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Ultrastructural changes of the olfactory bulb in manganese-treated mice

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Key words: metal toxicity, toxicity, neurodegeneration, electron microscopy.

ABSTRACT: The effect of manganese toxicity on the ultrastructure of the olfactory bulb was evaluated. Male albino mice were injected intraperitoneally with $MnCl_2$ (5 mg/Kg/day) five days per week during nine weeks. The control group received NaCl (0.9%). The olfactory bulbs of five mice from each group were processed for transmission electron microscopy after 2, 4, 6 and 9 weeks of manganese treatment. On week 2, some disorganization of the myelin sheaths was observed. After 4 weeks, degenerated neurons with dilated cisternae of rough endoplasmic reticulum and swollen mitochondria appeared. A certain degree of gliosis with a predominance of astrocytes with swollen mitochondria, disorganization of the endomembrane system, dilation of the perinuclear cisternae and irregularly shaped nuclei with abnormal chromatin distribution were observed after 6 weeks. Some glial cells showed disorganization of the Golgi apparatus. On week 9, an increase in the number of astrocytes, whose mitochondrial cristae were partially or totally erased, and a dilation of the rough endoplasmic reticulum were found. Neurons appear degenerated, with swollen mitochondria and a vacuolated, electron dense cytoplasm. These changes seem to indicate that the olfactory bulb is sensitive to the toxic effects of manganese.

Introduction

Manganese is an essential metal for humans. It is an integral part of a series of metalloproteinases such as hydrolases, kinases, decarboxylases and transferases. It is also a co-factor of a series of enzymes in both lipid and carbohydrate metabolism (Bonilla, 1986).

Manganese is widely used for industrial purposes including the manufacture of paints, soaps, batteries and insecticides. Currently, methylcyclopentadienyl manganese tricarbonyl is used as an anti-knock agent in gasoline (Cooper, 1984).

Although manganese has low toxicity, it may cause intoxication in humans exposed to high concentrations of this metal or to low doses for long periods of time (Barbeau, 1984). Both mine and metal workers are frequently exposed to this element (Huang *et al*., 1998). Manganese intoxication in humans follows a biphasic course: the acute phase is characterized by alterations in motor activity, hallucinations, and compulsive behavior ("manganese madness"), which resembles schizophrenia (Mena *et al*., 1967). This phase is followed by a predominance of neurological symptoms, such as rigidity, muscular weakness, and tremor, which are similar

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to those found in Parkinson´s disease (Cotzias *et al*., 1971). Neuropathological (Canavan *et al*., 1934), biochemical (Bernheimer *et al*., 1973), and pharmacological (Cook *et al*., 1974) studies strongly support the resemblance between both pathologies. In fact, chronic manganese poisoning has been proposed as an experimental model for the study of Parkinson´s disease (Bernheimer *et al*., 1973).

The pulmonary route is the main entrance of manganese into the body. But the brain has the capability for retaining it for longer periods of time than in other organs, probably due to its difficulty to get rid of the excess of this metal (Bonilla, 1986). Manganese may cross the blood brain barrier, and it is delivered to different brain regions, via an axonal transport system (Bonilla, 1986).

Diverse studies performed in manganese poisoned patients and animals treated with manganese have shown a decrease in the striatal levels of dopamine (Autissier *et al*., 1982; Bird *et al*., 1984). However, some researchers have found an increase (Cotzias *et al*., 1971) or no alteration (Shukla and Chandra, 1979) in the concentration of this amine. These contradictory results possibly reflect the biphasic nature of the alterations produced by manganese during the intoxication process.

Manganese treatment has also been found to cause changes in the metabolism of several neurotransmitters such as serotonin, gamma-aminobutyric acid (GABA), and norepinephrine (Kimura *et al*., 1978; Chandra and Shukla, 1981; Chandra *et al*., 1982). But the alterations seem to be milder in the metabolism of acetylcholine (Martinez and Bonilla, 1981). Increases, decreases, or no changes on ligand binding to dopaminergic (Bhargava, 1987; Villalobos *et al*., 2001), gabaergic (Chandra *et al*., 1982; Moreno and Bonilla, 1984), muscarinic (Bhargava, 1987; Leung *et al*., 1986; Villalobos *et al*., 1994) and adenosine receptors (Villalobos *et al*., 2001) have been described in manganese-treated animals.

Post-mortem studies in humans and experimental animals such as monkeys, mice, guinea pigs, and rabbits exposed to manganese showed neuronal degen-

