# NEUROTOXIC EFFECTS OF MANGANESE OXIDE NANOPARTICLES IN RATS

## PhD Thesis

**Leila Sárközi**

**Department of Public Health**

**University of Szeged Faculty of Medicine**

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## Introduction

The state of the environment and human health are in close connection. Among its main elements, air is the most sensitive to pollution and the most mobile carrier medium, able to quickly transport the particles far away from the place of emission, so that air pollution concerns the biggest part of the human population.

Entrance of materials into the air can occur from both natural and man-made sources. The fate of pollutants in the air depends on physical and chemical factors, whereby particle size is one of the most determinative properties. Airborne particles can by their size be grouped as dusts (>10 μm, quick sedimentation), fine dusts or fumes (100 nm-10 μm) and ultrafine particles or Nanoparticles (UFPs, NPs, <100 nm). In this study, the effects of the UFPs were investigated, because these are the most persistent substances in the air. Airborne UFPs may contain several toxic heavy metals, emitted into the air by industrial processes, transportation and utilization of agrochemicals, and from household articles. Chronic exposure to certain heavy metals (e. g. manganese, mercury, cadmium, lead) can lead to functional impairment of the nervous system. In this study we investigated the neurotoxic effects of nanosized Mn.

Normal background level of UFPs in the urban atmosphere is in the range of $1\text{-}4x10^4/cm^3$; however, their mass concentration is normally not greater than 2 $mg/m^3$. Naturally NPs are formed during gas-to-particle conversion or forest fires, but exposure to airborne NPs has increased intensively due to the appearance of several anthropogenic sources. In workplace atmospheres, NPs occur regularly in metal fumes and polymer fumes. Due to their small size, high number concentration, and large specific surface area, NPs have greater biological activity per given mass than larger particles, including oxidative stress induction, increased adsorption of organic molecules, and enhanced ability to penetrate cellular targets in the lung and systemic circulation. So they can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs. High ambient UFP level is epidemiologically associated with adverse respiratory and cardiovascular effects. Even low solubility and low toxicity materials are more toxic and inflammogenic in ultrafine form. NPs generate reactive oxygen species more intensely than larger particles, leading to increased synthesis of pro-inflammatory mediators. Exposure to NPs also modifies C-reactive protein, fibrinogen, factor VII, RBC count, neutrophil and platelet count etc.

Deposition and clearance mechanisms of NPs in the respiratory tract are considerably different from that of larger particles. Deposition of inhaled NPs in the respiratory tract is mainly due to free diffusion driven by collision of the particles with air molecules and is most likely either in the nasopharynx or in the alveoli. Once deposited, NPs – in contrast to larger-sized particles – appear to translocate readily to extrapulmonary sites and reach other target organs by different transfer routes and mechanisms. This involves transcytosis (caveola formation) across epithelia of the respiratory tract into the interstitium, further access to the blood circulation directly or via the lymph drainage, and finally distribution throughout the body. Extrapulmonary effects of NPs depend on particle solubility, size, site of deposition, and integrity of the epithelial lining. Mn-containing NPs can, e.g., reach the brain from the blood through the capillary endothelial cells in the blood-brain barrier, and through the choroid plexuses. In the olfactory epithelium, the primary sensory neurons provide a direct pathway by which intranasally instilled NPs can get into the CNS.

Manganese (Mn) is an essential trace element, required for the development and the normal working of the brain, and is mainly found in tissues rich in mitochondria. In the central nervous system, glutamine synthetase catalyzes the conversion of glutamic acid to glutamine. This enzyme requires Mn but is inhibited by its excess. Superoxide dismutase, another Mn-enzyme, detoxifies superoxide radicals. The neurotoxic effects of Mn were described first in mining, later also in welding, smelting etc. Environmental exposure to this metal results from Mn in fungicides (maneb, mancozeb), use of methylcyclopentadienyl manganese tricarbonyl (MMT) as anti-knock petrol additive, communal waste incineration. In certain geographical locations, drinking water was high in Mn. Ingested Mn is mainly removed by biliary excretion. The amounts not excreted can pass the blood-brain barrier in transferrin-bound form, and as free $Mn^{2+}$ ion via a cation transporter, and deposit in the brain. After inhalation Mn can reach cerebral target sites directly, before hepatic clearance, especially in case of very fine particles. Overexposure to Mn was found to trigger manganism, a set of extrapyramidal disorders akin to Parkinson's disease. The most important cellular effect of Mn is impairment of energy metabolism, resulting from mitochondrial disorder and free radical production. Disturbed energy production can alter excitatory transmission by the abnormal release of glutamate, by blocking glutamate reuptake, and by increasing postsynaptic responses to glutamate receptor activation. $Mn^{2+}$ was found to interfere with Ca-channels of neurons and presynaptic endings.

