Manganese Taken Up into the CNS via the Olfactory Pathway in Rats Affects Astrocytes

Jörgen Henriksson and Hans Tjälve

Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, BMC, Box 573, SE-751 23 Uppsala, Sweden

Received October 29, 1999; accepted February 10, 2000

Manganese (Mn), administered intranasally in rats, is effectively taken up in the CNS via the olfactory system. In the present study, Mn (as MnCl₂) dissolved in physiological saline, was instilled intranasally in rats at doses of 0 (control), 10, 250, or 1000 µg. At the start of the experiment each rat received an intranasal instillation. Some rats were killed after one week without further treatment (the 1-w group), whereas the remaining rats received further instillations after one and two weeks and were killed after an additional week (the 3-w group). The brains were removed and either used for ELISA-determination of the astrocytic proteins glial fibrillary acidic protein (GFAP) and S-100b or histochemical staining of GFAP and S-100b, microglia (using an antibody against the iba1-protein) and the neuronal marker Fluoro-Jade. There were no indications that the Mn induced neuronal damage. On the other hand, the ELISA showed that both GFAP and S-100b decreased in the olfactory cortex, the hypothalamus, the thalamus, and the hippocampus of the 3-w group. The only effect observed in the 1-w group was a decrease of S-100b in the olfactory cortex at the highest dose. The immunohistochemistry showed no noticeable reduction in the number of astrocytes. We assume that the decreased levels of GFAP and S-100b are due to an adverse effect of Mn on the astrocytes, although this effect does not result in astrocytic demise. In the 3-w group, exposed to the highest dose of Mn, increased levels of GFAP and S-100b were observed in the olfactory bulbs, but these effects are probably secondary to a Mn-induced damage of the olfactory epithelium. Our results indicate that the astrocytes are the initial targets of Mn toxicity in the CNS.

Key Words: manganese; intranasal administration; olfactory pathway; astrocytes; GFAP; S-100b.

We have previously shown that Mn administered intranasally in rats is effectively taken up in the CNS via the olfactory system (Henriksson et al., 1999; Tjälve et al., 1995, 1996). The transport and distribution of Mn initially followed the primary, secondary, and tertiary olfactory neurons resulting in high levels of the metal in areas such as the olfactory bulb, the olfactory cortex, the hypothalamus, the thalamus, the hippocampus, and the habenular complex. At later intervals, Mn was seen to migrate to all parts of the brain, and even into the spinal cord. Thus the olfactory route provides a pathway for Mn which comes in contact with the olfactory epithelium to pass directly to the brain, thereby circumventing the blood-brain barrier. The intranasal route of exposure was found to result in a much higher accumulation of the metal in the brain compared to intraperitoneal administration (Tjälve et al., 1996). We have proposed that the neurotoxicity of inhaled Mn is related to an uptake of the metal into the CNS via the olfactory pathway.

In the present study, we have examined whether Mn taken up in the CNS via the olfactory route in rats will induce toxic effects in the brain. As an index of neuronal toxicity, we used Fluoro-Jade histochemistry, which has been shown to be a sensitive and reliable method to detect degenerating neurons (Schmued et al., 1997). Changes in astrocytes can be used as markers for various types of damage to the CNS. In the present study, determination of the astrocytic proteins glial fibrillary acidic protein (GFAP) and S-100b, using enzyme linked immunosorbent assay (ELISA) and immunohistochemistry, were used to evaluate effects of Mn in the CNS. GFAP is the major intermediate filament protein of astrocytes (Eng, 1988). S-100b is a cytosolic, low-molecular-weight calcium-binding protein, which belongs to a family of closely related proteins thought to play a role in cell growth and differentiation (Fano et al., 1995). We also examined microglia. The most characteristic feature of this cell type is a rapid activation in response to even minor pathological changes in the CNS (Kreutzberg, 1996). This activation occurs as a graded response in vivo (Kreutzberg, 1996). We used immunohistochemistry of iba1 to examine if Mn will induce microglial activation; iba1 is a calcium-binding protein (ionized calcium-binding adapter mole-

1 To whom correspondence should be addressed. Fax: +46-18-504144. E-mail: jorgen.henriksson@farmtox.slu.se.
cule 1) present in resting, activated, and phagocytic microglia (Imai et al., 1996; Ito et al., 1998; Ohsawa et al., 1997).