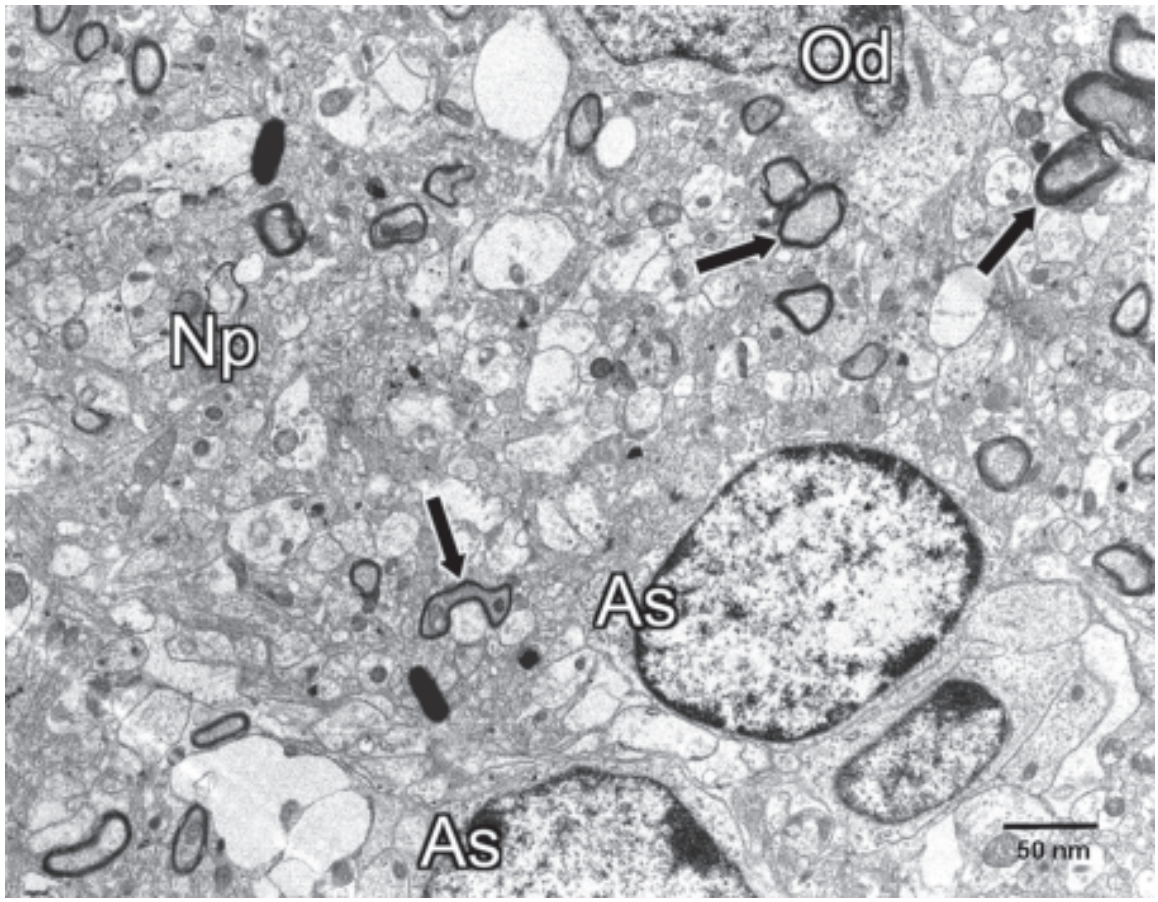

FIGURE 1. Electron micrograph of a normal mouse olfactory bulb. Two astrocytes (As) and one oligodendrocyte (Od), along with some myelinated fibers in the neuropil can be observed. Also, somas and cellular processes displaying a normal aspect are present (arrows). Scale bar represents 50 nm.

eration of caudate nucleus, putamen, globus pallidus, cerebellum and substantia nigra (Gupta *et al*., 1980; Bonilla, 1986) along with morphological alterations in neurons from frontal cortex, hippocampus, midbrain and pons (Cardozo and Bonilla, 1985; Yamada *et al*., 1986).

In rat cerebral cortex, structural changes have been described in mitochondria, synaptic vesicles, endoplasmic reticulum, Golgi apparatus, and lysosomes (Bikashvili *et al*., 2001). Besides, manganese affects the fluidity of the cell membrane (Kumar *et al*., 1996).

This research was designed to study the effects of manganese treatment on the ultrastructure of the olfactory bulb in mice during different periods of exposure to this metal in an attempt to determine the sensitivity of this organ to manganese treatment and its possible role in the pathophysiology of manganese poisoning.

Materials and Methods

Animal treatment

Male albino mice 25-30 g (NMRI-IVIC strain), were fed ad libitum with rat laboratory chow (Ratarina®) and distilled water. Animals were kept at 25ºC under a 12 h light/darkness cycle, and divided in two groups: The first group was inoculated intraperitoneally with a solution containing 5 mg $MnCl_2$ /kg/day (5 days/week). A control group was inoculated with saline. Both groups received a 0.1 ml dose of the appropriate solution during 9 weeks. Five mice from each group were sacrificed by cervical dislocation 2, 4, 6 and 9 weeks after inoculation. Brains were extracted, and the olfactory bulb dissected. The dose of manganese was selected according to previous studies (Bonilla *et al*., 1994).

