Mitochondrial-Mediated Apoptosis in Neural Stem Cells Exposed to Manganese

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Manganese is an essential nutrient for humans that has to be maintained at proper levels for normal brain functioning. However, manganese also acts as a toxicant to the brain, and several studies have linked exposure to excessive manganese to neurotoxicity in adults. A recent report has suggested that ingesting high doses of manganese via drinking water can impede intellectual functions in children. It is known that during development, the nervous system is particularly vulnerable to different types of injuries and toxicants. Neural stem cells (NSCs) play an essential role in both the developing nervous system and the adult brain where the capacity for self-renewal may be important. In the present study, we have used NSCs to investigate the molecular mechanisms involved in manganese developmental neurotoxicity. The results show that primary cultures of rat embryonic cortical NSCs as well as the murine-derived multipotent NSC line C17.2 undergo apoptotic cell death via a mitochondrial-mediated pathway in response to manganese. Exposed cells exhibit typical apoptotic features, such as chromatin condensation and cell shrinkage, mitochondrial cytochrome c release, activation of caspase-3, and caspase-specific cleavage of the endogenous substrate poly (ADP-ribose) polymerase. In addition, our data also show that reactive oxygen species formation plays a role in the onset of manganese toxicity in NSCs.

Key Words: C17.2; cell death; MnCl₂; ROS.

There is compelling evidence pointing to the unique susceptibility of the developing brain to toxic agents even at exposure levels that have no lasting effects in the adult (Rodier, 1995). In spite of the growing knowledge, there is still little evidence about how neurotoxicants affect the developing nervous system, especially at low-dose exposures. Brain development goes on from early embryonic life to puberty, and it is characterized by a sequence of orchestrated events such as cell division, programmed cell death, migration, differentiation, and formation of synaptic connections. Interference with any step of these controlled processes alters subsequent developmental stages and may have long-term effects later in life (Rice and Barone, 2000). In spite of the difficulties inherent in identifying neurodevelopmental effects of toxicants, a few compounds have been recognized as neurotoxicants after occupational or accidental exposure to high doses. Now a major challenge for neurotoxicologists is to identify the potential detrimental neurodevelopmental consequences that chemicals may have even at exposure to low levels (see Grandjean and Landrigan, 2006). Epidemiological studies certainly have a critical role in providing evidence for the association between toxicants and neurodevelopmental disorders. However, they have to be complemented by in vivo and in vitro experimental studies aimed at dissecting the cellular and molecular mechanisms of toxicity.

During the past years, we have implemented the use of neural stem cell (NSCs) cultures as in vitro models to study developmental neurotoxicity (Onishchenko et al., 2007; Sleeper et al., 2002; Tamm et al., 2004, 2006). NSCs are characterized by their ability to self-renew and differentiate into the three major cell types: neurons, astrocytes, and oligodendrocytes (Johe et al., 1996). They play a critical role in the development and maturation of the nervous system and may also be important for adult brain normal functions (Gage, 2000). Our recent studies on survival and cell death mechanisms in NSCs exposed to toxic insults, such as methylmercury (Tamm et al., 2006), have shown that these cells are more sensitive than differentiated neurons (Dare et al., 2000) or glia cells (Dare et al., 2001), and point to NSCs as a valuable model for evaluating substances with neurotoxic potential even at low-dose exposure.

Recently, the neurodevelopmental toxicity of manganese (Mn) has come out as a significant public health concern. Epidemiological studies of children have shown that manganese hair levels are associated with hyperactivity (Collipp et al., 1983; Pihl and Parkes, 1977). More recently, Wasserman et al. (2006) have reported that Bangladeshi children drinking well water with high concentrations of Mn have decreased intellectual functions (Wasserman et al., 2006). Similarly, a Canadian study (Bouchard et al., 2007) has shown that exposure to high levels of Mn in tap water is associated with elevated Mn hair levels in children, which is significantly correlated with hyperactivity and oppositional behaviors.

Experimental studies have shown an increased brain accumulation of Mn and a more pronounced brain pathology.