MATERIALS AND METHODS

Materials. MnCl₂ · 4 H₂O was obtained from Sigma (St. Louis, MO). Unless otherwise specified, other chemicals were purchased from Kebo AB (Stockholm, Sweden).

Animals. Male Sprague-Dawley rats weighing about 150 g were obtained from Bantin and Kingman (Sollentuna, Sweden). The animals were housed at 22°C with a 12-h light/dark cycle, with free access to tap water and a standard pellet diet (Lactamin AB, Vadstena, Sweden). The studies were approved by the Local Ethics Committee for Animal Research.

Animal treatment and dissection. Manganese (MnCl₂), dissolved in physiological saline, was instilled intranasally in the right nostril in rats at doses of 0 (control = NaCl), 10, 250, or 1000 µg. Each group consisted of 9 (Mn 10 and Mn 250) or 11 animals (NaCl and Mn 1000). Six animals of each group were used for biochemical studies and 3 for histopathological examination of the nose. In addition, 2 animals of the NaCl- and Mn-1000 groups were used for immunohistochemistry. Two survival periods were used: 1 and 3 weeks. The rats were anesthetized by pentobarbital sodium (30 mg/kg body weight [bw] ip), and 10 µl of the manganese solution was instilled (0.5 µl/s) in the nostril by means of a polyethylene tubing connected to a Hamilton syringe, as described previously (Tjälve et al., 1996). At the start of the experiments, each rat received an intranasal instillation. Some animals were killed after one week without further treatment (the 1-w group), whereas the remaining rats received further instillations after one and two weeks and were killed after an additional week (the 3-w group). All animals were killed by CO₂ asphyxiation, and the brains were removed and used either for histochemical or biochemical studies. For the biochemical studies the brains were quickly removed and dissected according to the procedure described by Henrikkson et al. (1999). The following brain parts were taken: the olfactory bulb, the olfactory cortex, the corpus striatum, the hippocampus, the hypothalamus, and the thalamus. The right brain parts were collected in 1.5 ml Eppendorf vials, weighed, quickly frozen in CO₂-ice, and stored at –80°C, until further treatments. For histochemical and histopathological examination, the rats were killed by CO₂ asphyxiation and the brains were removed, fixed in formaldehyde solution, and processed for paraffin embedding. For the histopathology, the nasal region was dissected and the nasal passages were gently flushed with the formaldehyde solution. Following the fixation, the tissues were dehydrated in 5.5% EDTA for 2 weeks and then embedded in methacrylate (Technovite 7100, Heraeus Kulzer, Wehrheim, Germany).

ELISA. Brain-parts were homogenized in their respective Eppendorf vials, using an Eppendorf micromill, in 15 vol. of 50-mM phosphate buffer, pH 7.4, containing 0.15% sodium dodecyl sulphate (SDS), 0.02% EDTA, and 0.5-mM phenylmethylsulfonyl fluoride (PMSF), mainly as described by Griffin et al. (1993). The homogenates were then centrifuged at 4°C at 10,000 × g for 15 min, and the supernatants were collected for protein determination and ELISA of GFAP and S-100b. Aliquots of the supernatants were stored at –80°C until they were analyzed. A sandwich ELISA method, based on the procedures described by O’Callaghan (1991) and Green et al. (1997), was used to measure the content of GFAP and S-100b. Purified bovine GFAP (code 77–106–2; ICN) and monoclonal mouse-anti-bovine S-100b (code 814369; Boehringer Mannheim; 1:500) or rabbit-anti-bovine S-100 conjugated to horseradish peroxidase (HRP) (code U29; DAKO; 1:1000)—both antibodies being diluted with 1% BSA-Wash solution—the plates were incubated for 1 h at room temperature. For the determination of the GFAP, 4 additional washes were performed with Wash solution, and the plates were then incubated for 30 min at 37°C with 100 µl HRP-conjugated goat-anti-mouse Ig (code P447; DAKO) diluted with 1% BSA-Wash solution. Phenylenediamine dihydrochloride (code S2045; DAKO) was used as substrate for the HRP. Thus, after washing the HRP-treated plates 3 times with Wash solution, 100 µl of freshly prepared 1.2-phenylenediamine dihydrochloride was added and the plates were incubated for 20 min at room temperature. Finally, 100 µl of 0.5 M H₂SO₄ was added to stop the color reaction. The optical density (OD) was measured at 490 nm using a Victor-3™ Multilabel Counter (WALLAC, Sweden). The OD values were corrected by subtracting the background OD of wells receiving nothing but antigen. The microtiter plates were organized to contain duplicates from the same brain parts from all animals, with the same survival interval as well as standards. All measurements were made in duplicate, and repeated if the difference within the duplicates was more than 10%.

