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Oxidative damage and neurodegeneration in manganese-induced neurotoxicity

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Abstract

Exposure to excessive manganese (Mn) levels results in neurotoxicity to the extrapyramidal system and the development of Parkinson's disease (PD)-like movement disorder, referred to as manganism. Although the mechanisms by which Mn induces neuronal damage are not well defined, its neurotoxicity appears to be regulated by a number of factors, including oxidative injury, mitochondrial dysfunction and neuroinflammation. To investigate the mechanisms underlying Mn neurotoxicity, we studied the effects of Mn on reactive oxygen species (ROS) formation, changes in high-energy phosphates (HEP), neuroinflammation mediators and associated neuronal dysfunctions both *in vitro* and *in vivo*. Primary cortical neuronal cultures showed concentration-dependent alterations in biomarkers of oxidative damage, F₂-isoprostanes (F₂-IsoPs) and mitochondrial dysfunction (ATP), as early as 2 hours following Mn exposure. Treatment of neurons with 500 μM Mn also resulted in time-dependent increases in the levels of the inflammatory biomarker, prostaglandin E₂ (PGE₂). *In vivo* analyses corroborated these findings, establishing that either a single or three (100 mg/kg, s.c.) Mn injections (days 1, 4 and 7) induced significant increases in F₂-IsoPs and PGE₂ in adult mouse brain 24 hours following the last injection. Quantitative morphometric analyses of Golgi-impregnated striatal sections from mice exposed to single or three Mn injections revealed progressive spine degeneration and dendritic damage of medium spiny neurons (MSNs). These findings suggest that oxidative stress, mitochondrial dysfunction and neuroinflammation are underlying mechanisms in Mn-induced neurodegeneration.

Keywords

Manganese; oxidative stress; medium spiny neurons; neurodegeneration; spines; manganism

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INTRODUCTION

Manganese (Mn) is an essential nutrient and it functions as a critical cofactor in many key enzymes (Takeda, 2003; Aschner and Aschner, 2005). However, elevated occupational exposure to Mn poses a health risk. Elevated brain Mn concentrations are associated with occupational exposure to high levels of inhaled Mn such as in ferroalloy smelting, welding, mining, battery assembly and the manufacture of glass ceramics (Srivastava et al., 1991; Mergler et al., 1994; Bader et al., 1999; Bast-Pettersen et al., 2004; Racette et al. 2001, 2005; Bowler et al., 2007; Montes et al., 2008). Mn exposure also occurs in patients with liver disease, immature neonates with compromised liver function and individuals receiving contaminated well-water and parenteral nutrition therapy (Iinuma et al., 2003; Dobson et al., 2004). Mn exposure has been also associated with use of the fuel additive, methylcyclopentadienyl manganese tricarbonyl (MTT) (Kaiser, 2003; Bolte et al., 2004).

Excessive accumulation of Mn in the striatum, globus pallidus (GP) and the substantia nigra (SN) can result in a neurodegenerative disorder, commonly referred to as manganism. Epidemiological studies have suggested a causal relationship between elevated environmental Mn exposure and increased risk for Parkinsonian-like symptoms (Cotzias et al., 1968). Movement disorders inherent to manganism or Parkinson's disease (PD) become progressive and are irreversible, reflecting damage to neuronal structures (Aschner et al., 2007). In addition to targeting similar brain areas, PD and manganism share common mechanisms leading to dopaminergic (DAergic) neurodegeneration, namely, mitochondrial dysfunction, aberrant signal transduction, oxidative stress and the activation of cell death pathways (Dobson et al., 2004; HaMai and Bondy, 2004; Latchoumycandane et al., 2005; Kitazawa et al., 2005).

