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Mitochondrial-dependent manganese neurotoxicity in rat primary astrocyte cultures

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Abstract

Chronic exposure to excessive levels of Mn results in a movement disorder termed manganism, which resembles Parkinson's disease (PD). The pathogenic mechanisms underlying this disorder are not fully understood. Several lines of evidence implicate astrocytes as an early target of Mn neurotoxicity. In the present study, we investigated the effects of Mn on mitochondrial function. Primary astrocyte cultures were prepared from cerebral cortices of one-day-old Sprague–Dawley rats. We have examined the cellular toxicity of Mn and its effects on the phosphorylation of extracellular signal-regulated kinase (ERK) and activation of the precursor protein of caspase-3. The potentiometric dye, tetramethylrhodamine ethyl ester (TMRE), was used to assess the effect of Mn on astrocytic mitochondrial inner membrane potential ($\Delta\Psi_m$). Our studies show that, in a concentration-dependent manner, Mn induces significant ($p < 0.05$) activation of astrocyte caspase-3 and phosphorylated extracellular signal-regulated kinase (p-ERK). Mn treatment (1 and 6 hrs) also significantly ($p < 0.01$) dissipates the $\Delta\Psi_m$ in astrocytes as evidenced by a decrease in mitochondrial TMRE fluorescence. These results suggest that activations of astrocytic caspase-3 and ERK are involved in Mn-induced neurotoxicity *via* mitochondrial-dependent pathways.

Keywords

Astrocytes; ERK signaling pathway; Manganese; Manganese neurotoxicity; Mitochondria; Oxidative stress

1. Introduction

Mn is required for normal growth, development and cellular homeostasis, and it is an essential metal for the function of multiple enzymes, such as Mn-superoxide dismutase (Mn-SOD), pyruvate carboxylase, arginase and glutamine synthase (GS). Mn can also substitute for magnesium in many enzymatic reactions catalyzed by kinases (Takeda and Avila, 1986; Baly et al., 1985; Brock and Walker, 1980). Although an essential element, exposure to high levels of Mn in occupational or environmental settings and therapeutic or disease conditions (hepatic

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encephalopathy) (Krieger et al., 1995; Spahr et al., 1996; Layrargues et al., 1998) lead to excessive Mn accumulation in the basal ganglia (Dorman et al., 2006), resulting in decreased dopamine (DA) levels and cell death in a syndrome commonly referred to as manganism (Barbeau, 1984; Sliot et al., 1994). *In vivo*, intrastriatal Mn injections result in the loss of dopaminergic neurons, resembling toxicity caused by mitochondrial poisons, such as aminooxyacetic acid and 1-methyl-4-phenyl-pyridinium (MPP⁺) (Brouillet EP et al., 1993).

Excess brain Mn represents a risk factor for idiopathic Parkinson's disease (IPD) (Gorell et al., 1999a; 1999b; Kim et al., 1999; Racette et al., 2001). The strongest correlation between any type of environmental exposure and increased susceptibility to PD is observed in Mn-exposed populations (Gorell et al., 1999a; 1999b; Hudnell, 1999). Occupational exposure to Mn for >20 years or combined long-term exposures to Mn and Al (>30 years) are associated with the increased prevalence of PD (Gorell et al., 1999a; Normandin et al., 2002). Mn toxicity results in symptoms similar to those seen in patients with PD, including rigidity, tremors, dystonia and bradykinesia (Calne et al., 1994). Recent studies suggest that the prevalence of Parkinsonism is higher among welders vs. age-standardized individuals in the general population (Racette et al., 2001; 2005a). Finally, positron emission tomography (PET) in a welder with Parkinsonism confirms that the clinical features of Mn-induced Parkinsonism overlap with those of PD, characterized by prolonged L-dopa responsiveness and reduced [¹⁸F]fluorodopa uptake (Racette et al., 2005b).