In the work presented, the function of the nervous system was primarily investigated on the basis of spontaneous and evoked cortical activity. The cerebral cortex, serving the highest level processing of information, is uninterruptedly active throughout the whole life of the organism. This is reflected in continuous electrical signals which can be recorded from the human head skin (electroencephalogram, EEG) or, in animal experiments, from the exposed surface of the cortex (electrocorticogram, ECoG). ECoG is the result of spatiotemporal summation of postsynaptic potentials of the cortical pyramidal cells, and can be defined as a stream of electrical deflections, characterized by its spectral composition. The spectrum of the ECoG is influenced, among others, by the ascending reticular activation which in turn is driven by afferent impulses.

The cerebral cortex as a whole can be divided into functionally different areas. Sensory cortical areas are the central terminals of sensory pathways. The barrel cortex, in particular, is where sensory inputs from the whiskers on the contralateral side of the body are represented. The cortical area occupied by the vibrissal afferents is large, corresponding to the importance of the whiskers as sensory organs. The innervation of the tail (also involved in the methods applied) includes a pair of dorsolateral and ventrolateral mixed nerve bundles which enter the spinal cord between the 3$^{rd}$ sacral and 3$^{rd}$ caudal segment. This pathway has synapses in the medulla (nu. gracilis) and in the thalamus (nu. ventralis posterolateralis) before it reaches the somatosensory cortical area. In visual perception, afferent discharges are first produced by the retinal ganglion cells. Their axons form the optic nerve, which projects to the lateral geniculate nucleus of the thalamus. Finally the afferents from the lateral geniculate nucleus enter the primary visual cortex. The hair cells within the organ of Corti accept mechanical stimulation by the sound waves. They are innervated by bipolar neurons of the spiral ganglion, the central process of which make up the auditory nerve. The central auditory pathways extend from the cochlear nucleus to the primary auditory cortex. The function of the pathways described above was examined by eliciting sensory evoked potentials (EPs). EPs appear as extreme deflections in the continuous cortical electrical activity, and result from the spatiotemporal coincidence of postsynaptic potentials of many cortical cells under the influence of a volley of afferent pulses arriving to the cortex phase-locked with an artificial external stimulus. The magnitude, shape and delay of an EP can reflect a number of influences on the sensory pathway in question and/or on the state of the cortex.

In contrast to cellular and molecular mechanisms, behaviour is an integrated output of a vast array of chemical and electrophysiological changes. Open field test are widely used to study

locomotor and other activities that comprise exploratory behaviour of the rat, controlled by its motor systems. Motor systems comprise the cortex, basal ganglia, cerebellum, the spinal cord and the peripheral nerves Basal ganglia damages (generated among others by Mn exposure) can appear in changes of muscle tone, of speed and quantity of movement, and in incoordination.

According to the literature, lung and brain are principal target organs for such NPs. Therefore, investigating their neurotoxic effects is of high importance. NPs have several potential portals of entry (skin, gastrointestinal tract, respiratory tract, olfactory bulbs) and target tissues; and various proven or suspected negative effects on human health. Metals, including Mn, are typical components in environmental pollutant NPs, and contribute to their pathogenicity. In this study we modelled the intratracheal and intranasal routes of exposure, because these are the main routes for airborne NPs. The behavioural, electrophysiological and general toxicological consequences of exposure by Mn-containing NPs, in different doses and ways of administration, were examined.