Upon entering the brain, Mn can be taken up into astrocytes and neurons. Astrocytes serve as the major homeostatic regulator and storage site for Mn in the brain (Aschner et al., 1999). However, increased accumulation of Mn in astrocytes may alter release of glutamate and elicit excitatory neurotoxicity (Erikson and Aschner, 2003). Neuronal uptake of Mn involves transferrin (Suarez and Eriksson, 1993) and utilization of specific transporter system such as the dopamine transporter (DAT) (Anderson et al., 2007; Chen et al., 2006a). At the cellular level, Mn preferentially accumulates in mitochondria, where it disrupts oxidative phosphorylation and increases the generation of reactive oxygen species (ROS) (Gunter et al., 2006). Increased striatal concentrations of ascorbic acid and glutathione (GSH), antioxidants that when increased signal the presence of an elevated burden from ROS, as well as other markers of oxidative stress, have been previously reported (Desole et al., 1994; Dobson et al., 2004; Erikson et al., 2007). Excessive production of ROS induces the oxidation of membrane polyunsaturated fatty acids, yielding a multitude of lipid peroxidation products. One such family of products is the F₂-isoprostanes (F₂-IsoPs), prostaglandin-like molecules produced by free radical-mediated peroxidation of arachidonic acid (AA) (Morrow and Roberts, 1999). These biomarkers of oxidative stress have been investigated in several *in vitro* and *in vivo* models, but not within the context of Mn-induced neurotoxicity *in vivo*. In addition to decrease in mitochondrial membrane potential and the depletion of high-energy phosphates, ROS generation is also associated with inflammatory responses and release of inflammatory mediators, including prostaglandins. Increasing reports demonstrate that inflammation contributes to neuronal damage and death (Liu et al., 2002; Milatovic et al., 2003, 2004). An uncontrolled or chronic inflammation response may cause irreversible tissue damage, fueling a self-propelling cycle of neuronal death. ROS generation is also associated with release of apoptogenic factors into the cytosol (Green and Reed, 1998). These interconnected pathways of oxidative stress, inflammation and apoptosis have been linked to the pathophysiology of neurodegenerative disease (Tansey et al., 2007).

Studies of postmortem brains of humans, non-human primates and rodents have indicated that Mn-induced neuronal damage is prominent in the striatum and other structures of the basal ganglia (Aschner et al., 2007; Perl and Olanow, 2007). Manganese is associated with alterations in integrity of DAergic striatal neurons and DA neurochemistry, including decreased DA transport function and/or striatal DA levels. Integrity of DAergic neurons in the substantia nigra pars compacta which are preferentially targeted in PD, is thought to be spared in Mn-induced parkinsonism (Aschner et al., 2007; Perl and Olanow, 2007). However, integrity of the striatal neurons that receive dopaminergic input has received relatively little attention. The medium spiny neurons (MSN) are the target of the dopaminergic innervation of the striatum, comprising more than 90% of striatal neurons (Deutch et al., 2007). MSNs have radially projecting dendrites that are densely studded with spines, synapsing with dopamine and glutamate axons and providing the site of integration of several key inputs and outputs of the striatum (Day et al., 2006). Consequently, alterations in dendritic length and dendritic spine number may destabilize the structural basis of synaptic communication and thus compromise MSN function.

Given the shared symptoms characterizing manganese and PD, the present study was conducted to test the hypothesis that Mn neurotoxicity is associated with oxidative damage, neuroinflammation and altered integrity of DAergic striatal neurons. We have used biochemical and morphological approaches to investigate novel markers of oxidative stress and neuroinflammation and quantify synaptodendritic degeneration of striatal MSNs in mice exposed to Mn.

MATERIALS AND METHODS

Materials

Manganese chloride (MnCl_2) and ATP standards were purchased from Sigma Chemical Co. (St Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) with heat-inactivated horse serum, penicillin, streptomycin and cytosine arabinoside were purchased from Invitrogen (Carlsbad, CA). $15\text{-F}_2\alpha\text{-IsoP-d}_4$ (internal standard for $\text{F}_2\text{-IsoPs}$ that contains four deuterium atoms), PGE_2 internal standard and prostaglandin F_2 , E_2 and D_2 methyl esters were purchased from Cayman chemicals (Ann Arbor, MI).

Animals

All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committees. C57Bl/6 female mice (obtained from Jackson laboratories, Bar Harbor, Maine) between 6 and 8 weeks of age, were housed at $21 \pm 1^\circ\text{C}$, humidity $50 \pm 10\%$, and light/dark cycle 12 h/12 h, and had free access to pelleted food (Rodent Laboratory Chow, Purina Mills Inc., St Louis, MO, USA) and water. Mice were exposed to Mn by a single or three subcutaneous (s.c.) injections at the scruff of the neck. While the first group of mice received a single injection of 0 or 100 mg/kg Mn, a second group of mice received three identical injections of 0 or 100 mg/kg on days 1, 4 and 7. Both groups (4–6 mice in each group) were sacrificed 24 hours after the last injection. Cerebral hemispheres were immediately removed and processed for evaluation of $\text{F}_2\text{-IsoPs}$ and PGE_2 or processed with Golgi impregnation for evaluation of synaptodendritic changes of striatal MSNs. The dose and route selections of Mn administration for this study are based on observations by Dodd and co-workers (2005), showing that the three Mn injection protocol produced 647% increase in striatal Mn levels relative to vehicle control mice.

