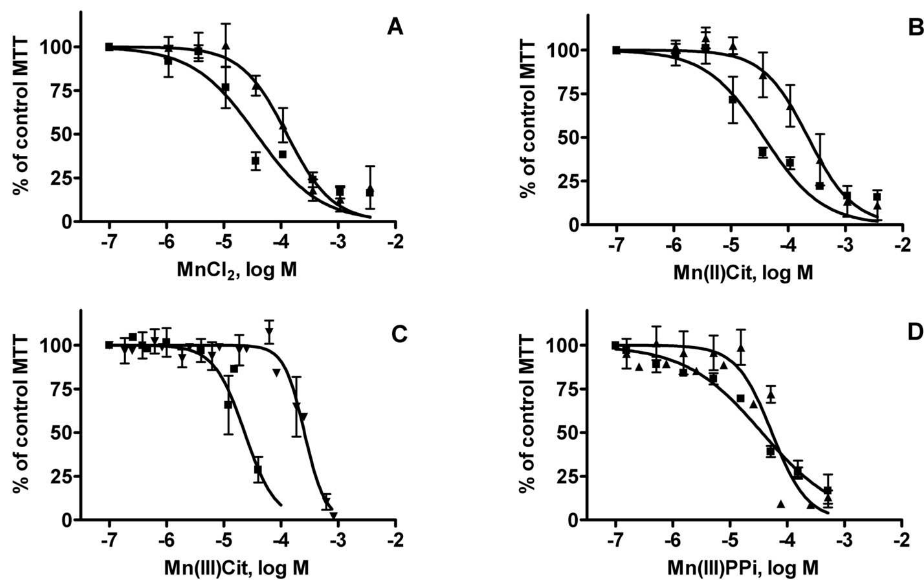

Figure 5: Concentration-response curves for the effects of Manganese species on cell viability in primary cultures of cerebellar granule neurons ( ) and of cortical neurons ( ). Cells were exposed for 120 h (from div 1 to div 6). Values are means SEM of at least 3 independent experiments, each performed in triplicate.
130x83mm (300 x 300 DPI)

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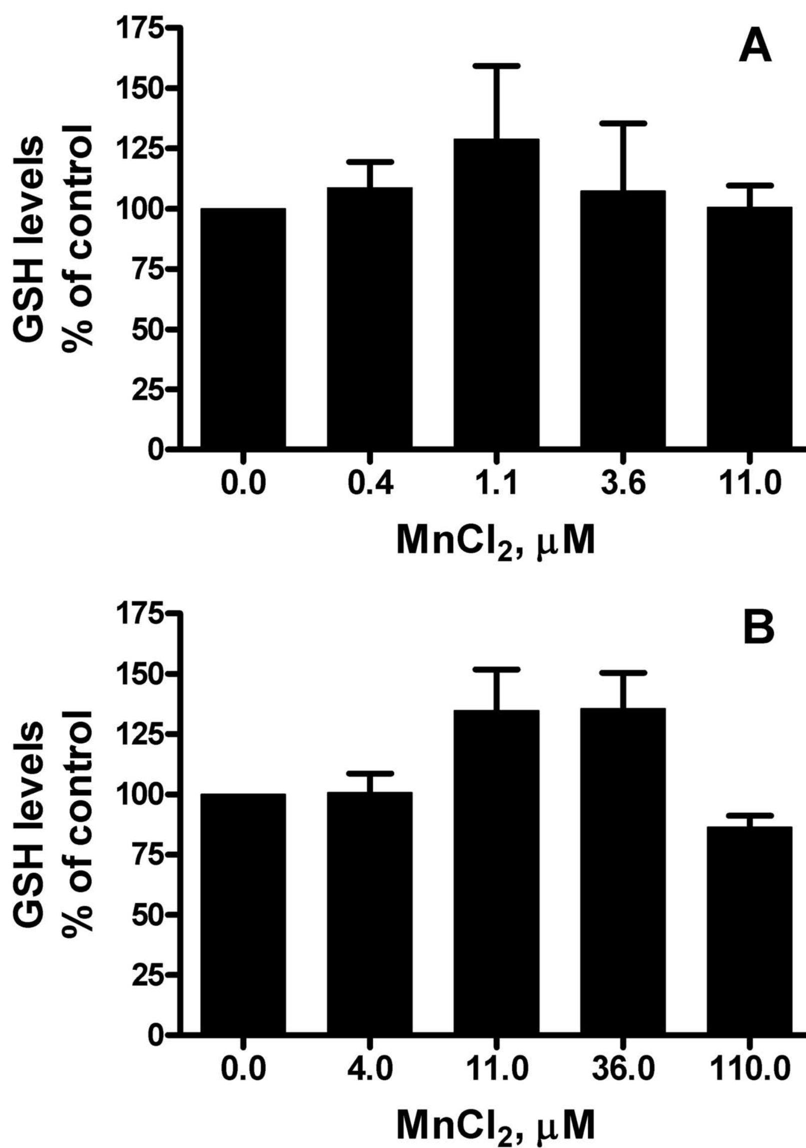