Protein assay. Total protein in the supernatants of the brain was assayed by the method of Smith et al. (1985), adapted for microplate readers. BSA served as standard.

Histochemistry. Rabbit-anti-cow GFAP (code Z334; DAKO; 1:1500) and monoclonal mouse-anti-bovine S-100b (code S2532; Sigma; 1:200) were used for immunohistochemistry of the astrocytic markers GFAP and S-100b. For microglial immunohistochemistry, we used a rabbit antibody against iba1. The iba1-antibody was kindly provided by Dr. Y. Imai, Keio University Japan, via Dr. J. Isaksson, Department of Pathology, Uppsala University Sweden; the dilution used was 1:800. All immunohistochemical reactions were performed according to the Avidin-Biotin technique. Deparaffinized 5-µm sagittal sections of the brain were treated in a microwave oven for 2 × 5 min in 0.01-M citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with 1% H₂O₂ in phosphate-buffered saline containing 0.3% Triton X-100 (PBS-T). After one rinse in PBS, the nonspecific binding was blocked by incubating the sections for 30 min in PBS, containing 4% BSA. All antibody dilutions were made in 0.1 M PBS, pH 7.4, containing 4% BSA and the antibody exposure was performed at 4°C overnight. Following 3 rinses in PBS-T, and 1 rinse in PBS, a secondary antibody was applied for 30 min at room temperature. The secondary antibodies were used were swine-anti-rabbit Ig (code E353; DAKO; 1:500) for the GFAP and the iba1 whereas rabbit-anti-mouse Ig (code E464; DAKO; 1:1500) was used for the S-100b. Then the sections were incubated with the avidin-biotin-peroxidase complex for 30 min at room temperature, according to the suppliers recommendations (code K355; ABComplex/HRP, DAKO). Following 3 rinses in PBS-T, and 1 rinse in PBS, the sections were treated with 3,3’-diaminobenzidine (DAB)-H₂O₂ for color development. In order to visualize neuronal degeneration, deparaffinized 5-µm sagittal sections of the brain were applied for histochemistry using the fluorochrome Fluoro-Jade (Histo-Chem Inc., AR, USA), according to the method described by Schmued et al. (1997). Fluoro-Jade is an anionic tribasic fluorescent derivative with a specificity for degenerating neurons. These neurons presumably express a strongly basic molecule, since they have an affinity for the strongly acidic Fluoro-Jade (Schmued et al., 1997). In the staining procedure, formaline-fixed tissue sections were treated with Fluoro-Jade solution and examined with an epifluorescence microscope, using a filter system suitable for visualizing fluoroescein or FITC.

For each of the GFAP-, S-100b-, iba1- and Fluoro-Jade histochemistry, 3 sections from the right side of the brain were taken from each of 3 different levels of each animal. The localization of the levels were as follows: a sagittal plane close to the midline of the brain; a sagittal plane in the middle of the right
olfactory bulb; and a sagittal plane immediately lateral to the right olfactory bulb. The slides were coded, randomized, and evaluated with regard to the different methods used. Serving as negative controls, sections were treated as described above, with the exception that the primary antibody or Fluoro-Jade was omitted. All negative controls were negative. Positive controls for the Fluoro-Jade consisted of sections from an animal treated with kainic acid (9 mg/kg, ip) and killed after 4 days. Sections of the positive control displayed a staining, in accord with the literature (Schmued et al., 1997).