Primary neuronal cultures

Primary cultures of cortical neurons were obtained from 17- to 18-day-old fetal Sprague–Dawley rats. Briefly, the cerebral cortex was isolated and placed into Hanks' balanced salt

solution (HBSS) containing 0.125% trypsin. After removal of the meninges, the cerebral cortices were digested with bacterial neutral protease for 30 min at room temperature, followed by mechanical trituration with pipettes. Subsequently, cells were plated on glass coverslips placed in 6-well plates coated with poly-L-ornithine at a density of 6.7×10^5 cells / well. The neurons were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum and F12 with penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 95% air – 5% CO₂. Two days after plating, non-neuronal cell proliferation was inhibited by the addition of cytosine arabinoside (10 µM), and the next day, the culture media was changed to NeuroBasal supplemented with B27, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Experiments were carried out in 10- to 14-day-old cultures.

Mn concentrations (100 µM, 500 µM or 1 mM) in this study with primary cultures of cortical neurons were determined based on the relevant toxic Mn effect on mammalian cells as described in the literature. Weekly injections of Mn over a 3-month period (0, 2.25, 4.5, or 9 mg/kg) in monkeys have been shown to produce dose-related clinical signs, which are more severe in the higher dose ranges (Suzuki et al., 1975). While the basal ganglia represent the main target for Mn neurotoxicity, reflecting upon its preferential accumulation in this region (Dorman et al. 2006), Mn is also well-known to affect the cerebral cortex (Guilarte and Chen 2007), thus providing a rationale for examining cells derived from this brain region. Concentration-dependent neurotoxic effect of 100 µM, 500 µM or 1 mM Mn is also confirmed in our previous study with primary astrocytes cultures (Milatovic et al., 2007).

Biochemical assays

Quantitation of F₂-IsoPs—Upon completion of the experiments, primary neuronal cultures and brains were rapidly harvested, flash frozen in liquid nitrogen, and stored at –80 °C until analysis. Total F₂-IsoPs were determined with a stable isotope dilution method with detection by gas chromatography/mass spectrometry and selective ion monitoring as previously described (Morrow and Roberts, 1999; Milatovic et al., 2007). Total F₂-IsoPs were measured in primary neuronal cultures exposed to 100 µM, 500 µM or 1 mM of MnCl₂ for 2 hours (exposure time with the most significant increase in F₂-IsoPs as indicated in previous studies). Briefly, cells were resuspended in 0.5 ml of methanol containing 0.005% butylated hydroxytoluene, sonicated and then subjected to chemical saponification using 15% KOH to hydrolyze bound F₂-IsoPs. The cell lysates were adjusted to a pH of 3, followed by the addition of 0.1 ng of 15-F_{2α}-IsoP-d4 internal standard. F₂-IsoPs were subsequently purified by C₁₈ and silica Sep-Pak extraction and by thin layer chromatography. They were then analyzed by pentafluorobenzyl ester, a trimethylsilyl ether derivative, via gas chromatography, negative ion chemical ionization mass spectrometry. Quantification of F₂-IsoPs from brains of mice exposed to saline or Mn followed the same procedure with exception that prior to chemical hydrolyses with KOH, cerebral hemispheres were homogenized in Folch solution and lipids extracted from chloroform layer by evaporation (Milatovic and Aschner, 2009).

Measurement of adenosine 5'-triphosphate (ATP)—Levels of ATP were analyzed by an isocratic reversed-phase high performance liquid chromatography (HPLC) method (Yang et al., 2004). ATP was extracted from control and Mn-exposed neurons by adding 950 µl of ice-cold perchloric acid (0.2 M) containing Na-EDTA (1 mM) to the primary astrocyte culture plates immediately after the medium was removed. The cells were then scraped off the bottom of the plates, and the acid extract was transferred to a microcentrifuge tube. The acid extract was neutralized with 170 µl of potassium hydroxide (KOH; 2 M) and centrifuged at 9000 g for 5 min to remove fine precipitates of perchlorate (KClO₄). The supernatants were stored at –20°C before being subjected to ATP determination. The concentration of ATP was determined in a 15 µl sample extract injected into HPLC with UV detector and 0.1 M of

ammonium dihydrogen phosphate (pH 6.0) containing 1% methanol as a mobile phase. Using the Symmetry Shield C-18 column and a flow rate of 0.6 ml/min, the peak of ATP was eluted at a retention time of 3.462 min and recorded at 206 nm.