Figure 6: Effect of MnCl2 on intracellular GSH levels in primary cultures of cerebellar granule neurons (A) and of cortical neurons (B). Cells were exposed for 120 h (from div 1 to div 6). Values are means ± SEM of at 4 - 6 independent experiments, each performed in triplicate.
122x168mm (300 x 300 DPI)

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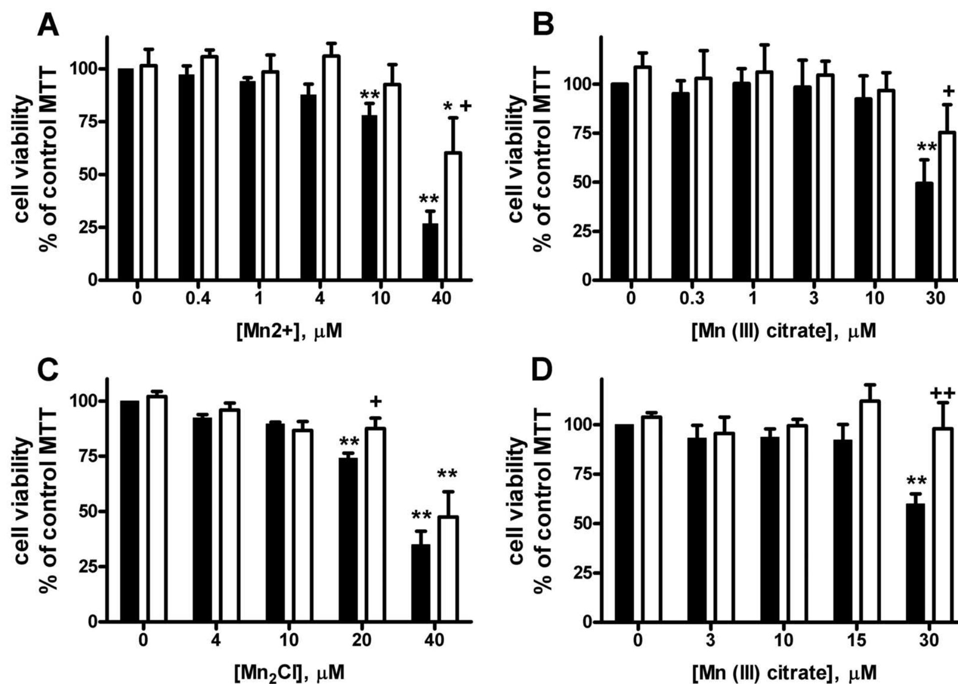

Figure 7: Protection by ascorbic acid (A and B) and lactate (C and D) of manganese-induced neurotoxicity in cerebellar granule cells. Cells were exposed to MnCl2 (A and C) or Mn(III)Cit (B and D) for 120 h (from div 1 to div 6), in the absence (black bars) or in the presence (white bars) of 200 μM ascorbic acid (A and B) or 5 mM lactate (C and D). Values are means ± SEM of at least 3 independent experiments, each performed in triplicate. Two-way ANOVA showed significant differences for manganese concentrations and the absence/presence of ascorbic acid or lactate. * P < 0.05 and ** P < 0.01 with respect to corresponding controls; + P < 0.05 and ++ P < 0.01 with respect to corresponding manganese concentration (one-way ANOVA followed by Dunnet’s test).
141x98mm (300 x 300 DPI)