**Histopathology of the nose.** Transversal sections of the olfactory region of the nose (2-μm thick; 3 sections at 3 different regions) were cut and stained with hematoxylin/eosin and toluidine blue (to show the general tissue-morphology), and periodic acid-Shiff (PAS) (to visualize mucopolysaccharides and mucoproteins). Slides were coded, randomized, and evaluated morphologically.

**Statistics.** All data are expressed as mean ± standard deviation. All data analyses were performed on untransformed values. The concentrations of the astrocytic proteins GFAP and S-100b were analyzed by a 1-way analysis of variance (ANOVA) for each brain part at each survival interval. Significant differences between the groups were evaluated using Fischer’s protected least-significant-difference as a post hoc analysis. Statistical analyses were carried out with StatView 5.0 statistical software (SAS Institute, Inc., USA). The level of statistical significance was set at $p < 0.05$.

**RESULTS**

**ELISA**

**GFAP.** At 1 week, there were no significant changes of GFAP between the Mn-treated animals and the controls (Fig. 1).

At 3 weeks, there was a significant decrease of GFAP, compared to controls, in the olfactory cortex and the thalamus in the Mn-250 and Mn-1000 groups and the hypothalamus and the hippocampus in the Mn-10, Mn-250, and Mn-1000 groups (Fig. 2). There was a significant increase of GFAP in the olfactory bulb in the Mn-1000 group.

**S-100b.** At 1 week, the only effect observed was a significant decrease of S-100b in the olfactory cortex in the Mn-1000 group (Fig. 1).

At 3 weeks, there was a significant decrease of S-100b compared to the controls in the hypothalamus, the hippocampus, and the thalamus in the Mn-250 and Mn-1000 groups (Fig. 2). There was a significant increase in the olfactory bulb in the Mn-1000 group.

**Immunohistochemistry**

**GFAP.** The GFAP-immunohistochemistry showed cells with a predominant labeling of the fibrillary processes, as described previously (Boyes et al., 1986; Raivich et al., 1999). The general appearance of the immunopositive cells, including the areas covered by the individual cells, were similar in the 2 exposure groups (control and Mn 1000) at both survival intervals (1 week and 3 weeks). In the corresponding brain parts, the number of immunopositive astrocytes was also similar. The only difference observed was a slightly decreased intensity of the staining of the cells in the hypothalamus and the hippocampus and a slightly increased intensity of the staining of the cells in the granular layer of the olfactory bulb in the Mn-1000 group, as compared to the controls at the 3-week survival interval (data not shown).

**S-100b.** The S-100b immunohistochemistry showed a predominant cytoplasmic staining of astrocytes, as described previously (Boyes et al., 1986; Haan et al., 1982). As for the GFAP, there was no difference in the general appearance or number of stained S-100b-positive cells between the different groups. Neither was it possible to detect any differences in the intensity of the staining of the astrocytes between the groups.

**Microglia.** The iba1 immunohistochemistry showed a selective staining of the microglial cells. Only cells with a typical resting-cell appearance were seen. No significant differences
could be observed between the controls and the Mn-1000 group.

Fluoro-Jade. No signs of damaged neurons were seen in the controls or in the Mn-1000 groups.

Histopathology

At 1 week, the morphology of the olfactory mucosa of the various groups of rats instilled with Mn solution did not differ from the controls. In all animals, the outermost surface of the epithelium on the side of the application was detached in a few limited areas of the septum, the roof of the nasal cavity, and the medial aspects of the endoturbinates. We consider that this is a mechanical injury of the epithelium at the application of the solution.

At 3 weeks, the histopathological examination showed an attenuation and a disorganization of the olfactory mucosa in some areas of the septum, the roof of the nasal cavity, and the medial aspects of the endoturbinates in the Mn-1000 group. The appearance of the olfactory mucosa in the control, the Mn-10, and the Mn-250 groups was similar to that observed at the 1-week survival interval.