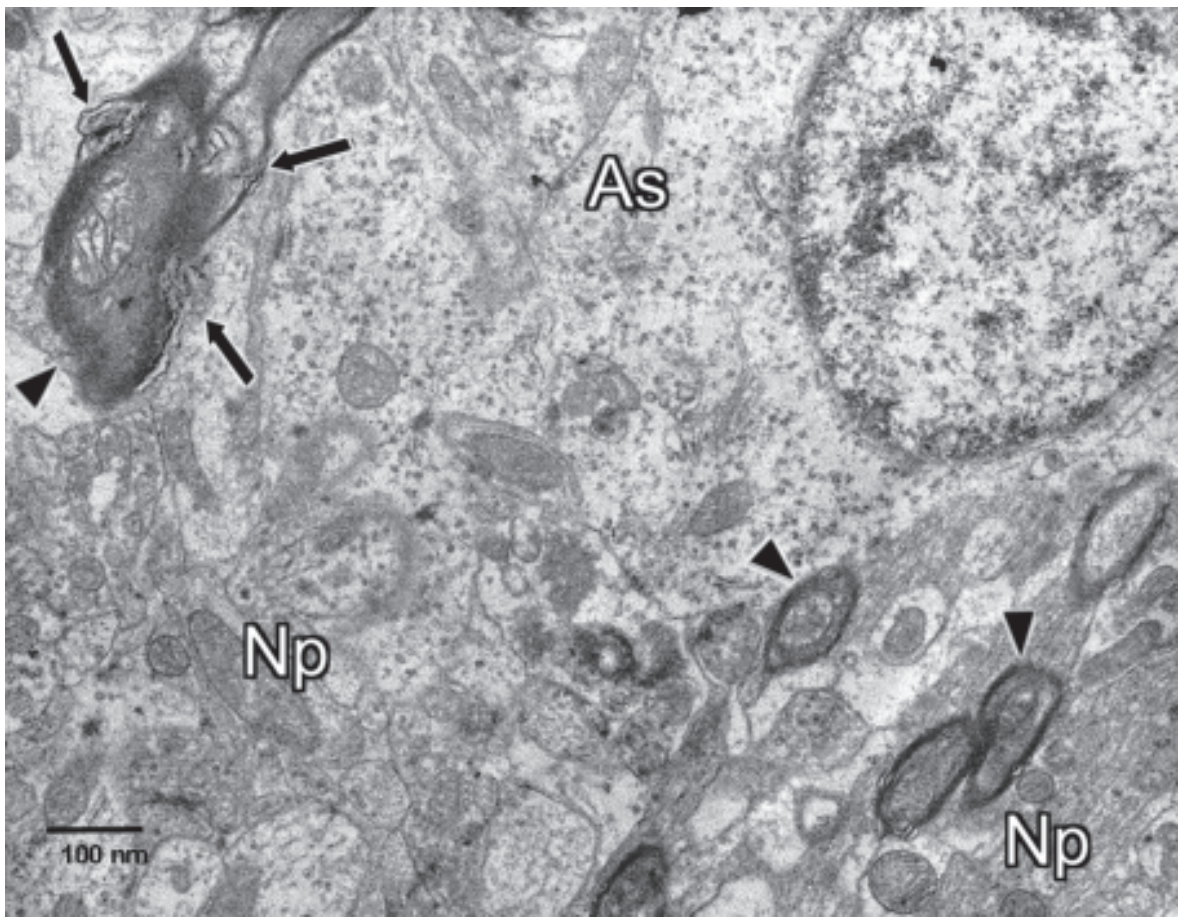

FIGURE 2. Electron micrograph of olfactory bulb from a two weeks manganese-treated mouse, showing a protoplasmatic astrocyte (As), and the surrounding neuropil elements (Np). Disorganization of the myelin periodic pattern (arrows) is seen in some myelinated fibers (arrow heads). Scale bar represents 100 nm.

Electron microscopy

The olfactory bulbs were dissected out from both groups of rats at different stages of intoxication and fixed in glutaraldehyde at 3% in cacodylate buffer 0.1 M; pH 7.4 at 4ºC for 1 to 3 h; they were washed with the same solution during 10 minutes and were then post-fixed in osmium tetraoxide at 1% in cacodylate buffer during 1 hour. The samples were then washed in cacodylate buffer solution 0.1 M, pH 7.4 during 10 minutes. The tissues were dehydrated in increasing concentrations of ethanol (50, 70, 80, 90 and 100%) and in two successive passages of propylene oxide. Afterwards, the samples were embedded in Araldite 502.

Thick sections of approximately 0.5 to 2.0 µm were stained with toluidine blue and examined with a Zeiss® photomicroscope. Ultra thin sections obtained with a Sorvall® MT-2B ultra microtome were stained with 3% uranyl acetate and lead citrate and observed in a Phillips® EM-208 electron microscope.

Manganese determinations

The manganese content in the olfactory bulb was determined by flameless atomic absorption spectrophotometry using a Perkin-Elmer® model 2380AAS with an HGA-2100 graphite furnace. Samples were analyzed by the method of standard additions (Bonilla, 1978). Only the olfactory bulbs obtained from 5 mice of each group after 9 weeks of manganese treatment were examined.

Results

Control group

Electron micrographs of the olfactory bulb in untreated mice showed neuronal somas and processes, along with a normal neuropil with myelinized and unmyelinized fibers. Normal protoplasmic astrocytes and oligodendrocytes were also seen (Fig. 1).