Astrocytes make up approximately 50% of the human brain volume (Chen and Swanson 2003) and serve many critical pathophysiological roles essential for normal neuronal activity, including glutamate uptake (80% of synaptic glutamate), glutamine release, K⁺ and H⁺ buffering and volume regulation and membrane-membrane mediated trophic cell signaling (Aschner and Gannon 1994; Kimelberg and Aschner 1998; Aschner et al., 1999; Perea and Araque 2006; Volterra and Steinhauser 2004). Unlike neurons, astrocytes have the ability to concentrate Mn to levels at least 50-fold higher than the culture media (Aschner et al., 1992); this provides a mechanism by which Mn concentrations in astrocytic cytosol could attain the range required for maximum activity of GS, an enzyme found predominantly in astrocytes.

Mitochondria are intracellular target for Mn toxicity (Gunter et al., 2006; Milatovic et al., 2007; Rama Rao et al., 2007). Intracellular Mn²⁺ is sequestered by mitochondria *via* the Ca²⁺ uniporter (Mela and Chance, 1968; Gunter et al., 1975; Gavin et al., 1999). Mn²⁺ can be oxidized to the strong pro-oxidizing agent, Mn³⁺, followed by oxidation of important cell components by Mn³⁺ (Archibald and Tyree, 1987; Donaldson, 1987); these reactions take place within the mitochondrial matrix. The formation and accumulation of a redox-active Mn³⁺ complex in brain mitochondria from oxidation of Mn²⁺ result in Mn toxicity (Gunter et al., 2006).

In many apoptotic processes, mitochondria play an important role in coordinating caspase activation by releasing proapoptotic factors, such as cytochrome c, which open the permeability transition pore of mitochondria, an irreversible step toward apoptosis. Little is known about the effect of Mn on ERK phosphorylation, particularly in astrocytes, a preferential site for Mn accumulation (Aschner et al., 1992; Wedler et al., 1989; Zwingmann et al., 2003). Extracellular signal-regulated kinases (ERK1/2), one of the members of mitogen-activated protein kinase (MAPK), respond to several extracellular stimuli and are activated by MAPK/ERK kinase1/2 (MEK1/2) by phosphorylating threonine and tyrosine residues (Seeger and Krebs 1995). It is known that oxidative stress activates MAPK cascades (Herrlich and Böhmer, 1999; Allen and Tresini 2000). ERK activation can contribute to neurotoxicity, particularly in the context of oxidative insults (Chu et al., 2004). ERK1/2 stimulation by ROS has been described in neurons (Samanta et al., 1998) and neuroprotection by MEK inhibition against oxidative stress in both neurons and in astrocytes (Satoh et al., 2000; Rosenberger et al., 2001). Although numerous

reports have implicated ERK in neuronal cell survival (Xia et al., 1995; Yujiri et al., 1998; de Bernardo et al., 2003), it has been proposed that the precise pattern of ERK1/2 activation ultimately determines whether the kinase participates in cell death-promoting or cell survival pathway (Stanciu et al., 2000; Chang and Karin 2001; Stanciu and DeFranco 2002; Chu et al., 2004). Moreover, the effect of the ERK pathway on cellular vulnerability to Mn-mediated oxidative stress has yet to be fully investigated. In this study, we tested the hypothesis that the cytotoxicity of Mn in astrocytes is mediated *via* mitochondrial control of cell death.

2. Results

2.1. Effects of manganese on cell viability

Mn-induced cytotoxicity was evaluated using the MTT and LDH assays upon treatments with Mn (100, 500, and 1000 μ M). Mn caused a concentration-dependent increase ($p < 0.05$) in LDH released from astrocytes into the media (Fig. 1 A and B). The effect of Mn was maintained for at least 24 hours (Fig. 1 C and D). In a concentration-dependent manner, Mn also led to a significant reduction in cell viability as assessed by the MTT assay ($p < 0.05$) (Fig. 2 A and B), with a maximal effect occurring between 2 to 6 h of exposure (Fig. 2 C and D).