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in neonatal rats than in adults after exposure to similar doses (Chandra and Shukla, 1978; Dorman et al., 2000; Seth et al., 1977; Shukla et al., 1980), which can possibly be due to a general higher vulnerability of the developing nervous system, an incomplete blood-brain barrier, lower bile excretion, high gastrointestinal absorption, and a homogenous diet (Kostial et al., 1978; Miller et al., 1975; Rehnberg et al., 1982). As an essential nutrient, manganese is needed by the fetus to support normal growth and development (Dorman et al., 2006). Additionally, Mn is required for normal amino acid, lipid, protein, and carbohydrate metabolism, as well as utilized by various antioxidant enzymes such as superoxide dismutase and glutamine synthetase (Cotzias, 1958; Takeda, 2003; Wedler and Denman, 1984). However, exposure to high levels of Mn may potentially pose a risk for the developing nervous system (see Erikson et al., 2007) in light of the higher susceptibility to neurotoxic agents of the developing brain and the well-known toxic action of Mn on the adult brain.

The cellular and molecular mechanisms of Mn neurotoxicity are not well understood. Generally, Mn is alleged to exert cellular toxicity via a number of mechanisms, including a direct or indirect formation of reactive oxygen species (ROS) (Ali et al., 1995; Brouillet et al., 1993; Milatovic et al., 2007), the direct oxidation of biological molecules (Archibald and Tyree, 1987), and the disruption of Ca2+ and iron homeostasis (Gavin et al., 1990; Kwik-Uribe et al., 2003; Zheng and Zhao, 2001). An imbalance between ROS generation and the antioxidant defense mechanisms with subsequent oxidative stress (Betteridge, 2000) can initiate apoptosis and/or necrosis (Orrenius et al., 2007), and oxidative stress is in fact one of the putative mechanisms by which Mn induces cell death in neuronal and glial cell lines (Dukhande et al., 2006).

In the present study, we have investigated the effects of Mn on NSCs used as an in vitro neurodevelopmental model to dissect the mechanisms of neurotoxicity. Our attention was particularly focused on the identification of the pathways leading to NSCs' apoptotic death.

MATERIAL AND METHODS

Cell culture procedures and experimental treatments. As experimental models, we have used the murine-derived multipotent NSC line, C17.2, and primary cultures of cortical NSCs (cNSC) obtained from E15 rat embryos. Both cell types are cultured while maintaining an undifferentiated state but can be let to differentiate into neurons, astrocytes, and oligodendrocytes (Hernemann et al., 2002; Joh et al., 1996; Snyder et al., 1992, 1997). The C17.2 cell line was grown in cell culture dishes (Corning Inc., Corning, NY) in Dulbecco’s modified Eagle medium (DMEM) (Life Technologies, Gibco BRL, Grand Island, NY) containing supplemented 10% fetal bovine serum (FBS) and 5% horse serum (Life Technologies) in a humidified atmosphere of 5% CO2 and 95% air at 37°C. For experiments, cells were grown in either cell culture dishes or on poly-L-lysine (Sigma, St Louis, MO) (50 µg/ml)-coated glass coverslips. At the time of the experiments, cells were nestin positive, confirming their proliferative and undifferentiated status. The primary cultures of NSCs were obtained from embryonic cortices dissected in Hanks’ balanced salt solution (HBSS) (Life Technologies) from timed-pregnant Sprague-Dawley rats (B&K, Sol lentuna, Sweden) at E15 (the day of copulatory plug defined as E0). The tissue was mechanically dispersed, and meninges and larger cell clumps were allowed to sediment for 10 min. The cells were plated at a density of 0.6 × 10^6 cells per 100-mm cell culture dish precoated with poly-l-ornithine and fibronectin (both from Sigma). Cells were maintained in enriched N2-medium (Bottenstein and Sato, 1979), with 10 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN) and 570 nm using a multiwell spectrophotometer (Molecular Devices, Sunnyvale, CA). The experiments were performed three times in triplicates, and the results are expressed as percentage of MTX reduction in the control cells.