The questions, investigated in particular in this thesis, were as follows:

- Does subacute application (up to 9 weeks) of Mn-containing NPs to rats cause any neurotoxic and general toxic effect?
- Does it cause changes in the spontaneous and stimulus-evoked cortical electrical activity?
- Does it cause changes in the rats' open field exploratory behaviour?
- If yes, how do the effects depend on the dose, time and way of administration, and is there a difference between effects obtained with identical doses of nanoparticulate and solute Mn?

Is any deposition of Mn detectable in the treated rats' organs, first of all in their brain, and is there any relationship between the tissue Mn levels and the observed functional alterations?

## Materials and Methods

Adult male Wistar rats (200-250 g body weight) were used. The Mn-containing nanosuspension, used for the treatment of the rats, consisted of $MnO_2$ particles of ca. 23 nm mean diameter (Fig. 1). It was produced at the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics, by a technique combining sonication and hydrothermal treatment. For administration to the rats, the NPs were suspended in a viscous medium in experiments I and II, and in distilled water in experiments III and IV (see Table 1).

**Table 1.** Summary of the experimental parameters: time scheme, doses, way of application tests performed.

| **Application method** | **INTRANASAL** | | | | | | **INTRATRACHEAL** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Experiments** | **I** | | | **II** | | | **III** | | | | **IV** | | | |
| **Groups** | *Nano* | *Solute* | *Con* | *Nano* | *Solute* | *Con* | *n1v2x* | *n2v1x* | *n1v1x* | *Con* | *HD* | *LD* | *W* | *Con* |
| **Materials** | $MnO_2$ | $MnCl_2$ | VM | $MnO_2$ | $MnCl_2$ | VM | $MnO_2$ | | | DW | $MnO_2$ | | DW | - |
| **Duration of treatment** | Once | | | For 3 and 6 weeks, 5 days per week, once a day (am.) | | | For 3 weeks, 5 days per week | | | | For 3, 6 and 9 weeks 5 days per week once a day (am.) | | | |
| **Total dose (mg Mn/rat)** | 2.53 | | | 3 weeks: 37.9<br>6 weeks: 75.9 | | | n1v1x: 9.86<br>n2v1x: 17.75<br>n1v2x: 17.75 | | | | 3 w: 19.72 3 w: 9.86<br>6 w: 39.44 6 w: 19.72<br>9 w: 59.16 9 w: 29.58 | | | |
| **Investigations at the end of treatment** | 5 days after the treatment: Open field and electrophysiology**.** | | | The next day after the treatment period: OF, el. phys., dissection and organ weighing. | | | The next day after the treatment period: el. phys., dissection and organ weighing. | | | | The next day after the treatment periods: OF, el. phys., dissection and organ weighing. | | | |

(VM: viscous medium, DW: distilled water, OF: Open Field test)

Intranasal instillation in Experiments I and II was done in ether anaesthesia. The material was administered into the left nasal cavity by means of a pipette tip pulled out to ca. 0.2 mm diameter and attached to a 100 µl Hamilton syringe. In Experiments III and IV, intratracheal instillation was performed. The animals, anaesthetized, were suspended on an oblique board, the tongue was pulled forward with a pair on non-traumatic forceps, and a custom-made laryngoscope was used to gain access to the glottis. The nanosuspension (or vehicle for the controls) was instilled into the trachea by means of a 1 ml syringe and 1.2 mm diameter plastic tubing, inserted between the vocal chords.
Body weight, as a general indicator of the rats' health state, was measured every day in experiment II. Later, in experiments III and IV, weekly weighing proved to be sufficient. At the end of experiments, the rats were sacrificed, dissected, organs removed and weighed, and the relative organ weight of the brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals was determined.

The rats' spontaneous locomotor activity was measured in an open field box of 48x48x40 cm size, equipped with two arrays of infrared movement detectors. The test was performed in experiments I, III and IV, once, after the last treatment, between 08:00 and 11:00.. Before the test the animals were allowed to acclimate in the test room for 30 minutes. The animals were placed individually into the centre of the box, and the instrument was recording their horizontal and vertical motor activity in 10 min sessions, based on the interruptions of the infrared beams. From these data, counts, time and run length of the activity forms (ambulation, local activity, immobility, rearing) were automatically calculated.