2.2. Involvement of ERK activation in the Mn-induced cell death

We evaluated the possible involvement of the MAPK signaling pathway in Mn-induced cell death. The activation of the MAPK subfamily was studied by measuring the phosphorylation levels of extracellular signal-regulated kinase (p-ERK). Mn exposure caused a robust activation of ERK ($p < 0.05$) in a concentration-dependent manner (Fig. 3 A and B). This activation was apparent as early as 30 min after exposure and peaked at 2 h (500 μ M) and 6 h (100 μ M) after exposure (Fig. 3 C and D). The phosphorylation of ERK is consistent with the cell viability patterns assessed by MTT (Fig 2 C and D).

2.3. Caspase-3 cleavage in Mn-induced cell death

To test if Mn exposure in astrocytes leads to apoptosis, we investigated the activation of caspase-3. The results demonstrated that the precursor of caspase-3 was cleaved by Mn treatment in a concentration-dependent manner (Fig 4 A and B). This effect was statistically significant ($p < 0.05$), with peak cleavage of caspase-3 occurring between 2 and 6 h after exposure to Mn (Fig 4 C and D).

2.4. Alteration of mitochondrial membrane potential ($\Delta\Psi_m$) in Mn- induced cell death

To determine whether mitochondrial dysfunction contributes to Mn-induced neurotoxicity, we treated astrocytes for 1 h with Mn (100, 500 μ M or 1 mM) and measured the $\Delta\Psi_m$ as a function of Mn exposure, which resulted in dissipation of the $\Delta\Psi_m$ in cultured astrocytes, as illustrated by decreased mitochondrial TMRE fluorescence (Fig. 5 A). Quantification of TMRE fluorescent intensities revealed that all investigated Mn concentrations induced significant dissipation of the $\Delta\Psi_m$ ($p < 0.05$) (Fig. 5 B).

3. Discussion

The present study, for the first time, demonstrates that exposure of primary cultured astrocytes to Mn triggers apoptosis and cell death via the ERK signaling pathway and the activation of caspase-3. Activation of the ERK signaling pathway and the collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$) represent early events in Mn-induced neurotoxicity and likely are responsible for further deterioration in cellular homeostasis, such as the generation of reactive oxygen species, ultimately associated with cell death. These events occur in astrocytes, and it has yet to be determined whether the inability of astrocytes to maintain control over the

extracellular fluid is associated with neuronal demise, or whether events analogous to those described here also occur within neurons, possibly having even more pronounced effects on the neurons, as they are known to have significantly lower redox potential compared with the astrocytes.

Mn is known to cause mitochondrial dysfunction (Gavin et al., 1990), including the inhibition of the enzymes of the tricarboxylic acid (TCA) cycle (Malthankar et al., 2004; Zheng et al., 1998) and a reduction in the activities of the electron transport chain (Malecki, 2001; Rama Rao and Norenberg, 2004), ultimately resulting in ATP depletion (Brouillet et al., 1993; Verity, 1999). Some of these mitochondrial events are significantly blocked by antioxidants (Chen and Liao, 2002), suggesting the involvement of oxidative stress in the mechanism of Mn-induced mitochondrial dysfunction. Mitochondrial Mn-SOD uses Mn for scavenging and detoxifying free radicals. Furthermore, Mn-SOD is critical in preventing or limiting apoptosis and necrosis resulting from cellular damage caused by reactive oxygen species (ROS) (Raha and Robinson 2000; Halliwell 2001; Kitazawa et al., 2002; Milatovic et al., 2007). Changes in Mn-containing proteins have been observed in many neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis and Parkinsonian-like syndrome (Frankel et al., 2000; Malecki 2001; Recette et al., 2001; Zelko et al., 2002; Olanow 2004; Petrozzi et al., 2007).