Apoptotic nuclear morphology and necrotic plasma membrane integrity assessment with Hoechst 33342 and propidium iodide. To evaluate the nuclear morphology, C17.2 cells and cNSCs were grown on coated coverslips. After exposure, cells were washed with phosphate-buffered saline (PBS), and aliquots of solution were pipetted into a 96-well microplate. The plates were then shaken to ensure that all the formazan crystals were dissolved, yielding purple formazan crystals, which are insoluble in aqueous media. At the 1983). Briefly, cells were grown in 12 well plates and exposed to MnCl2. An hour before the end of incubation time, 0.5 mg/ml of MTT was added to each well. Cell membrane stained blue, while healthy or apoptotic cells with intact plasma membrane stayed unstained. All experiments were performed in triplicates and repeated at least three times. Assessment of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is carried out to evaluate cell viability (Mosmann, 1983). Briefly, cells were grown in 12 well plates and exposed to MnCl2. An hour before the end of incubation time, 0.5 mg/ml of MTT was added to each well. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals, which are insoluble in aqueous media. At the end of the incubation, the crystals were dissolved by the addition of a solubilization solution (0.04M HCl in anhydrous isopropanol). The culture plates were then shaken to ensure that all the formazan crystals were dissolved, and aliquots of solution were pipetted into a 96-well microplate. The absorbance of the resulting purple solution was measured at a wavelength of 570 nm using a multwell spectrophotometer (Molecular Devices, Sunnyvale, CA). The experiments were performed three times in triplicates, and the results are expressed as percentage of MTT reduction in the control cells.
Olympus BX60 fluorescence microscope. Condensed nuclei were identified by the smaller size, irregular shape, and higher intensity of chromatin staining with Hoechst 33342. Cells were counted scoring at least 300 cells in five microscopic fields randomly selected on each coverslip. The experiments were performed three times in triplicates.

**ATP determination.** ATP levels were determined according to the instructions for the ATP Bioluminescence Assay Kit CLS II (Boehringer Mannheim, Mannheim, Germany). Briefly, one million cells were collected and centrifuged at 1000 x g for 5 min. The pellet was subsequently resuspended in boiling buffer (100mM Tris, 4mM ethylenediaminetetraacetic acid [EDTA], pH 7.75) and further boiled for 2 min. The suspension was then centrifuged at 10 000 x g for 60 s. ATP was measured in the supernatant by adding an equal volume of luciferase reagent. Sample ATP levels were rectified against a standard curve. The experiments were performed two times in triplicates.

**Measurements of mitochondrial Ca2+ uptake rate.** For measurement of the mitochondrial sequestering rate of calcium, 106 cells were collected, washed with PBS, and suspended in 400 µl of buffer (150mM KCl, 5mM KH2PO4, 5mM succinate, 1mM MgSO4, 5mM Tris, pH 7.4). Cells were permeabilized with 0.05% digitonin, and 2 µM rotenone was added in order to maintain pyridine nucleotides in a reduced form. Mitochondrial calcium uptake was induced by sequential additions of calcium to the cells. Calcium concentration changes were registered using a calcium-sensitive electrode (Thermo Orion, Beverly, MA) and visualized with a chart recorder. The experiments were performed three times.

**Measurement of caspase activity.** To evaluate caspase activity, we assessed the cleavage of Ac-Asp-Glu-Val-Asp-(7-Amino-4-methylcoumarin) (DEVD-AMC) and Ac-Ile-Glu-Thr-Asp-(AMC) (IETD-AMC) (both from Peptide Institute) using a fluorometric assay (previously described in Gorman et al., 1999). Cells were pelleted and washed once with ice-cold PBS. Cells were resuspended in 25 µl of PBS, added to a microtiter plate, and mixed with substrates dissolved in a standard reaction buffer (100mM Hepes, pH 7.25, 10% sucrose, 10mM dithiothreitol [DTT], 0.1% 3(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate). Caspase activity and cleavage of the fluorogenic peptide substrates was monitored by AMC discharge with a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using 355-nm excitation and 460-nm emission wavelengths. Fluorescent units were converted to pmoles of AMC released using a standard curve generated with free AMC and subsequently related to amount of protein in each sample. The experiments were performed three times in triplicates.