For electrophysiological recording, the animals were prepared on the day indicated in Table 1. In urethane anaesthesia (1000 mg/kg b.w ip), the animal's head was fixed in a stereotaxic frame, and the left hemisphere was exposed by removing the majority of the parietal bone. The wounds were sprayed with 10% lidocaine, and the exposed dura was protected by a thin layer of petroleum jelly. After 30 minutes recovery, the rat was laid into the stereotaxic frame of the recording instrument, and silver electrodes were placed on the primary somatosensory (SS), visual (VIS) and auditory (AUD) areas. During the measurement, the animal's body temperature was stabilized by a thermostated base plate. The recording sequence started with 2 x 3 minutes of ECoG from the mentioned areas. Then, sensory EPs were recorded by applying the sensory stimuli in trains of 50. For somatosensory stimulation, 2 needles were

inserted into the contralateral whisker pad to deliver square electric pulses (3-4 V, 0.05 ms, 1-10 Hz). Visual stimulation was produced by a high-luminance white LED aimed directly at the rat's right eye, driven by 0.2 ms pulses. The acoustic stimuli were clicks (40 dB) of a small earphone guided into the animal's right ear via the hollow ear bar. The frequency of stimulation was 1 Hz in all modalities, plus 2 and 10 Hz for SS stimulation in order to observe frequency-dependent changes. Finally, compound action potential form the rat's tail nerve was recorded. Two stimulating needle electrodes (delivering 4 -5 V, 0.05 ms, 1 Hz pulses) were inserted into the tail base; and another two, for recording, 50 mm distally. At first, 10 single stimuli were applied, at 1 Hz rate to determine action potential latency; and at higher rates to see the frequency dependence of latency (and amplitude). Then, double stimuli with different inter-stimulus intervals were given for refractory period calculation. From the ECoG records, a software automatically calculated the relative band powers (classical human EEG bands: delta, theta, alpha, beta1, beta2, and gamma). The cortical EPs and tail nerve potential were averaged, and latency and duration of the EP was measured manually. In case of the tail nerve action potential, the amplitude was measured peak-to-peak. From the tail nerve latency data, conduction velocity was obtained, using the distance of the electrodes. From the data with double stimulation, refractory periods were calculated.

From the general toxicological, electrophysiological and behavioural data, group means (±SD) were calculated and plotted. After normality check by the Kolmogorov-Smirnov test, the statistical analyses were carried out by one-way ANOVA and the post hoc analysis was done by Scheffe' test. The confidence level was set to $p<0.05$ in every case. For the statistical analyses SPSS 15.0 for Windows software package was used.

During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed.

## Results and Discussion

A single intranasal instillation of $MnO_2$ NPs (*Nano* group) and $MnCl_2$ (*Solute* group; 2.53 mg Mn/kg b.w. in both) in Experiment I had only slight effects, but on extension of the same dose for 3 and 6 weeks (Experiment II) decreased body weight gain was seen in the *Solute* group. In the OF, ambulation time was significantly diminished in the *Solute* group, local activity and immobility were significantly enhanced, but only in *Solute*, hardly in *Nano*. The general trend of changes of the ECoG was increase of the fast (beta2, gamma) and decrease of the slow (mainly delta) bands, stronger after 6 weeks than 3 weeks, and in the *Solute* vs. *Nano*

group. EPs had lengthened latency, and here the effect was significant also in the *Nano* group. Increased frequency-dependent change of the somatosensory EP was also seen. The relative refractory period of the tail nerve increased in the treated groups, significantly in *Solute*.

In Experiment III and IV, the Mn-containing NPs were applied into the trachea of the rats. In Exp. III, 2.63 and 5.26 mg Mn/ kg b.w. was given in NP form once or twice daily, for 3 weeks (control/*Con*: distilled water instillation). The highest dose caused massive mortality but also the lower doses induced significant body weight loss in the 3 weeks. The most pronounced organ weight change was increase of the lungs weight, which organs were also macroscopically damaged (emphysematic). There were no significant changes in the power spectrum of the ECoG. Latency of SS EPs was significantly longer than in *Con*. In the group treated twice daily with the lower dose, frequency-dependent increase of the latency was also significantly stronger than in *Con*. Latency lengthening was observed also on the auditory EPs, whereas all changes of the visual EP remained below significance. Absolute refractory period of the tail nerve was lengthened in each treated group; relative refractory period and conduction velocity changed accordingly, but less clearly. The frequency dependence in the parameters of the tail nerve action potential indicated increased fatigability of the nerve, which was in accordance with the increased frequency-dependent latency lengthening of the somatosensory EP.