Quantification of prostaglandin E₂ (PGE₂)—PGE₂ was also measured by using a stable isotope dilution gas chromatographic/negative ion chemical ionization-mass spectrometric assay (Awas et al., 1996). Briefly, [⁴H₂]-PGE₂ (1.28 ng) was added to neuronal homogenates or aqueous layer of brain homogenates after Folch extraction. The sample was then acidified to pH 3 with 1 N HCl and extracted on a C18 Sep-Pak. PGE₂ was eluted with ethyl acetate:heptane and evaporated under a stream of N₂. PGE₂ in methoxylamine solution was extracted with ethyl acetate and evaporated with N₂. The pentafluorobenzyl esters were purified by thin layer chromatography (PGE₂ and PGD₂ methyl esters are used as TLC standards), converted to O-methyloxime pentafluorobenzyl ester trimethylsilyl derivatives, and PGE₂ dissolved in undecane that is dried over a bed of calcium hydride. Gas chromatographic/negative ion chemical ionization-mass spectrometric analysis was performed as described previously with the M-181 ions for PGE₂ (m/z 526) and the [⁴H₂]-PGE₂ as internal standard (m/z 528).

Quantitative morphology of medium spiny neurons—Quantitative neuronal analysis is conducted on tissue stained with Golgi impregnation that is uniform throughout the section. Length of dendrites and spine number counts of MSN were evaluated in Golgi impregnated 50 microns thick striatal sections from paraffin-embedded blocks prepared according to the manufacturer's specifications (FD Rapid GolgiStain Kit). MSN were recognized by their soma size and dendritic extensions. Six or more MSN with no breaks in staining along the dendrites were selected by observer blinded to experimental procedures and spines counted according to the published methods (Leuner et al., 2003; Zaja-Milatovic et al., 2005). Reconstruction of the three-dimensional dendritic tree by tracing each neuron in a two-dimensional plane and counting are done using a Neurolucida system at ×100 under oil immersion (MicroBrightField, VT).

Statistical analysis

Measurements of F₂-IsoPs, ATP and PGE₂ from primary neuronal cultures were conducted in duplicate or triplicate wells/experiment, and the mean from three to four independent experiments was used for statistical analysis. The data from *in vivo* experiments are presented as means ± SEM of 4–6 mice in each group. 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 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Effects of Mn on F₂-IsoPs, ATP and proinflammatory prostaglandine E₂ (PGE₂) formation in cultured neurons

We tested the ability of Mn to induce oxidative stress in primary cortical neuronal cultures by measuring the levels of F₂-IsoPs, a lipid peroxidation biomarker of oxidative injury. Rat primary neurons exposed to 500 μM or 1 mM of MnCl₂ for 2 hours showed significant increases in F₂-isoPs (Fig. 1). Neuronal cultures exposed to 1 mM of MnCl₂ for 2 hours showed a two-fold increase in F₂-IsoPs compared to the control level (40.74 ± 5.4 pg/mg protein). The lowest MnCl₂ concentration (100 μM) did not induce statistically significant increase in markers of oxidative stress compared to control.

As an additional indicator of cellular toxicity and mitochondrial dysfunction, we have evaluated cellular ATP levels in control and MnCl₂-exposed neuronal cultures. Primary neuronal cultures showed concentration-dependant decrease in ATP levels following graded exposure to Mn for 2 hours (Fig. 2). Analogous to the changes in biomarkers of oxidative stress (F₂-IsoPs), depletion of ATP did not reach statistical significance following exposure to 100 µM of MnCl₂; however, compared to controls, higher MnCl₂ concentrations (500 µM or 1 mM) led to significant ATP depletion in primary neuronal cultures.

PGE₂, a biomarker of proinflammatory responses, was also evaluated. Treatment with 500 µM for 1 or 6 hours had no effect on neuronal PGE₂ levels (Fig. 3). However, 24 hour exposure to 500 µM MnCl₂ resulted in a significant increase in PGE₂ levels, indicating delayed inflammatory response.