DISCUSSION

The Fluoro-Jade histochemistry, which reveals neuronal degeneration (Schmued et al., 1997), showed no signs of damaged neurons. In addition, our results showed absence of microglial activation, which is considered to be a sensitive marker of neuronal damage (Raivich et al., 1999). Based on these data, we conclude that the Mn in our study induces no detectable toxicity towards the neurons.

In contrast to the lack of effect of Mn on the neurons, the ELISA showed a decrease of GFAP and S-100b in several brain areas, such as the olfactory cortex, the hypothalamus, the thalamus, and the hippocampus. The immunohistochemistry showed no noticeable reduction in the number of astrocytes. It is considered that immunohistochemistry of S-100b is a reliable method to evaluate the number of astrocytes (Boyes et al., 1986; Haan et al., 1982). On the other hand, it may be difficult to perform quantitative evaluations of the S-100b and GFAP contents in the cells based on the intensity of the immunohistochemical staining. This may explain why some of the quantitative effects observed by the ELISA were not always apparent in the immunohistochemistry. We conclude that the effect of Mn on the astrocytes does not result in cell death. This conclusion is further supported by the absence of microglial activation, since microglia have been reported to respond to degenerating astrocytes or astrocytes undergoing apoptosis (Khurgel et al., 1996; Lafarga et al., 1998; Penkowa et al., 1997, 1999).

It is well known that neuronal injuries induce secondary hypertrophy of the astrocytes, with enhanced expression of GFAP (O’Callaghan et al., 1995). Decreased levels of GFAP are not so commonly reported but have been taken as evidence of astrocytic damage. This has, e.g., been observed after excessive ammonia accumulation within the CNS such as occurs in hepatic encephalopathy, or congenital and acquired hyperammonemia (Albrecht, 1996), an astrocytic infection by a herpes virus in mice (Itoyama et al., 1991), acute glial cell damage after status epilepticus induced by pilocarpine in rats (Schmidt-Kastner and Ingvar, 1994), and intracerebral injection of the astrocytic toxin α-aminoacidic acid in rats (Khurgel et al., 1996). In addition to causing a decrease of GFAP, α-aminoacidic acid induces a loss of S-100b and it was proposed that this reflects a damage of the integrity of the astrocytes (Khurgel et al., 1996). Astrocytes play an important role in normal brain functions, such as regulation of ion homeostasis, uptake of neurotransmitters and contribution to the CNS immune system and direct effects of chemicals on astrocytes may contribute to abnormal brain functions (Aschner, 1998).

FIG. 2. Concentrations of GFAP and S-100b in various parts of the brain in rats killed 1 week after 3 successive weekly intranasal administrations of physiological saline only (control) or different amounts of Mn dissolved in physiological saline. The different groups are NaCl (only physiological saline), Mn 10 (10 mg Mn), Mn 250 (250 mg Mn) and Mn 1000 (1000 mg Mn). The results show the means ± SD of 5–6 rats. *p < 0.05, **p < 0.01, as compared to NaCl (statistical significance evaluated by ANOVA and Fischer’s protected least-significant difference as post hoc analysis).
Damage to astrocytes by Mn may be due to a high uptake of the metal into these cells. It is known that about 80% of the Mn in the brain is present in astrocytes as a functional component of glutamine synthetase (Wedler and Denman, 1984). A specific transport system for Mn, with a relatively high capacity, has been shown in cultured rat astrocytes (Aschner et al., 1992). Results of a recent study at our laboratory have indicated that manganese, during its transport in the olfactory system and the brain, is taken up to a considerable extent by the astrocytes (Henriksson et al., 1999). Studies by Sloot and Gramsbergen (1994) have shown that following striatal injections of Mn, more metal will be taken up into a quinolinic acid-lesioned striatum, which is depleted of intrinsic nerve cells and contains an abundant amount of glial cells, than into a non-lesioned striatum.