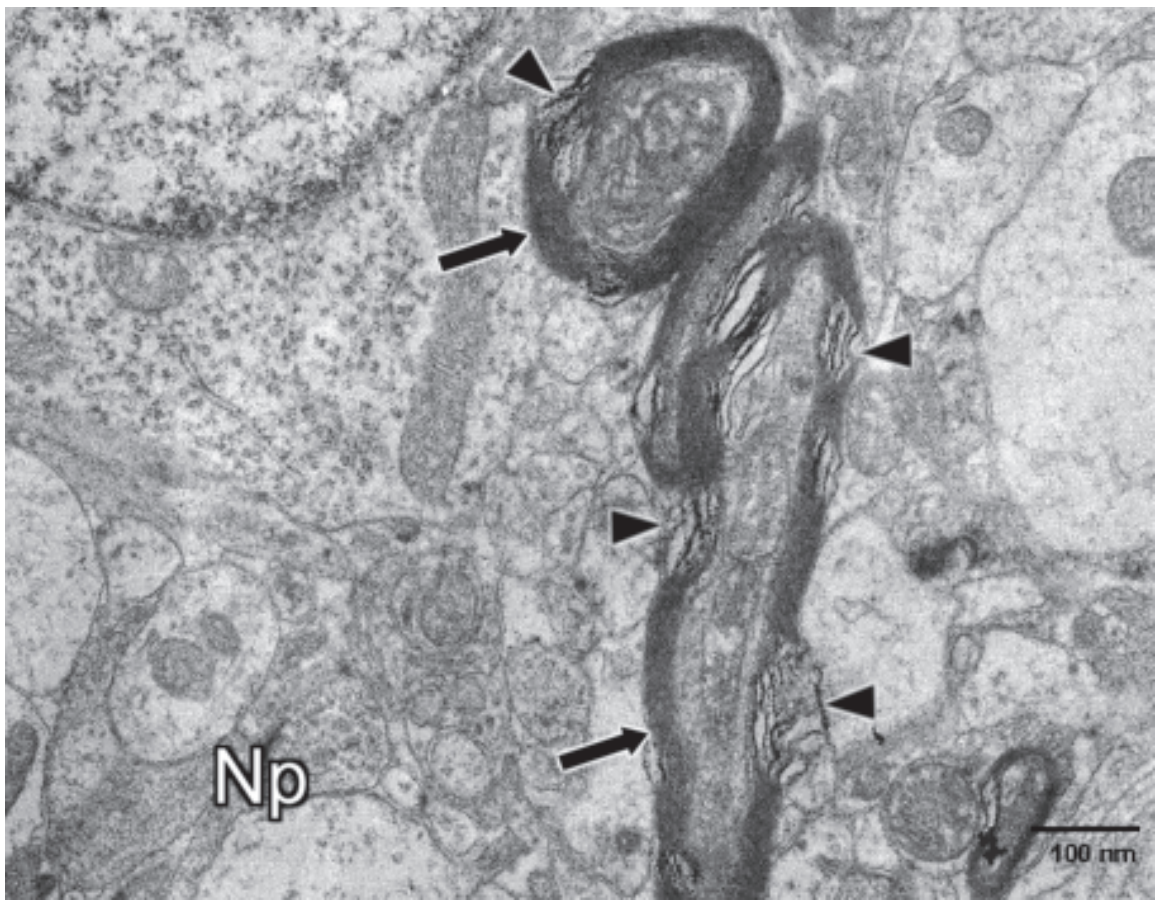

FIGURE 3. Electron micrograph of olfactory bulb from a 2 weeks manganese-treated mouse, where fibers with areas of myelin disorganization can be seen in the neuropil (Np). Scale bar represents 100 nm.

Experimental groups

Ultrastructural analysis of the olfactory bulb in manganese-treated mice showed slight conformational changes in the myelin sheaths of 2-week treated mice (Figs. 2-3).

After four weeks of treatment, altered astrocytes showing a decrease in cytoplasmic organelles were observed (Fig. 4). An electron dense neuronal cytoplasm showing signs of degeneration, dilated cisternae of rough endoplasmic reticulum and swollen mitochondria were also observed (Fig. 5).

Six weeks after treatment a reactive gliosis with predominance of astrocytes (Fig. 6) and a few microglial cells could be observed (Fig. 7). The cytoplasm of these cells contained swollen mitochondria, disorganization of the endomembrane system, and enlargement of the perinuclear cistern. Nuclei showing an irregular contour and an altered pattern of chromatin distribution could also be seen (Fig. 7). The cytoplasm of some glial cells appeared electron dense, with altered mitochondria and disorganization of the inner membrane system (Fig. 8).

Nine weeks after treatment the concentrations of manganese in the olfactory bulb increased 275% from 2.5 ± 0.56 (mean ± SE) ug/g dry weight in controls to 9.38 ± 1.45 in manganese-treated mice ($p<0.01$). The neuronal degeneration increased and the highly vacuolated electron dense cytoplasm with swollen mitochondria, and disorganization of the inner membrane system could be observed. Myelin sheaths presented a marked disorganization of their periodic pattern. An increase in the number of astrocytes whose mitochondrial cristae were partially or totally erased and a dilation of the rough endoplasmic reticulum were found (Figs. 9-10).

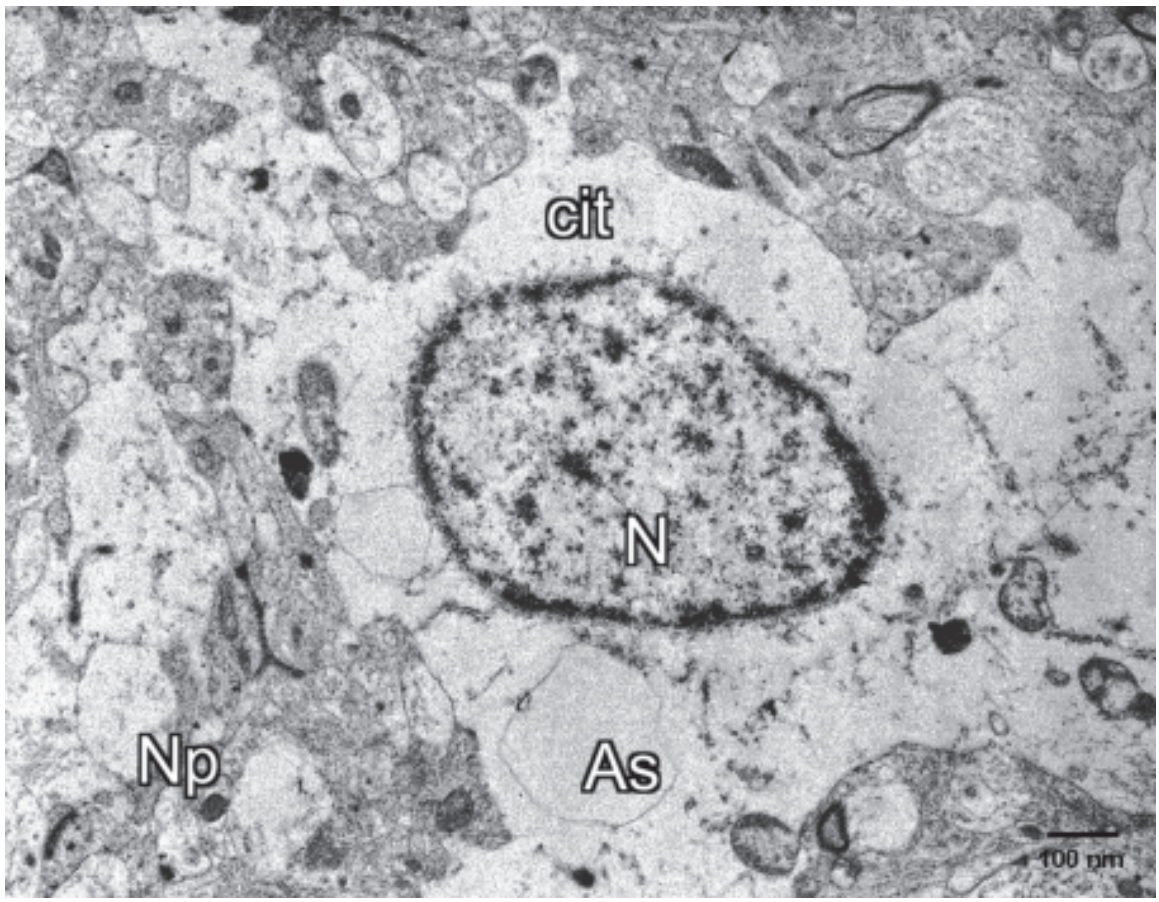

FIGURE 4. Electron micrograph of olfactory bulb from a 4 weeks manganese-treated mouse, where an altered astrocyte (As) showing a large cytoplasm (Cit) with a notorious lack of organelles is seen. The nucleus presents a normal aspect. In the periphery, the neuropil (Np) elements, can be seen. Scale bar represents 100 nm.