**Immunocytochemistry.** Cells were fixed with 4% paraformaldehyde (Sigma) for 60 min and then washed with PBS. Primary antibodies were diluted in PBS with 0.3% Triton-X100 and 0.5% bovine serum albumin (Boehringer Mannheim, Bromma, Sweden). The following primary antibodies were used: mouse anti-cytochrome c (1:100) (BD Biosciences, Stockholm, Sweden), rabbit anti-Bax (1:400) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and rabbit anti-actin (1:1000) (Chemicon, Temecula, CA), mouse anti-poly (ADP-ribose) polymerase (PARP) (1:1000) (Santa Cruz Biotechnology), mouse-anti cytochrome c (1:2500) (BD Biosciences), and rabbit anti-GAPDH (1:1000) (Nordic Biosite, Taby, Sweden). The membranes were rinsed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:10 000, Pierce) for 2 h at room temperature. Following the secondary antibody incubation, the membranes were rinsed and developed with enhanced chemiluminescence reagent (Amersham, Little Chalfont, Bucks, UK) and exposed to X-ray autoradiography films (Fuji, Japan). All experiments were repeated at least three times.

**Visualization of ROS in live cells.** The Image-iT live green ROS detection system (Molecular Probes) was used to visualize ROS in live C17.2 cells and cNSCs exposed for 18 h to 250µM MnCl2, a dose inducing apoptosis in 20–30% of the cells after 24 h. This new assay approach is based on (and-(6)-carboxy-2,7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) (Armeni et al., 2004; Minami et al., 2005). The nonfluorescent carboxy-H2DCFDA permeates live cells and is deacylated by nonspecific intracellular esterases. In the presence of ROS, the reduced fluorescein compound is oxidized and emits bright green. Fluorescence microscopy (Axiovert 200M; Zeiss, Oberkochen, Germany) was performed to capture images of nuclei (blue fluorescence; Hoechst 33342) and oxidized fluorescein. Cells were examined using a Zeiss LSM 510 Meta confocal microscope (Zeiss).

**Statistical analysis.** Data were presented as mean ± SEM. Statistical analyses were carried out with the one-way ANOVA and Fisher’s protected least significant difference test.

**RESULTS**

**Neural Stem Cells Are Highly Susceptible to Manganese Toxicity**

To investigate the toxic effects of manganese on NSCs, we began by comparing the NSC line C17.2 with the hippocampal neuronal cell line HT22 and the astrocytoma cell line D384, to see whether NSCs are more sensitive to Mn exposure than more differentiated cells. All three cell lines were exposed to MnCl2 (50–250µM) for 24 h. C17.2 cells exposed to 100 or 250µM MnCl2 for 24 h exhibited clear morphological changes, becoming rounded, shrunken, and more loosely attached to the cell culture dish surface (Figs. 1A–C) in contrast to the HT22 (Figs. 1D–F) and D384 cell lines (Figs. 1G–I) that showed morphological alterations only in scattered cells. The MTT assay showed a significant dose-dependent decrease in cell viability only in the C17.2 cells exposed to MnCl2 (Fig. 1J). In the HT22 cell line, a significant toxic effect was detected when
cells where exposed to doses ranging from 0.5 to 1mM, while the D384 cells were not affected (data not shown). The low amount of Trypan blue positively stained cells (Fig. 1K) indicates that the decrease in C17.2 cell viability detected by the MTT assay is not caused by necrotic cell demise. Overall, C17.2 cells seem to be more sensitive to MnCl₂ than more differentiated neuronal and glial cell lines.

**Manganese Induces Apoptosis in NSCs via a Caspase-Dependent Intrinsic Mitochondrial Pathway**

To examine nuclear morphology, C17.2 cells exposed to 100 or 250µM MnCl₂ for different periods of time (12–48 h) were stained with Hoechst 33342 and PI. Increasing amounts of cells with nuclear condensation and intact cell membrane, considered morphological hallmarks of apoptosis, could be detected in a time- and dose-dependent manner (Fig. 2). Conversely, after 48-h exposures to MnCl₂, the Hoechst 33342/PI vital staining showed a significant amount of necrosis, in agreement with the Trypan blue exclusion experiments (data not shown).