Effects of Mn on biochemical and structural parameters of neurotoxicity in vivo

Initial *in vivo* experiments investigated the extent of cerebral oxidative damage and neuroinflammation in C57Bl/6 mice exposed to Mn. Analysis of cerebral biomarkers of oxidative damage revealed that both a single as well as three injections of 100 mg/kg MnCl₂ (day 1, 4 and 7) led to significant increase in F₂-IsoPs in adult mouse brains. Thus, a one-time challenge of mice with MnCl₂ was sufficient to induce oxidative stress and produce an equivalent increase in cerebral biomarkers of oxidative damage (F₂-IsoPs) as multiple Mn exposure when examined 24 hours following the last injection (Fig. 4). One-time injection of mice with MnCl₂ also led to a significant increase in PGE₂ levels (Fig. 5). Results from these experiments also showed that a week exposure and multiple MnCl₂ injections induced further increase in PGE₂, indicating more pronounced inflammatory reaction in mice.

We next investigated if cerebral oxidative damage and neuroinflammation is accompanied by altered integrity of the striatal dendritic system of mice exposed to MnCl₂. Dendritic degeneration of MSNs was observed in the striatum of mice exposed to a single or multiple injections of MnCl₂. Representative images of Golgi impregnated striatal sections with their traced MSN from control and Mn-exposed animal are presented in Figure 6. Neuroexplorer assisted neuronal morphometry revealed progressive spine degeneration (total number of spines per neuron) of MSN in mice with increase in time and dose of MnCl₂ exposure (Fig. 7a). Consistent with these effects, MnCl₂ also induced dose- and time-dependent progressive dendritic damage (total dendritic length per neuron) of MSN (Fig. 7b).

DISCUSSION

The present study provides a potential direct link between oxidative stress, mitochondrial dysfunction, inflammation and neurodegeneration due to Mn neurotoxicity. To our knowledge, this is the first study to investigate neuronal oxidative stress by quantifying *in vitro* and *in vivo* biomarkers of oxidative damage (F₂-IsoPs) and quantification of spines and dendritic length in Golgi-impregnated striatal tissues of mice exposed to Mn. Our results show that increased cerebral lipid peroxidation, depletion of ATP and inflammation are potentially associated with Mn-induced neuronal dysfunction and synaptodendritic degeneration of striatal MSNs.

Studies on the effects of Mn have failed to fully elucidate the primary mechanisms of Mn-mediated neurodegeneration. However, generation of ROS and altered mitochondrial activities are closely linked to Mn neurotoxicity (Dobson et al., 2004; Milatovic et al., 2007). We have used both *in vitro* and *in vivo* models of Mn neurotoxicity to test the hypothesis that Mn-induced ROS generation and neuroinflammation mediates neuronal injury. Results from the *in vitro* study showed that Mn induced concentration-dependent oxidative stress (F₂-IsoPs) and depletion of ATP in primary cortical neuronal cultures. These results corroborate previous

findings in astrocytes exposed to Mn showing significant elevations in F₂-IsoPs levels and depletion of ATP (Milatovic et al., 2007). Therefore, increases in ROS, which are generated by electron leak from the electron transport chain (Turrens and Boveris 1980), potentially damaging mitochondria directly or through the effects of secondary oxidants like superoxide, H₂O₂ or peroxynitrite (ONOO⁻), mediate Mn-induced oxidative damage. Moreover, superoxide produced in the mitochondrial electron transport chain may catalyze the transition shift of Mn²⁺ to Mn³⁺ through a set of reactions similar to those mediated by superoxide dismutase and thus lead to the increased oxidant capacity of this metal (Gunter et al., 2006). In addition, perturbation in energy metabolism and depletion of ATP may affect intracellular Ca²⁺ in these primary cultures through mechanisms involving the disruption of mitochondrial Ca²⁺ signaling. This assertion is supported by data that clearly show that Mn inhibits Na⁺-dependent Ca²⁺ efflux (Gavin et al., 1990) and respiration in brain mitochondria (Zhang et al., 2004), both critical to maintaining normal ATP levels and optimal inter-mitochondrial signaling. In addition, these deleterious effects may cause structural and functional derangement of the phospholipids bilayer of membranes, disrupt metabolite biosynthesis and initiate apoptosis (Uchida, 2003; Attardi and Schatz, 1988; Yang et al., 1997). Results from our *in vitro* study also showed that Mn exposure induced time-dependent increase in PGE₂. Recent studies have shown inflammatory response of glial cells following Mn exposure (Chen et al., 2006b; Zhang et al., 2009; Zhao et al., 2009). Mn potentiates lipopolysaccharide-induced increases in proinflammatory cytokines in glial cultures (Filipov et al., 2005) and increase in nitric oxide production (Chang and Liu, 1999). An increase in proinflammatory genes, such as tumor necrosis factor- α , iNOS and activated inflammatory proteins such as P-p38, P-ERK and P-JNK have been measured in primary rat glial cells after Mn exposure (Chen et al., 2006b). However, data from the present study indicate that release of proinflammatory mediators following Mn exposure is not only associated with glial response but neurons as well (Fig. 5).