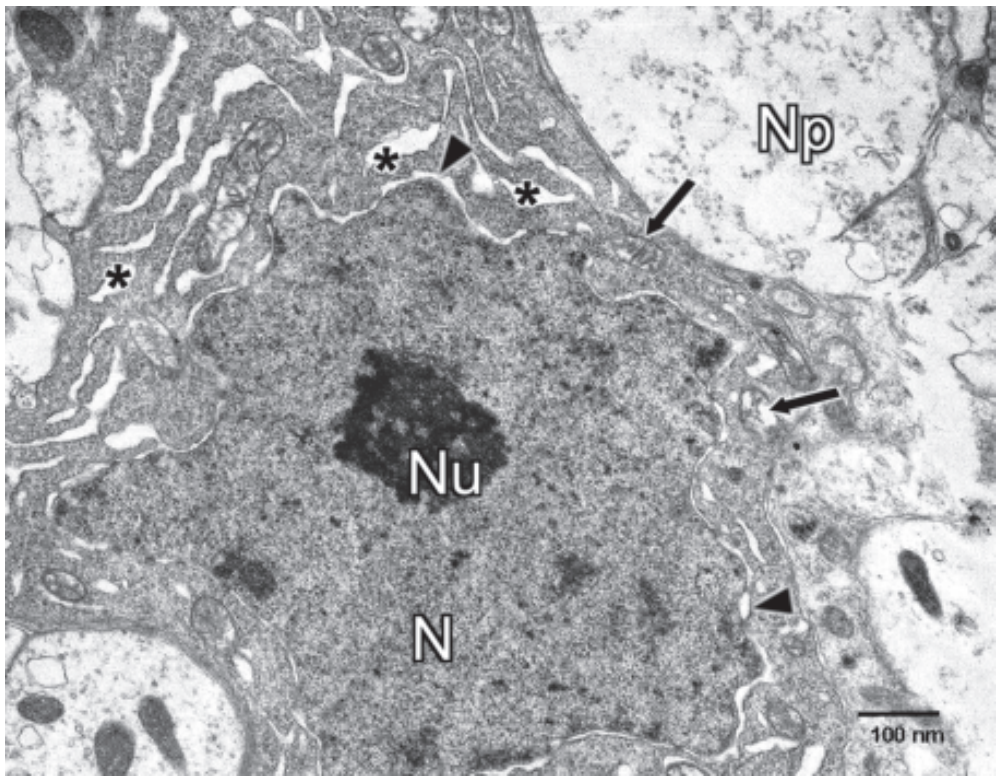

FIGURE 5. Electron micrograph of olfactory bulb from a 4 weeks manganese-treated mouse showing a large degenerated neuron. Swollen mitochondria (arrows), distended cisternae of rough endoplasmic reticulum (*), and the neuropil (Np) can be observed in its highly electron dense cytoplasm. The nucleus (N), and nucleolus (Nu) are surrounded by a barely distended perinuclear cistern (arrow heads). Scale bar represents 100 nm.

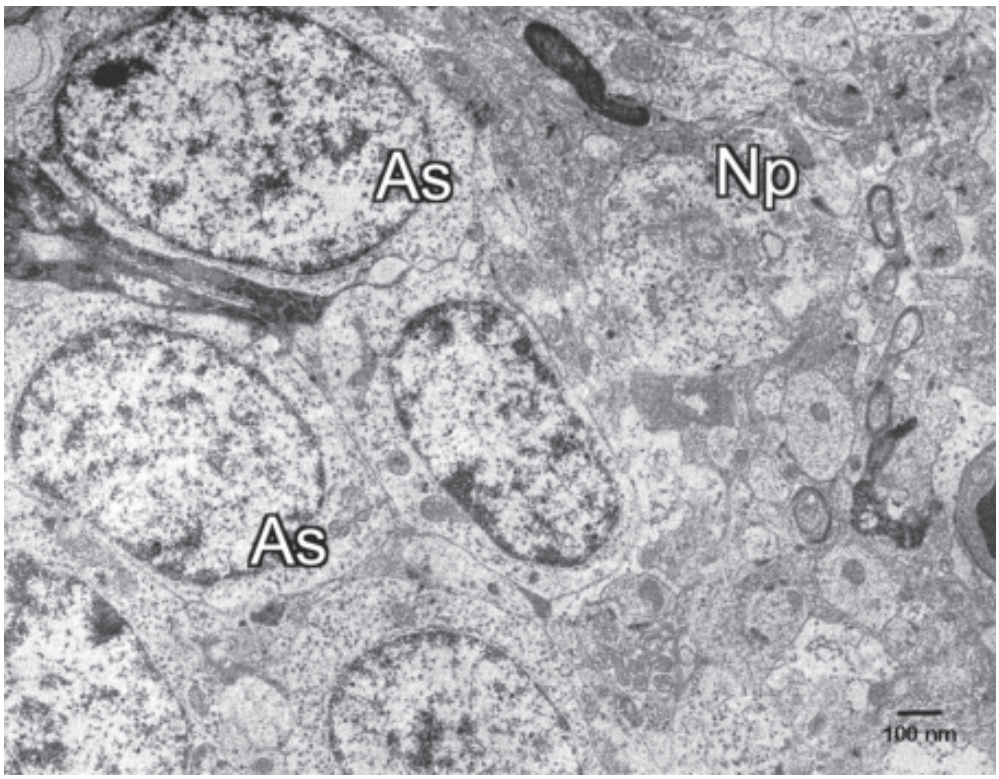

FIGURE 6. Electron micrograph of olfactory bulb from a 6 weeks manganese-treated mouse, where an area of reactive gliosis and astrocyte (As) predominance can be observed. Five astrocytes (As) and surrounding neuropil (Np) are shown. Scale bar represents 100 nm.