One of the lessons of Experiment III was that massive general toxicity may have masked some specific effects. In Experiment IV, the treatment was prolonged for 3, 6 and 9 weeks and the most extreme doses were omitted. The same nanosuspension as in the previous experiment was administered to the rats intratracheally once a day, 5 days per week, 2.63 and 5.26 mg Mn/ kg b.w. (low and high dose; *LD* and *HD*, respectively). To see if the manipulation itself had any effect on the studied neuro-functional and general toxicological parameters, a vehicle control (*W*) group (instillation of distilled water), and the untreated *Con* group. These had normal body weight gain, and compared to that, weight gain in *W* was still undisturbed; indicating that the manipulation (anaesthesia and instillation) alone was not responsible for the alterations observed. In both treated groups body weight ceased to increase in the 6$^{th}$ week. The relative weight of the lungs increased strongly in *LD* and *HD* but distilled water instillation alone (group *W*) had no effect on the lung weight. By the 9$^{th}$ week, significant decrease of the liver relative weight developed also. By the 9$^{th}$ week, the dose- and time-dependently increased weight of the adrenals (probably due to stress) also became

significant. The treated rats' open field activity showed a shift to less and less motility. Ambulation time decreased already after 3 weeks. Local activity and immobility began to increase in *HD* by the end of the 6$^{th}$ week. There was some decrease of ambulation also in the two controls during the 9 weeks, probably due to increasing age of the rats. In the somatosensory area, slight decrease of the theta band was seen in both treated groups by the 3$^{rd}$ week.. By the 6$^{th}$ week, also the beta1 and beta2 bands began to increase dose-dependently. In the visual cortex, gamma band was significantly increased in both treated groups after 6 weeks, and gamma and beta2 was enhanced, and delta diminished, after 9 weeks in both treated groups. In the auditory area the changes were similar. Lengthening of latency was again the most prominent change of evoked potentials, and developed in a dose- and time-dependent manner. In the somatosensory area, the increase was significant in both treated group vs. *Con*, but only at more frequent stimulation which may have indicated increased fatigability of the cortex in the treated rats. In the tail nerve, conduction velocity was significantly decreased by the end of the 9 weeks in both treated groups. The Mn level determination in brain and other tissue samples has not yet been completed. Preliminary data indicated Mn deposition in brain, lung and liver samples, and supported the conclusion that the functional changes observed in the treated rats were indeed due to manganese.

Literature data show that excess Mn causes various functional abnormalities in humans Airborne exposure by welding fumes resulted in epilepsy and parkinsonism; and altered EEG and event-related potentials. Similar cortical electrophysiological changes were seen in or laboratory previously, in rats following acute and subchronic oral exposure to Mn. Significant behavioural alterations and an increase in tissue Mn levels were also seen. This time, two novel administration methods (intranasal and intratracheal) and a different physicochemical form of Mn (oxide NPs) were applied.

It has been hypothesized that inhaled UFPs accumulate and cause effects in the cardiovascular system and the CNS because of their propensity to translocate across epithelial barriers. The deposited Mn can be translocated to extrapulmonary organs by an indirect way: transcytosis across epithelia of the respiratory tract into the interstitium and access to the circulation via lymphatics, resulting in distribution throughout the body. In the direct way, sensory nerve endings embedded in the epithelia of the airways take up Mn. In our study, intratracheal administration was used to model indirect absorption route of Mn particles; while with intranasal instillation, the effects of the directly absorbed NP Mn were investigated. It has been suggested that solubility highly influences the uptake across the olfactory epithelium. In our Experiment II, the administered Mn caused decreased locomotor activity and lengthened

evoked potential latencies in both treated groups ($MnCl_2$ and nano-$MnO_2$), but the solute form had stronger effects.