Next, we investigated cerebral oxidative damage and neuroinflammation in a mouse model of Mn neurotoxicity. Analyses of cerebral biomarkers of oxidative damage and proinflammatory prostaglandin revealed that one-time challenge of mice with Mn (100 mg/kg) was sufficient to produce significant increases in F₂-IsoPs (Fig. 4) and PGE₂ (Fig. 5) 24 hours following the last injection, respectively. While elevations in F₂-IsoPs levels remained at the same level, more pronounced inflammatory response and further increase in PGE₂ were noted following longer and multiple Mn exposures. Results from this study corroborate previous findings from the mouse model of activated innate immunity (Milatovic et al., 2003;2004) and confirm a relationship between changes in biomarkers of oxidative damage and neuroinflammation. We have previously used intracerebroventricular (ICV) injection of low dose lipopolysaccharide (LPS) and showed that despite the lack of adaptive immune cell infiltrate, or detectable neuron loss, there was significant, reversible free radical damage to neuronal membranes following ICV LPS. In combination, these data suggest that these two events are mechanistically related, with neuroinflammation either alone or in combination with activated glial response contributing to oxidative damage and consequent cell injury. DAergic neurons possess reduced antioxidant capacity, as evidenced by low intracellular GSH, which renders these neurons more vulnerable to oxidative stress and glial activation relative to other cell types (Sloot et al., 1994; Greenamyre et al., 1999). ROS may act in concert with reactive nitrogen species (RNS) derived from astroglia and microglia to facilitate the Mn-induced degeneration of DAergic neurons. Therefore, the over-activation of glia and release of additional neurotoxic factors may represent a crucial component associated with the degenerative process of DAergic neurons.

Additional studies investigated the effects of Mn on degeneration of striatal neurons. Representative images of Golgi impregnated striatal sections with their traced MSNs from control and Mn-exposed animal are presented in Figure 6. Images of neurons with Neurolucida-assisted morphometry show that Mn-induced oxidative damage and neuroinflammation

targeted the dendritic system with profound dendrite regression of striatal MSNs. When administered to adult rodents, excess Mn accumulates in various basal ganglia structures, including the striatum (caudate and putamen), globus pallidus, and brain stem (Autissier et al., 1982; Dorman et al., 2000; Calabresi et al., 2001; St-Pierre et al., 2001). The striatum is a major recipient structure of neuronal efferents in the basal ganglia. It receives excitatory input from the cortex and dopaminergic input from substantia nigra and projects to the internal segment of the globus pallidus (Dimova et al., 1993; Saka et al., 2002). Nigrostriatal dopamine neurons appear to be particularly sensitive to Mn-induced toxicity (Defazio et al., 1996; Slood and Gramsbergen, 1994; Slood et al., 1994), because a prevalence of studies have shown that intense or prolonged Mn exposure in adulthood causes long-term reductions in striatal DA levels and induces a loss of autoreceptor control over DA release (Autissier et al., 1982; Komura and Sakamoto, 1992). Nigrostriatal DA axons synapse onto striatal MSNs, and these neurons have radially projecting dendrites that are densely studded with spines (Wilson and Groves, 1980). Postmortem studies of PD have revealed a marked decrease in MSNs spine density and dendritic length (Stephens et al., 2005; Zaja-Milatovic et al., 2005). Similar morphological changes in MSNs were seen in animal models of Parkinsonism (Arbuthnott et al., 2000; Day et al., 2006). Results of the dendritic morphology of randomly selected striatal MSNs from control and Mn-exposed animals revealed that even single Mn exposure altered the integrity of dendritic system and induced significant decrease in spine number (Fig. 7a) and total dendritic lengths (Fig. 7b) of MSNs. Even though, prolonged Mn exposure led to further reduction in spine number and dendritic length (Fig. 7), MSNs neurodegeneration could result from loss of spines, removing the pharmacological target for DA-replacement therapy, without overt MSNs death (Stephens et al., 2005; Zaja-Milatovic et al., 2005).