In our investigation of the link between Mn toxicity and intracellular molecular events leading to apoptosis or cell death, we focused on the phosphorylation of ERK and the activation of caspase-3 precursor protein in primary cultured astrocytes. Our results show that Mn can activate the phosphorylation of ERK in a concentration-dependent manner (Fig 3A, B), and that this activation is sustained for at least 24 hr, with peak activation occurring between 2 and 6 h (Fig 3C, D). Extracellular stimuli are communicated to the cell interior by cascades of kinase reactions. Although signal transduction pathways are often activated by ligand-dependent events, a growing body of evidence suggests that these pathways may also be directly activated by oxidants (Herrlich and Böhmer, 1999; Allen and Tresini, 2000). This phenomenon has been most fully characterized for the extracellular signal-regulated kinase (ERK) pathway. ERK is integrally involved in regulating pivotal processes including proliferation, differentiation, adaptation, survival and even cell death. The phosphorylation of ERK in the induction of apoptosis has also been reported in HeLa cells, neuroblastoma cells, and human glioma cells (Woessmann et al., 2002; Choi et al., 2004). ERK can modulate mitochondrial functions, particularly those associated with cell death, and can promote oxidative neuronal injuries (Chu et al., 2004; Kulich and Chu, 2001; 2003; Gomez-Santos et al., 2002; Kuperstein and Yavin, 2002). Also, activation of ERK is involved in the induction of apoptosis of brain cortical astrocytes (Blazquez et al., 2000; Oh et al., 2006). *In vivo*, brain ERK activation was observed after ischemia and hypoglycemia (Hu and Wieloch, 1994; Kurihara et al., 1994); MEK inhibitors decreased lesion volume and improved behavioral outcome (Alessandrini et al., 1999).

Caspases are a family of cysteine proteases that mediate cell death and are critical regulators of apoptosis. Caspase-3 plays a central role in mediating apoptosis, chromatin condensation and DNA fragmentation (Riedl and Shi, 2004). Therefore, caspase-3 is considered one of the major executioners of apoptosis and has classically been viewed as a terminal event in the process of programmed cell death. Accordingly, caspase-3 activation has been described in neuronal cells following specific types of central nervous system (CNS) insults, including traumatic brain injury and ischemic/excitotoxic damage (Beer et al., 2000; Brecht et al., 2001; Manabat et al., 2003; Nath et al., 2000). In each of these conditions, the participation of executioner caspase activation and subsequent apoptosis in the neuronal cell death process has been established both in the adult and postnatal brain. Similarly, *in vitro* studies have also suggested that caspase-3 proteolytic activity plays a crucial role in excitotoxin-induced

neuronal apoptosis (Allen et al., 1999; Du et al., 1997; Tenneti and Lipton, 2000). In addition, expression of caspase-3 has been described in oligodendrocytes (Beer et al., 2000; Nottingham and Springer, 2003) and astrocytes following CNS damage (Beer et al., 2000; Benjelloun et al., 2003; Mouser et al., 2006; Su et al., 2000). The present study demonstrates that following Mn exposure, activation of the caspase-3 precursor protein is observed in cultured astrocytes. Caspase-3 is significantly activated by Mn after exposure for 30 min, with peak activation occurring between 2 and 6 hours (Fig 4).