At cellular level, the most important effect of Mn exposure is the impairment of mitochondrial energy production; leading to disturbed ion movements and transmitter turnover. The effect on dopaminergic mechanisms were indicated by altered open field behaviour, and on glutamatergic functions, by altered cortical electrical phenomena. Excess glutamate due to astrocytic glutamine synthetase inhibited by Mn may have caused increased ascending reticular activation of the cortex, and dampened, desensitized specific afferentation.

Oxidative stress most probably contributed to the alterations observed on the dissected organs. Chronic airways inflammation and fibre production most likely caused the emphysema seen on the removed lungs and also the enlargement of heart (due to increased flow resistance).

## Conclusion

Investigations towards the environmental presence and health effects of NPs has become technically feasibly relatively recently, which also means that a number of questions are likely to come up yet. Among these, exposure to airborne Mn will remain, most probably, a current problem of occupational and environmental hygiene. By means of studies like that presented above, the health consequences can be better understood.

The questions pointed out as aims can finally be answered as follows:

- Subacute (3 to 9 weeks) application of Mn-containing NPs to rats had clear general toxic (body and organ weights) and neurotoxic effects.
- Significant alterations in the numerical parameters of spontaneous and stimulus-evoked cortical and peripheral nervous activity were observed.
- The rats' open field behaviour was altered.
- These effects showed dependence on cumulative dose (daily doses and treatment time), and there was no qualitative difference between the effects of Mn in NP or solute form.
- No conclusive data could be obtained as yet on deposition of Mn in the brain and other organs. The preliminary data support that the observed neuro-functional changes were in fact due to Mn.

## The Applicant's Relevant Publications

*Sárközi, L.,* Szabó, K., Hornyik, T., Horváth, E., Szabó, A.: The effects of manganese administered in nanoparticle or in ionic form on behaviour and electrophysiology. **Proceedings of the 14th Symposium on Analytical and Environmental Problems** (Galbács, Z., ed.), Szeged, 2007, pp. 184-187.

Papp, A., *Sárközi, L.*: Consequences of subacute intratracheal exposure of rats to cadmium oxide nanoparticles: electrophysiological and toxicological effects. **Proceedings of the 8th International Symposium on Metal Elements in Environment, Medicine and Biology** (Silaghi-Dumitrescu, I., Garban, Z., Dragan, P., eds.), Temesvár, 2008, pp. 67-73.

*Sárközi, L.*, Horváth, E., Kónya, Z., Kiricsi, I., Szalay, B., Vezér, T., Papp, A.: Subacute intratracheal exposure of rats to manganese nanoparticles: Behavioral, electrophysiological and general toxicological effects. **Inhalation Toxicology**, közlés alatt.
*Impakt faktor: 1,831*

*Sárközi, L.*, Horváth, E., Szabó, A., Horváth, E., Sápi, A., Kozma, G., Kónya, Z., Papp, A.: Neurotoxic effects of metal oxide nanoparticles on the somatosensory system of rats following subacute intratracheal application. **Central European Journal of Occupational and Environmental Medicine**, közlésre elfogadva.

Papp, A., *Sárközi, L.*: General and nervous system effects of lead applied in nanoparticulate form into the trachea of rats. **Proceedings of the 10th International Symposium of Interdisciplinary Regional Research**, Vajdahunyad, 2009.

**Abstracts:**

*Sárközi, L.*, Szabó, K., Hornyik, T., Horváth, E., Szabó, A.: Behavioral and electrophysiological effects of manganese given to rats intranasally in different chemical forms. **Acta Physiologica** 191 Suppl 658:60, 2007. (Joint Meeting of the Slovak Physiological Society, the Physiological Society and the Federation of European Physiological Societies, 2007., Pozsony.)

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*Sárközi, L.*, Horváth, E., Szalay, B., Papp, A., Vezér, T. :General and neurotoxicological effects in rats evoked by subacute intratracheal administration of manganese nanoparticles. **Acta Physiologica Hungarica** 96:122-123, 2009. (Magyar Élettani Társaság 72. vándorgyűlése, 2008, Debrecen.)

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