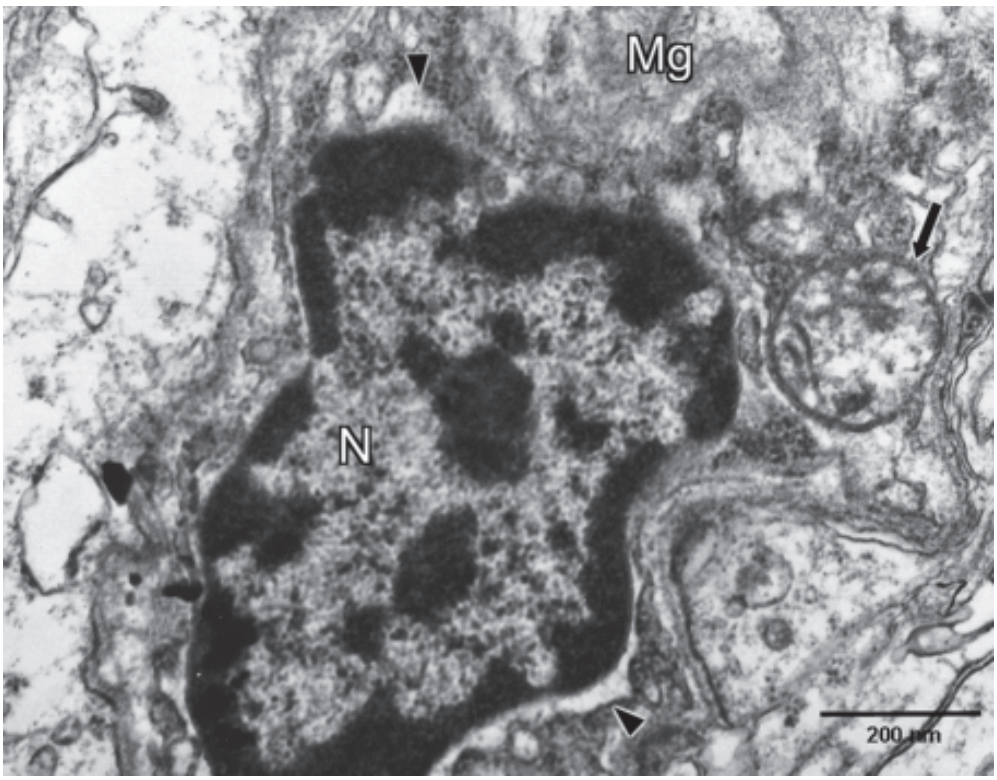

FIGURE 7. Electron micrograph of olfactory bulb from a 6 weeks manganese-treated mouse where an altered glial cell similar to a microglia cell (Mg) may be observed. Notice the distended perinuclear cistern (arrow head) and the presence of swollen mitochondria (arrow) in the cytoplasm. Scale bar represents 200 nm.

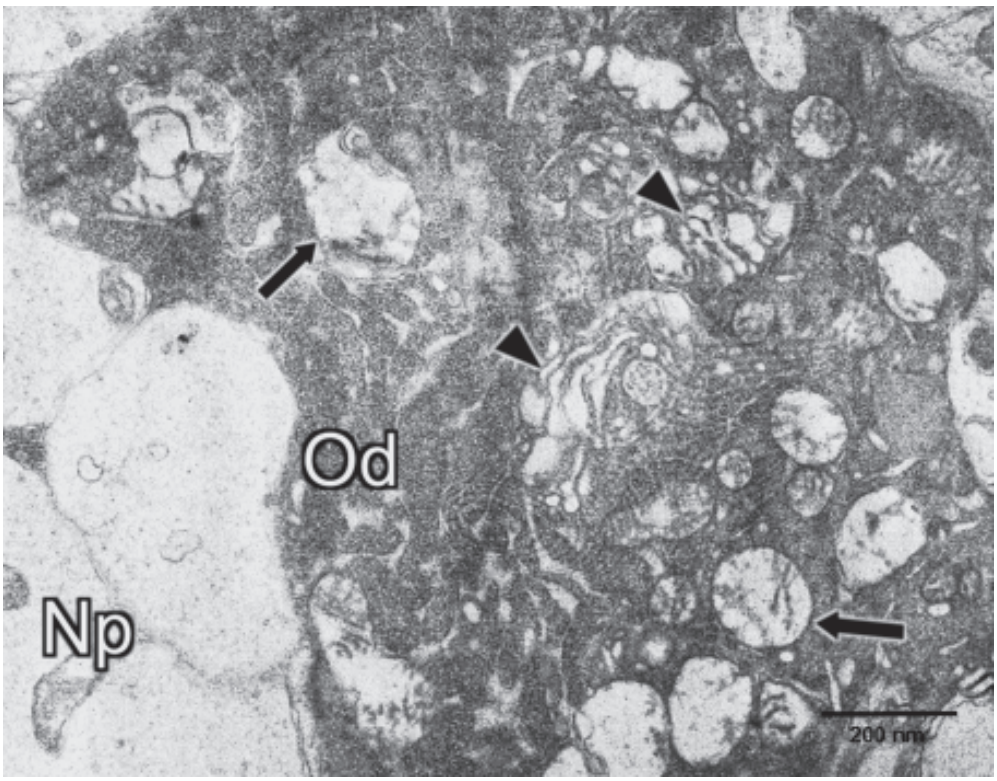

FIGURE 8. Electron micrograph of olfactory bulb from a 6 weeks manganese-treated mouse showing a degenerated glial cell, probably an oligodendrocyte (Od). See the extent of disorganization of the inner membranes, rough endoplasmic reticulum, Golgi (arrowarrowheads), and altered mitochondria (arrows). Scale bar represents 200 nm.