In this study, we also evaluated the toxic effects of Mn by measuring the release of LDH from damaged cells to culture media as well as the reduction of MTT. The results reveal that Mn exposure induces a concentration-dependent increase of LDH release and the reduction of MTT (Figs 1A, B and 2A, B). Astrocytes exposed to the high concentration of 1000 μ M of Mn for 30 min or 2 hr demonstrated two- to three-fold increases in LDH release and an approximate 30% decrease in MTT compared to the control. The effects of the Mn-induced release of LDH and reduction of MTT can be sustained for 24 hr, with the peak occurring between 2 and 6 hr. Our previous study demonstrated that Mn-induced oxidative stress leads to mitochondrial dysfunction in astrocytes (Milatovic et al., 2007). Other authors have also reported that Mn exerts oxidative stress and increases free radical production in experimental models of Mn neurotoxicity (Brenneman et al., 1999; HaMai and Bondy 2004). Mn also causes mitochondria-dependent cell death in human B cells, including lymphoma B cell lines (El McHichi et al., 2007; Schrantz et al., 1999). In an *in vivo* study, researchers reported that weekly injections of Mn over a 3-month period in the striatum and globus pallidus of monkeys (0, 2.25, 4.5 and 9 gm), produce dose-related clinical signs, which are more severe in the higher dose ranges. At the highest dose, the Mn concentration is increased 12-fold in the striatum and 9-fold in the globus pallidus (Suzuki et al., 1975).

Another consequence of increased oxidative stress and mitochondrial energy failure is the induction of the mitochondrial permeability transition (MPT), a Ca^{2+} -dependent process characterized by the opening of the permeability transition pore in the inner mitochondrial membrane. This process results in increased permeability to protons, ions and other solutes (Zoratti and Szabo, 1995), which, consequently leads to a collapse of the mitochondrial inner membrane potential ($\Delta\Psi$ m). Loss of the $\Delta\Psi$ m results in the colloid osmotic swelling of the mitochondrial matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis and further generation of ROS. Our experiments demonstrate a concentration-dependent effect of Mn on the $\Delta\Psi$ m in cultured astrocytes (Fig 5). Studies by Zhang et al. (2004) have shown that high levels of MnCl_2 (1 mM) cause a significant dissipation of the $\Delta\Psi$ m in isolated rat brain mitochondria, consistent with induction of the MPT. Our results are also in agreement with an earlier study by Rama Rao and Norenberg (2004), which found that Mn causes a dissipation of the $\Delta\Psi$ m in a concentration- and time-dependent manner in astrocytes. The mechanism(s) by which Mn induces the MPT in astrocytes is not completely understood; however, increased production of ROS and associated oxidative stress are generally considered major contributing factors (Castilho et al., 1995; Rama Rao and Norenberg, 2004; Milatovic et al., 2007).

In summary, our studies demonstrate that the mechanisms of Mn-induced neurotoxicity are associated with the phosphorylation of ERK pathway, which consequently triggers the cleavage of caspase-3, thereby inducing cell death. The activation of ERK might be associated with Mn-induced collapse of the mitochondrial membrane potential and increase of ROS generation. We propose that oxidative stress generated through mitochondrial perturbation plays a key role in Mn-induced astrocytic dysfunction. Further studies to determine whether antioxidants can block or attenuate Mn toxicity by preserving mitochondrial membrane potential integrity and/or inhibition of phosphorylation of ERK and/or cleavage of caspase-3 should prove a profitable area of future investigations.

4. Materials and Methods

4.1. Materials

Manganese chloride (MnCl_2), LDH and MTT assay kits were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium (MEM) with Earle's salts, heat-inactivated horse serum, penicillin, streptomycin and tetramethyl rhodamine ethyl ester (TMRE) were purchased from Invitrogen (Carlsbad, CA). Polyclonal anti-ERK was purchased from Stressgen Bioreagents (Ann Arbor, MI). Monoclonal anti-pERK and polyclonal anti-Caspase-3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-beta-actin antibody (clone AC15) was purchased from Sigma Chemical Co. (St. Louis, MO).