While apoptosis is an energy-dependent process, a feature of necrosis is ATP depletion. Mitochondrial ATP synthesis is mainly driven by membrane potential generated by electron transfer via the respiratory chain and the subsequent pumping of protons from the mitochondrial matrix. Sequestering of cytosolic Ca²⁺ is an important mitochondrial function, which is also supported by the membrane potential. We examined mitochondrial function in C17.2 cells exposed to MnCl₂ for 24 h and found no significant decrease in ATP levels (Table 1). In addition, no effect in the rate of mitochondrial Ca²⁺ uptake could be detected (Table 1). Overall, these data further support

![FIG. 1.](image-url) (A–I) Light phase-contrast images of C17.2 (A–C), HT22 (D–F), and D384 cells (G–I) after exposure to MnCl₂ for 24 h. As shown in B and C, a significant number of apoptotic cells was observed only in MnCl₂-exposed C17.2 cells, whereas only scattered apoptotic cells are detected in HT22 (F) and D384 (I) exposed to the highest dose (250µM). (J) The cell viability (MTT) assay shows a significant dose-dependent response in C17.2 cells but not in HT22 and D384 after 24 h of MnCl₂ exposure. (K) The Trypan blue exclusion test shows that within the dose range tested, MnCl₂ exposure for 24 h does not alter the cell membrane permeability in any of cell lines. Values are mean ± SD (n = 3). *p < 0.05 (ANOVA, Fisher’s protected least significant difference test).

![FIG. 2.](image-url) Quantification of C17.2 cell exhibiting nuclear condensation (apoptotic cells) after exposure to MnCl₂ (100 and 250µM) for 12, 24, and 48 h. Cells were stained with Hoechst 33342 and PI vital stainings. Values are mean ± SD (n = 3). p(*) < 0.05 (ANOVA, Fisher’s protected least significant difference test).
Manganese Induces ROS Formation in NSCs

We assessed ROS production in cNSCs by using a 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate–based assay, which allows ROS visual detection in live cells. A considerable increase in ROS formation could be observed following MnCl₂ exposure as compared to unexposed control cells (Figs. 7A and 7B). The dot-like pattern of the detected ROS suggested a mitochondrial localization. Notably, pretreatment with the antioxidant MnTBAP (25 μM) did protect cNSCs (data not shown) as well as C17.2 cells (Fig. 7C) from MnCl₂-induced toxicity.

DISCUSSION

We have used the murine NSC line C17.2 and primary cultures of NSCs from rat embryos as in vitro neurodevelopmental models to investigate the cytotoxic effects of Mn, a naturally occurring environmental element that is both an essential trace mineral and a potent neurotoxin. Our results show that NSCs are highly sensitive to Mn and that cells undergo apoptotic cell death via activation of the mitochondrial caspase–mediated pathway. In addition, we provide evidence for the occurrence of oxidative stress in Mn-exposed NSCs, which may play a critical role in the onset of NSC death.

The neurotoxicity of excess exposure to Mn is well documented in adults, mostly in exposed workers. Exposure to low levels of Mn may induce motor, cognitive, and psychological alterations, whereas high exposure has been associated to severe neurotoxic effects (manganism) characterized by an extrapyramidal syndrome similar to Parkinson’s disease (PD) (Dobson et al., 2004; Levy and Nassetta, 2003). However, the parkinsonism induced by Mn differs from PD...
because the damage occurs mostly in the pallidum and striatum rather than in the substantia nigra (Olanow, 2004). Several studies have suggested that Mn could have harmful effects on the developing nervous system because learning disabilities and hyperactive behavior has been reported in children with high hair Mn (Bouchard et al., 2007; Collipp et al., 1983; Pihl and Parkes, 1977). The association between Mn and hyperactivity is conceivable because the dopaminergic and the γ-aminobutyric acid circuitries, which are involved in children hyperactivity (Sagvolden et al., 2005), are affected by Mn (Fitsanakis et al., 2006). In addition, a magnetic resonance imaging study performed on children affected by attention-deficit hyperactivity disorder syndrome has shown a significant decrease in gray matter in a few brain regions including the pallidum (Overmeyer et al., 2001), that is a specific target for Mn accumulation (Uchino et al., 2007). High levels of Mn in drinking water (Bouchard et al., 2007) and infant formula with high Mn content (Collipp et al., 1983) have been considered as possible sources of exposure. While the Mn concentration in human breast milk is about 6 μg Mn/l, in infant soy-based formula it may be up to 300 μg Mn/l (Dorner et al., 1989; Lonnerdal, 1994). These factors, in light of the toxic actions of Mn on the adult brain and the higher susceptibility to neurotoxic agents of the developing nervous system justify the increasing concern about neurodevelopmental effects of Mn.