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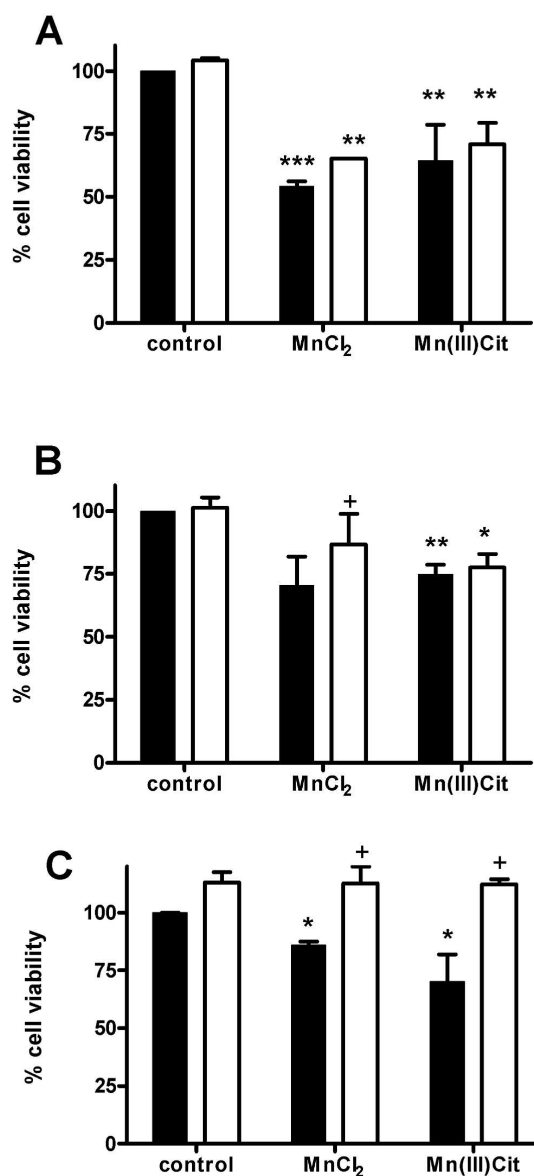

Figure 8: Effects of ascorbic acid (A), lactate (B) and ascorbic plus lactate (C) in manganese-induced neurotoxicity in Cortical Neurons. Cells were exposed to MnCl2 or Mn(III) citrate for 120 h (from div 1 to div 6), in the absence (black bars) or in the presence (white bars) of ascorbic acid, lactate or a combination of both. Values are means ± SEM of at least 3 independent experiments, each performed in triplicate. Two-way ANOVA showed significant differences for manganese species and the absence/presence of ascorbic acid or lactate. * $P < 0.05$ and ** $P < 0.01$ with respect to corresponding controls; + $P < 0.05$ with respect to corresponding manganese concentration (one-way ANOVA followed by Dunnet's test).
182x373mm (300 x 300 DPI)

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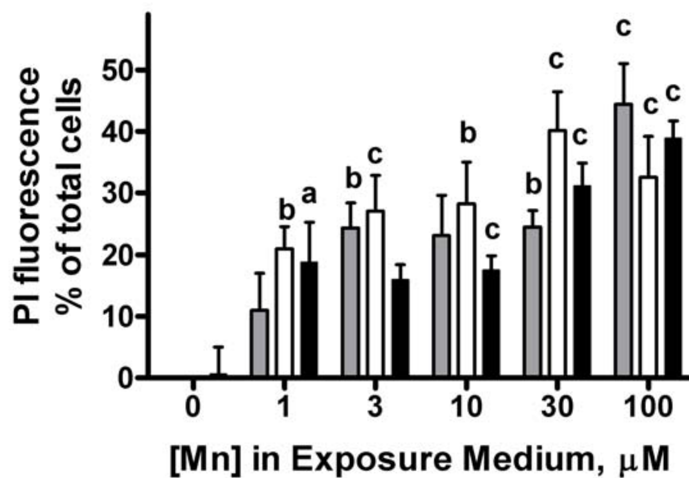
Supplementary Data

Mn-induced cytotoxicity was assessed by means of the incorporation of propidium iodide through damaged cell membrane. Both MnCl2, Mn(II)Cit and Mn(III)Cit induced a concentration-dependent cell death in primary cultures of cerebellar granule cells exposed to Mn species from div 1 to div 6.



Cytotoxic effects of manganese species on primary cultures of cerebellar granule neurons. Cells were exposed for 120 h (from div 1 to div 6). Values are means ± SEM of at 3 - 4 independent experiments, each performed in triplicate. A) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$ vs control cells, after significant two-way ANOVA, based on Bonferroni post hoc analyses.