Discussion

Studies performed in humans and rats have shown that the olfactory bulb is one of the brain regions with the highest manganese concentration (Bonilla *et al*., 1982; Bonilla *et al*., 1994; Normandin *et al*., 2002; Normandin and Hazell, 2002), and it represents an important route for manganese transport, storage, and release in the brain, via a retrograde and saturable mechanism (Tjalve *et al*., 1996; Gianutzos *et al*., 1997; Dorman *et al*., 2002). It has been shown that manganese may be taken by piriform olfactory neurons and released near the terminals of the secondary olfactory neurons, and then it is transported to the secondary olfactory cortex (Henriksson *et al*., 1999; Takeda *et al*., 1998). Magnetic resonance imaging with group-wise statistical analysis has demonstrated bilateral transsynaptic manganese transport to secondary and tertiary neurons of the olfactory system (Cross *et al*., 2004).

In our study, alterations of the periodic pattern of myelin in manganese treated mice were found after only two weeks of treatment. Similar changes of the myelin sheath have been described in human brain edema (Castejón, 1985).

Towler *et al*. (2000) showed that manganese treatment caused membrane alterations in the Golgi apparatus and the endoplasmic reticulum of mammalian cells, although the microtubular cytoskeleton was not affected. This observation led these authors to speculate on a more important role of manganese in the regulation of the interactions between intracellular membranes or vesicles and the cytoskeleton. They also observed fragmentation of the Golgi apparatus into disperse smaller structures after manganese treatment and concluded that this cation represents an important tool for the study of protein secretion and the membrane dynamics of the secretory pathway (Towler *et al*., 2000).

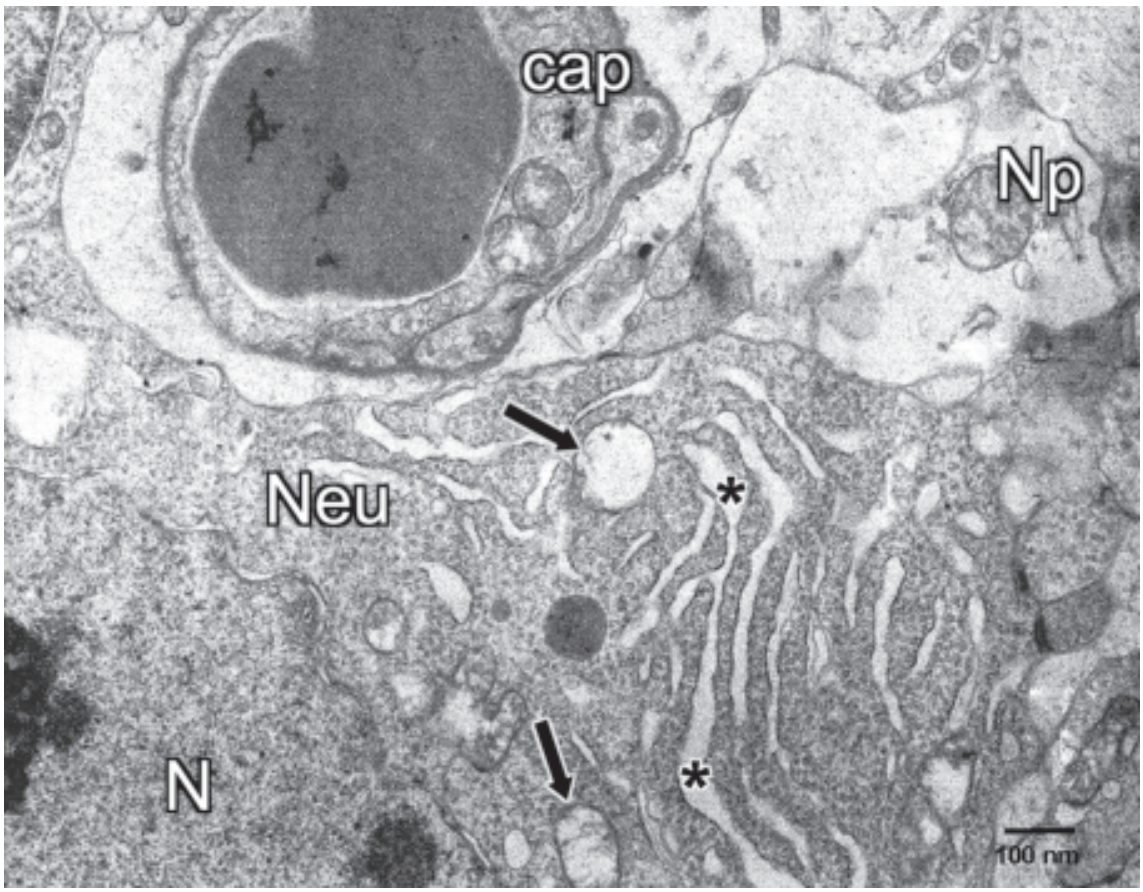

FIGURE 9. Electron micrograph of olfactory bulb from a 9 weeks manganese-treated mouse showing part of a degenerated neuron (Neu). Swollen mitochondria (arrows) and distended cisternae of the rough endoplasmic reticulum (*) can be observed in its highly electron dense cytoplasm. A capillary vessel (cap), and neuropil elements (Np) can be seen in the surrounding area. Scale bar represents 100 nm.

The degenerated neurons, with electron dense cytoplasm, enlarged rough endoplasmic reticulum and swollen mitochondria, seen after 4 weeks of manganese treatment have been described in brain tumors, trauma and congenital malformations (Long, 1970; Castejón, 1985; Shibata, 1989).

Bikashvili *et al*. (2001) reported hyperplasia of the vacuolar system and swollen mitochondria in the neurons of the cerebral cortex in rats treated with 50 mg Mn /Kg for 30 days. They also observed changes in the synapsis including a reduction in the number of synaptic vesicles and mitochondrial degeneration.