4.2. Primary astrocyte culture

Astrocytic cultures from cerebral cortices of newborn (1-day-old) Sprague–Dawley rats were established as previously described (Aschner et al., 1994). Briefly, rat pups were decapitated, and the cerebral cortices were removed. After removal of the meninges, the cerebral cortices were digested with bacterial neutral protease (dispase, Invitrogen), and the astrocytes were recovered by repeated removal of dissociated cells from brain tissues. Twenty-four hours after the initial plating in 6- and 12-well plates, the media were changed to preserve the adhering astrocytes and to remove neurons and oligodendrocytes. The cultures were maintained at 37°C in a 95% air/5% CO_2 incubator for 3–4 weeks in minimal essential medium (MEM) with Earle's salts supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The media were changed twice per week. The surface-adhering monolayer cultures were >95% positive for the astrocytic marker, glial fibrillary acidic protein (GFAP).

4.3. Cell viability assay

Cell viability was assessed by the measurement of formazan production after the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO). The number of surviving cells after treatment was determined by measuring the optical density (OD) of the dissolved formazan product at A_{490} nm after the addition of MTT for 1 h according to the manufacturer's instructions. Untreated negative controls were run together with the treated cells, and plates with reagent only served as background controls. After background OD subtraction, the results were expressed as a percentage of the average negative control. Each experiment was performed in triplicate plates and repeated three times in cultured astrocytes.

4.4. Cytotoxicity assessment

Cytotoxicity, as indicated by cell membrane integrity, was assessed by measuring the activity of lactate dehydrogenase (LDH) in the culture media by colorimetric detection of formazan, using a LDH diagnostic kit (Promega, Madison, WI, USA). After the experiments, the supernatant was transferred to a 96-well plate and incubated with the reaction mixture for 30 min at room temperature to develop color. The optical density was measured at 492 nm using a spectrophotometer (MRX, Dynatech Laboratories). LDH leakage was expressed as percentage of LDH in the medium over control. Each experiment was performed in triplicate and repeated three times in different groups of astrocytic cultures.

4.5. Western blotting and antibodies

The cells were exposed to Mn (100, 500 μM and 1 mM) for differing time periods. The cells were lysed with lysis buffer [Tris-HCl, pH 7.4, 20 mM, EDTA 2.5 mM, Triton X-100 1%, Sodium Deoxycholate 1%, SDS 0.1%, NaCl 100 mM, PMSF 1.0 mM, Leupeptin 10 $\mu\text{g}/\text{ml}$,

Pepstatin 10 $\mu\text{g/ml}$] and collected for protein concentration determination by BCA assay (Pierce, Rockford, IL). An equal amount of protein (30 μg) was loaded and run on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (PerkinElmer Life Sciences, Boston, MA). The primary antibodies (Abs) used were polyclonal anti-ERK1/2, monoclonal anti-phospho-ERK, polyclonal anti-caspase-3 and monoclonal anti- β -actin. The secondary antibody used was peroxidase conjugated (HRP) goat anti-rabbit IgG or goat anti-mouse IgG (Pierce). Supersignal West Pico (Pierce) was used for HRP detection on a Hyperfilm ECL system (Nickon). Stripping of the blot membrane was performed in Restore Western Blot Stripping Buffer (Pierce), as required. The levels of phosphorylated ERK (p-ERK) were expressed as arbitrary units of optical density, following the correction for content of total ERK (ERK1/2). Band intensities of caspase-3 were corrected for the content of β - actin. Densitometry measurement of band intensity was used for quantification (AlphaEaseFC Imaging System software, Alpha Innotech, San Leandro, CA).

4.6. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ was measured with the fluorescent dye, TMRE (Rama Rao and Norenberg, 2004). At the end of treatments, the culture medium was removed (duplicate plates per experiment; repeated three times using different astrocyte isolations), and the cells were loaded with TMRE at a final concentration of 50 nM in HEPES buffer for 20 min at 37°C in a 5% CO_2 incubator. Cells were rinsed with PBS and examined with a Zeiss inverted fluorescent microscope (Zeiss Axiovert S100, Carl Zeiss MicroImaging Inc., Thornwood, NY) equipped with a cooled digital camera (Photometrics CoolSNAP, Roper Scientific Photometrics, Tucson, AZ). Images of various fields in each plate were captured at 10 $\times$ magnification with the digital camera. Fluorescent intensities were calculated in 8 randomly selected fields per experiment and analyzed using NIH imaging software (Scion Incorporation, Frederick, MD). In each image field, the total number of pixels was quantified on a gray scale (0 – 255 counts), and the mean pixel value in each image field was expressed as mean $\pm$ S.E.M. The fluorescent intensities were expressed as percent fluorescence change over control. Each experiment was performed in duplicate plates and repeated three times in astrocytes isolated from independent isolations.