Our present data show that C17.2 NSCs are more vulnerable to Mn toxicity as compared to other in vitro neural models, such as neuronal and glial cell lines. Interestingly, the C17.2 cell line and rat embryonic cNSCs are almost equally susceptible to Mn in contrast to what was observed with methylmercury that is 10-folds more toxic in primary NSCs (Tamm et al., 2006). The different culture conditions characterized by the presence or absence of serum in the medium of the C17.2 and cNSCs, respectively, do not seem to

FIG. 3. (A) Positive staining for oligomerized Bax, shown with Hoechst 33342–stained nuclei, is detected in C17.2 cells after exposure to 250 μM MnCl2 for 24 h, but not in control cells. (B) Compared to the mitochondrial dot-like pattern of cytochrome c (Cyt c) immunoreactivity in the control, the diffuse cytosolic staining observed in C17.2 cells exposed to MnCl2 for 24 h implies release of cytochrome c into the cytosol. Scale bar 20 μM. (C) Western blotting of cytosolic and membrane-enclosed organelles (pellet) further illustrate the release of mitochondrial cytochrome c in C17.2 cells exposed to MnCl2 (50–250 μM) for 24 h. (D) Western blot analysis also detects cleavage of PARP (85 kD) in C17.2 cells exposed to MnCl2 for 24 h. (E) Exposure to MnCl2 (100–250 μM) for 24 h induces caspase-3-like activity in C17.2 cells, as measured by DEVD-AMC cleavage. Values are mean ± SD (n = 3), p(*) and p(#) < 0.05 (ANOVA, Fisher’s protected least significant difference [PLSD] test). (F) Pretreatment with the pan-caspase inhibitor zVAD-fmk (20 μM) induces a significant increase in cell viability of C17.2 cells exposed to 250 μM MnCl2 for 24 h, as detected by the MTT assay. Values are mean ± SD (n = 3), p(*) and p(#) < 0.05 (ANOVA, Fisher’s PLSD test).
influence the degree of cell susceptibility to Mn. Conversely, the presence of transferrin in both culture media may be critical considering the proposed role of the transferrin/Mn complex in Mn internalization (Aschner and Gannon, 1994; Davidsson et al., 1989; Suarez and Eriksson, 1993).

The decreased cell viability of NSCs exposed to Mn (100–250 μM) appears to be related to the occurrence of apoptotic cell death. Apoptosis is an ATP-dependent process that takes place when well-defined biochemical pathways are activated (Zimmermann et al., 2001). The release of mitochondrial proteins, such as cytochrome c, initiates the cascade of intracellular events that characterize the intrinsic pathway (e.g., Bratton et al., 2000; Liu et al., 1996). Cytochrome c interacts with the apoptotic protease–activating factor-1 in the cytosol, leading to the activation of procaspase-9 that in turn cleaves and activates procaspase-3 (Bratton et al., 2000).

Another pathway, receptor mediated, involves the binding of members of the death receptor family (e.g., Fas [Apo1/CD95], Tumor Necrosis Factor Receptor [TNFR] Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor [TRAIL-R] and their cognate ligands (Nagata, 1997). Exposure to Mn has been reported to lead to disruption of mitochondrial function (Gavin et al., 1992, 1999). However, we did not detect any significant change in ATP levels or in mitochondrial Ca²⁺ uptake in Mn-exposed NSCs, which indicate that in our models mitochondria are functional and can provide the energy required for the apoptotic process. NSCs exposed to Mn undergo apoptosis via activation of the mitochondrial pathway as shown by the presence of oligomerized Bax, cytochrome c release, activation of the effector caspase-3, and subsequent cleavage of PARP. In addition, preincubation with the pan-caspases inhibitor zVAD-fmk protects NSCs from Mn toxicity, as shown by the significant increase in cell viability. The occurrence of caspase-3–dependent apoptosis after exposure to Mn has been described also in PC12 cells (Hirata, 2002). Interestingly, overexpression of Bcl-2 can prevent Mn-induced apoptosis in human B cells (Schrantz et al., 1999). Our results showing Bax oligomerization in NSCs after Mn exposure provide further evidence for the involvement of the Bcl-2 protein family in Mn-induced apoptotic cell death.