Shukakidze *et al*. (2001) studied the ultrastructure of astrocytes, oligodendrocytes and microglia in the caudate nucleus, substantia nigra and cerebral cortex of rats receiving orally 20 mg MnCl2/Kg/day/30 days and reported mitochondrial destruction, enlargement of endoplasmic reticulum and glycogen accumulation. Oligodendrocytes were resistant to manganese. Using a higher dose of 50 mg of $MnCl_2$/Kg/day, they found an increase in the astrocyte phagocytic activity. Their findings are similar to those observed by us in the olfactory bulb after 6 weeks of manganese treatment. In fact, we found that nine weeks after treatment, the astrocytosis was increased and in the astrocytes as well as in markedly electron dense neurons great numbers of altered mitochondria were observed along with huge vacuolation and disorganization of the cytoplasmic inner-membrane system: rough endoplasmic reticulum and Golgi apparatus. These observations are also similar to those observed by Shukakidze *et al*. (2001) in other brain regions.

Manganese is accumulated in mitochondria and mitochondria-rich tissues *in vivo*. That is why this organelle is so important in the neurotoxicity of manganese (Gavin *et al*., 1992). Experimental evidence has shown that this metal causes neuronal degeneration through indirect excitotoxic processes secondary to an alteration of the oxidative energetic metabolism (Brouillet *et al*., 1993).

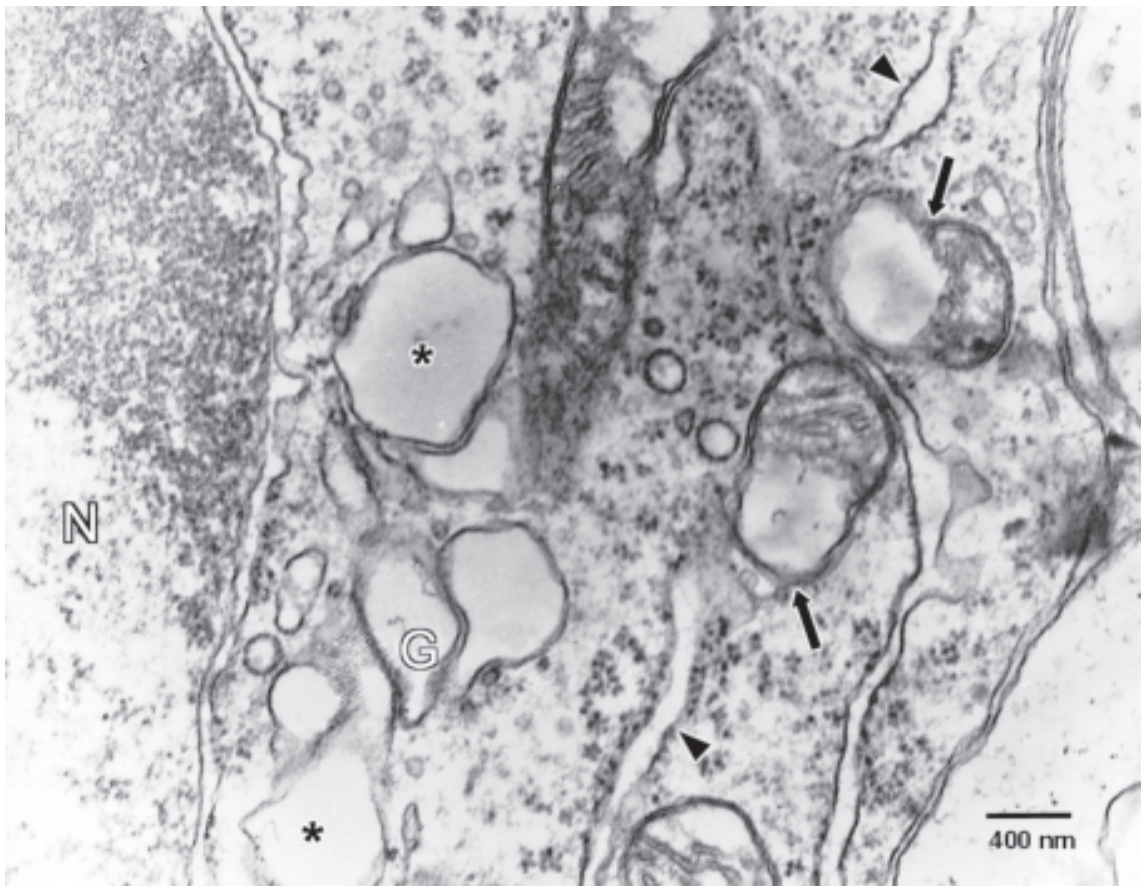

FIGURE 10. Electron micrograph of olfactory bulb from a 9 weeks manganese- treated mouse showing part of a degenerated glial cell. See the vacuolation of cytoplasm (*) and the extent of inner membranes disorganization (arrow heads). The Golgi (G) complex and altered mitochondria (arrows) are also shown. Scale bar represents 400 nm.

Changes in oxidative metabolism caused by manganese have been linked to cell apoptosis (Hirata *et al*., 1998). These neurotoxic properties are shared with polyvalent metals like zinc (Kim *et al*., 1999; Lobner *et al*., 2002).

Suzuki *et al*. (1984) have suggested that lysosomes play an important role in manganese metabolism and the development of this metal toxicity. In fact, their studies demonstrated that 24 h after receiving an intraperitoneal dose of 245 mg of manganese acetate most of the manganese taken by the mouse brain was recovered in the mitochondrial and lysosomal fractions, mainly in the latter.

Our results show that the olfactory bulb is highly affected by chronic exposition to manganese. In fact, it has been shown that it is very sensitive to the oxidative stress induced by elevated concentrations of this metal (Dobson *et al*., 2003; Erikson *et al*., 2005). The connections of olfactory bulb with the brain limbic region suggest the possibility that in humans the initial psychiatric symptoms of manganese poisoning may originate in the olfactory-limbic pathway.

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