The mechanisms by which Mn may induce astrocytic toxicity are not known. Mn has been shown to accumulate in mitochondria in cultured chick glial cells by an active-transport mechanism (Wedler et al., 1989). It has been proposed that Mn may interfere with mitochondrial function in neurons, both directly and by disrupting Ca\(^{2+}\) homeostasis (Gavin et al., 1999), and this may also apply to astrocytes. Another possibility is that Mn may induce formation of toxic levels of nitric oxide within the astrocytes. It has been shown that Mn, in contrast to Co, Cu, and Fe, induces nitric oxide synthase and augments nitric oxide synthesis in murine astrocytes in vitro (Spranger et al., 1998). There is circumstantial evidence from a variety of sources that implicate involvement of glial cells and free radicals, formed from the reaction between NO and superoxide to form peroxynitrite, in degenerative conditions of the CNS such as Parkinson's and Alzheimer's diseases (Merrill and Murphy, 1996).

It can be observed that the olfactory cortex, the hypothalamus, the thalamus, and the hippocampus, which all showed a decrease of GFAP and S-100b, are regions with prominent connections with the olfactory bulb and/or the olfactory cortex (Allison, 1953; Brodal, 1981; Cragg, 1961; Haberly, 1990; Price, 1985; Schwertdfeger et al., 1990). These areas have previously been shown to accumulate high amounts of Mn at short intervals after intranasal administration of the metal (Tjälv et al., 1996). It is interesting to notice that Pappas et al. (1997) observed a significant decrease of GFAP in the dentate gyrus of the hippocampus in rats exposed to Mn. In that study MnCl\(_2\) was dissolved in the drinking water of dams and their litters from conception until postnatal day 30. The decreased GFAP was detected at postnatal day 32. Actually, in this study, other effects of the Mn-treatment were minimal. In our investigation, no effect of Mn was observed in the corpus striatum. Similarly, the studies of Pappas et al. (1997) showed unaltered GFAP-levels in the caudate of the corpus striatum. In humans and non-human primates, the corpus striatum—especially the globus pallidus—appears to be a primary target of Mn neurotoxicity (Olanow et al., 1996; Shinotoh et al., 1997; Verity, 1999; Yamada et al., 1986). The reason for this discrepancy between primates and rodents is unclear.

Results of experimental studies indicate that rodents may be more resistant to Mn than non-human primates. In fact, it has been reported that it is difficult to induce clinical symptoms or pathological effects by Mn in rodents (Kristensson et al., 1986; Pappas et al., 1997). Most experiments in rodents have been performed during relatively short survival intervals and the interpretation of such studies is that acute or subacute Mn exposure is not particularly neurotoxic in these species (Kristensson et al., 1986; Pappas et al., 1997). In the work environment, symptoms appear after months or years of occupational exposure. In the present study, the longest survival period was 3 weeks, and pronounced reductions of GFAP and S-100b were not observed prior to this interval. Conceivably, a longer exposure period might have resulted in more marked effects, which in addition also might have involved neurons.

In contrast to the decrease of GFAP and S-100b in the olfactory cortex, the hypothalamus, the thalamus, and the hippocampus, significantly increased levels of these proteins were observed in the olfactory bulb in the 3-w group at the highest dose of Mn. It has been shown that injuries to the olfactory mucosa can cause reactive astrocytosis in the olfactory bulb (Bergman and Brittebo, 1999; Deamer et al., 1994; Genter et al., 1992). Our results showed attenuation and disorganization of the olfactory epithelium in some areas of the nasal cavity, following repeated high-dose Mn instillations (i.e., in this 3-week group). We therefore interpret the increase of GFAP and S-100b in the olfactory bulbs as being secondary to damage by Mn on the olfactory epithelium.

In conclusion, our results suggest that the first targets of Mn toxicity in the CNS are the astrocytes and that the adverse effects of the metal on neurons are secondary to an impaired function of the astrocytes.

ACKNOWLEDGMENTS

We thank Dr. Yoshinori Imai (Department of Neurochemistry, Keio University, Japan) for the kind gift of rabbit-anti-ib1, obtained via Dr. Jonas Isaksson (Department of Pathology, Uppsala University, Sweden). We also wish to thank Ms. Agneta Bostrom for excellent technical assistance. This study was supported by the Swedish Council for Work Life Research (RALF) and by the Foundation for Strategic Environmental Research (MISTRA).

REFERENCES