4.7. Statistical analysis

Measurements of the densitometries of caspase-3, p-ERK, total ERK and β -actin by immunoblotting, the mitochondrial membrane potential by TMRE fluorescent intensity conducted in duplicate or triplicate wells/experiment, and the mean value from three to four independent experiments were used for statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test with statistical significance set at $p < 0.05$. All analyses were carried out with GraphPad Prism 4.02 software for Windows (GraphPad Software, San Diego, CA, USA).

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Abbreviations

$\Delta\Psi_m$	mitochondrial inner membrane potential
ERK	extracellular signal-regulated kinase
Mn	manganese

TMRE

tetramethyl rhodamine ethyl ester

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Fig 1A

Fig 1B

Fig 1C

Fig 1D

Figure 1. Effects of Mn on LDH release from primary astrocyte cultures

Rat primary astrocyte cultures were incubated at 37°C in the absence or presence of Mn (100, 500 and 1000 μ M) and LDH release to the media was quantified at the indicated times. Figures 1A and 1B depict the concentration-dependent effects of Mn exposure for 30 min (A) and 2 h (B). Figures 1C and 1D show the time course from 30 min to 24 h at Mn concentrations of 100 μ M (C) and 500 μ M (D). Statistical analysis was carried out by one-way ANOVA followed by Bonferroni multi-comparison tests. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; $n = 6-10$; the values are mean $\pm$ S.E.M.).

Fig 2A

Fig 2B

Fig 2C

Fig 2D

Figure 2. Effects of Mn on astrocyte viability by MTT assay in primary astrocyte cultures

Astrocytes were treated with different concentrations of Mn (0, 100, 500 and 1000 μ M) and cell viability by MTT assay was quantified at the indicated times. Figures show exposure to Mn for 30 min (A) or 2 h (B) in a concentration-dependent manner and Mn exposure from 30 min to 24 h at 100 (C) or 500 μ M (D) in a time course. The values are mean $\pm$ S.E.M. of 4 different experiments. * $p < 0.05$, *** $p < 0.001$ versus control.

Fig 3A

Fig 3B

Fig 3C

Fig 3D

Figure 3. Effect of Mn on ERK phosphorylation in cultured astrocytes as determined by immunoblotting

Values are mean $\pm$ SEM of 4–7 experiments in each group. Statistical analysis was carried out by one-way ANOVA followed by Bonferroni comparison tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control.

Fig 4A

Fig 4B

Fig 4C

Fig 4D

Figure 4. Cleavage of caspase-3 precursor in cultured astrocytes exposed to Mn (1 hr and 24 hrs) as determined by immunoblotting

Values are mean $\pm$ SEM of 4–7 experiments in each group. Statistical analysis was carried out by one way ANOVA followed by Bonferroni multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control.

Fig 5A

Fig 5B

Figure 5. Effect of Mn on mitochondrial inner membrane potential ($\Delta\Psi_m$)

Control astrocytes show a prominent fluorescence indicating polarized mitochondria; astrocytes treated with Mn (100, 500 μ M and 1 mM) for 1 hr (A) show decreased TMRE fluorescence consistent with the depolarization of the $\Delta\Psi_m$. The fluorescent quantification (B) was determined as described in Section 2. Values are expressed as mean $\pm$ S.E.M. of 24 random fields in each group. * $p < 0.05$, *** $p < 0.001$ versus control.