In conclusion, these findings suggest that oxidative stress, mitochondrial dysfunction and neuroinflammation are underlying mechanisms in Mn induced vulnerability of MSNs. Mediation of any of these mechanisms and control of alterations in biomarkers of oxidative injury, neuroinflammation and synaptodendritic degeneration may provide a therapeutic strategy for suppression of dysfunctional dopaminergic transmission and slowing the neurodegenerative process. Further morphometric studies that interrogate MSN structures with suppression of oxidative injury and neuroinflammation and/or progression of Mn exposure are needed to investigate the significance of MSN degeneration in Mn-induced Parkinsonism. Future experiments should also address the selectivity of MSN degeneration and investigate if Mn exposure also results in spinodendritic degeneration of cortical and hippocampal pyramidal neurons in mouse brain.

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Figure 1.

Effect of MnCl₂ on F₂-IsoPs formation in cultured neurons. Rat primary neuron cultures were incubated at 37°C in the absence or presence of MnCl₂ (100 μM, 500 μM or 1 mM) and F₂-IsoPs levels were quantified at 2 h. Data represent the mean ± S.E.M. from three independent experiments. * p<0.05 versus control by one-way ANOVA followed by Bonferroni's multiple comparison tests.

Figure 2.

Effect of MnCl₂ on ATP concentration in cultured neurons. Rat primary neuron cultures were incubated at 37°C in the absence or presence of MnCl₂ (100 μM, 500 μM or 1 mM), and ATP levels were quantified at 2 h. Data represent the mean ± S.E.M. * $p < 0.05$ versus control by one-way ANOVA followed by Bonferroni's multiple comparison tests.

Figure 3.

Effect of MnCl₂ on PGE₂ formation in cultured neurons. Rat primary neuron cultures were incubated at 37°C in the absence or presence of MnCl₂ (500 μM) and PGE₂ levels were quantified at 2 h, 6 h and 24 h. Data represent the mean ± S.E.M. * p<0.05 versus control by one-way ANOVA followed by Bonferroni's multiple comparison tests.

Figure 4. Cerebral F₂-IsoPs concentrations in saline (control) or MnCl₂ (100 mg/kg, s.c) exposed mice. Brains from mice exposed once or three times (day 1, 4 and 7) to MnCl₂ were collected 24 hr post last injection. Values of F₂-IsoPs represent mean±SEM (n=4–6). * p<0.05 versus control by one-way ANOVA followed by Bonferroni's multiple comparison tests.

Figure 5.

PGE₂ levels in mouse brain following MnCl₂ (100 mg/kg, s.c.) exposure. Brains from mice exposed once or three times (day 1, 4 and 7) to MnCl₂ were collected 24 hr post last injection. Values of PGE₂ represent mean±SEM (n=4–6). * p<0.05 versus control by one-way ANOVA followed by Bonferroni's multiple comparison tests.

Figure 6.

Photomicrographs of mouse striatal sections with representative tracings of medium spiny neurons (MSNs) from mice treated with saline (control) (A) or MnCl_2 (100 mg/kg, s.c.) (B). Brain from mouse exposed three times (day 1, 4 and 7) to MnCl_2 was collected 24 hr post last injection. Treatment with Mn induced degeneration of striatal dendritic system, decrease in total number of spines and length of dendrites of MSNs. Tracing and counting are done using a Neurolucida system at $100\times$ under oil immersion (MicroBrightField, VT). Colors indicate the degree of dendritic branching (yellow= 1° , red= 2° , purple= 3° , green= 4° , turquoise= 5°).

Figure 7. Total number of spines (A) and total dendritic lengths (B) of MSNs from striatal sections of mice exposed to saline (control), single Mn injection (100 mg/kg, s.c.) or multiple Mn injections (100 mg/kg, s.c.) on day 1, 4 and 7. Mice were sacrificed 24 hours after the last injection. * $p < 0.01$ versus control by one-way ANOVA followed by Bonferroni's multiple comparison tests. ^ $p < 0.001$ for single Mn injection vs. multiple (8 days) Mn treatment by Bonferroni's multiple comparison tests.