It was recently shown that p38 MAPK signaling is implicated in Mn-induced apoptosis in different cell types. In HeLa and NIH3T3 cell lines, Mn induces apoptosis via a p38 MAPK and caspase-12–dependent pathway, independent of the mitochondria, while in human Burkitt lymphoma B cells Mn exposure induces a p38 MAPK–dependent activation of Mitogen- and stress-activated protein kinase 1 with subsequent Fas–associated death domain protein–independent activation of caspase-8 (El McHichi et al., 2007; Oubrahim et al., 2001, 2002). In the latter study, it was reported that caspase-8, via the cleavage of Bid, induced the mitochondrial-mediated apoptotic pathway with the release of cytochrome c and caspase-3.
activation (El McHichi et al., 2007). We did not detect activation of caspase-8 in the C17.2 cell line after exposure to MnCl₂, and in agreement, pretreatment with the specific p38 MAPK inhibitor SB203580 or the caspase-8–selective inhibitor zIETD-fmk did not exert any protective effects.

The formation of ROS derived from mitochondrial damage has previously been suggested in Mn-induced apoptosis (Kitazawa et al., 2002). In fact, at cellular level, Mn accumulates in mitochondria, disrupts oxidative phosphorylation, and increases the production of ROS with subsequent lipid peroxidation (Gunter et al., 2006). Oxidative stress seems to play a role also in our in vitro models, as shown by the protective effects that antioxidants have on NSCs exposed to Mn as well as by the presence of ROS in Mn-treated cells.

In conclusion, NSCs are highly vulnerable to the toxic effects of Mn, and the C17.2 NSC line appears to be more susceptible as compared to neuronal or glial cell lines. Cell death associated to oxidative stress and mitochondrial-mediated caspase activation is the major cause of the decrease in cell viability detected after Mn exposure. However, although the protection by the caspase inhibitor or the antioxidant was significant, none of the pretreatments could totally prevent the harmful effects of Mn. Thus, the mechanisms of Mn NSCs toxicity may engage other cellular pathways not identified yet. Forthcoming studies will attempt to fully elucidate this issue. The final goal is to identify mechanism-based end points that could be used in vivo to shed light on the impact of exposure to Mn during development and clarify whether the adult brain may be vulnerable to long-lasting effects from developmental Mn exposure. Exposure to neurotoxic agents affecting NSCs can obviously result in an unfavorable outcome for the development of the nervous system. Furthermore, it should be kept in mind that NSCs are also present in the adult brain where their capability for self-renewal may be important for...

FIG. 6. (A) Bax oligomerization and nuclear condensation as detected by Hoechst 33342 staining is detected in cNSCs after exposure to 250μM MnCl₂ for 24 h. (B) Compared to the mitochondrial network-like pattern of cytochrome c (Cyt c) immunoreactivity in control cells, a diffuse cytosolic staining was observed after exposure to 250μM MnCl₂ for 24 h suggesting release of cytochrome c into the cytosol. (C) The exposed cells also exhibit activated caspase-3. Scale bar 10μM. (D) Western blot analysis of cytosolic and membrane-enclosed organelles (pellet) further points up the release of cytochrome c from the mitochondria in cNSCs exposed to MnCl₂ (100–250μM) for 24 h. (E) Western blot analysis shows the caspase-specific cleavage product of PARP (85 kD) in cNSCs exposed to MnCl₂ for 24 h.
brain repair and normal functions, including learning, memory, and emotional responses (Santarelli et al., 2003; Schaffer and Gage, 2004). Thus, NSCs damage may have critical consequences even in adults.

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