

**PhD Thesis**

**NEUROTOXIC EFFECTS OF MANGANESE OXIDE  
NANOPARTICLES IN RATS**

**Leila Sárközi**

**Department of Public Health**

**University of Szeged**

**Szeged**

**2009**

## The Applicant's Relevant Publications

- I. *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 14<sup>th</sup> Symposium on Analytical and Environmental Problems** (Galbács, Z., ed.), Szeged, 2007, pp. 184-187.
- II. Papp, A., *Sárközi, L.*: Consequences of subacute intratracheal exposure of rats to cadmium oxide nanoparticles: electrophysiological and toxicological effects. **Proceedings of the 8<sup>th</sup> International Symposium on Metal Elements in Environment, Medicine and Biology** (Silaghi-Dumitrescu, I., Garban, Z., Dragan, P., eds.), Temesvár, 2008, pp. 67-73.
- III. *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*
- IV. *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.
- V. Papp, A., *Sárközi, L.*: General and nervous system effects of lead applied in nanoparticulate form into the trachea of rats.  
**Proceedings of the 10<sup>th</sup> 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.)

*Sárközi, L., Szabó, K., Hornyik, T., Horváth, E., Oszlanczi, G.:* Neurotoxic effects in rats following subacute manganese exposure in various forms.

**Clinical Neuroscience** 61 S1: 55-56 2008. (IBRO International Workshop, 2008., Debrecen.)

*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.)

## Summary

Particulate air pollutants can, by their size, be grouped as dusts ( $>10\ \mu\text{m}$ , quick sedimentation), fine dusts or fumes ( $100\ \text{nm}$ - $10\ \mu\text{m}$ ) and nano- or ultrafine particles (NPs, UFPs,  $<100\ \text{nm}$ ). Airborne NPs may contain several heavy metals, including toxic ones. In workplace atmospheres, NPs occur regularly in metal fumes and polymer fumes; both of which can induce acute inflammatory responses in the lung upon inhalation. 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 in the systemic circulation. NPs can contribute to adverse health effects of the respiratory tract and of extrapulmonary organs. From the airways, NPs appear to translocate readily to extrapulmonary sites and reach other target organs by transcytosis across epithelia of the respiratory tract into the interstitium. 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.

Manganese (Mn) is an essential trace element, mainly found in tissues rich in mitochondria (liver, muscles, brain etc.). Numerous publications demonstrated the occupational health risk of Mn exposure in mining, welding, smelting etc. Overexposure to Mn triggers extrapyramidal disorders akin to Parkinson's disease. The most important cellular effect of Mn is impairment of energy metabolism, which can alter excitatory transmission by the abnormal release of glutamate, by blocking the glutamate reuptake, and by increasing postsynaptic responses to glutamate receptor activation.  $\text{Mn}^{2+}$  was found to interfere with Ca-channels of neurons and presynaptic endings.

In this study we modelled the intratracheal and intranasal routes of exposure, because these are the main routes for airborne NPs. We tested the behavioural, electrophysiological and general toxicological consequences of Mn-containing NPs, in different doses and ways of administration. 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?

Adult male Wistar rats (200-250 g body weight) were used. In Experiments I and II, intranasal instillation was applied, and in Experiments III and IV, intratracheal application, both performed in short ether anesthesia. The nanosuspension used consisted of MnO<sub>2</sub> particles of ca. 23 nm mean diameter, and was produced at the Department of Applied Chemistry, University of Szeged. Body weight, as a general indicator of the rats' health state, was measured repeatedly during the treatment periods. Following electrophysiological recordings, the rats were dissected, organs were removed and weighed, and the relative organ weight of the brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals, related to 1/100 of body weight or to the brain weight, was calculated. The rats' spontaneous locomotor activity was measured in an open field (OF) box in 10 min sessions. After that, at the end of the treatment period, electrophysiological was done in urethane anesthesia (1000 mg/kg b.w ip.). The recording sequence started with 2 x 3 minutes of electrocorticogram (ECoG) recorded from the mentioned areas. Then, somatosensory, visual and auditory evoked potentials (EPs) were recorded by applying sensory stimuli in trains of 50. Finally, compound action potential from the rat's tail nerve was recorded.

A single intranasal instillation of MnO<sub>2</sub> NPs (*Nano* group) and MnCl<sub>2</sub> (*Solute* group; 2.53 mg Mn/kg b.w. in both) in Experiment I had only slight effects, but by 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 nano-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, there was only one application per day of . 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<sup>th</sup> 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<sup>th</sup> week, significant decrease of the liver

relative weight developed also. By the 9<sup>th</sup> week, the dose- and time-dependently increased weight of the adrenals (probably due to stress, see above) 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<sup>th</sup> 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<sup>rd</sup> week. By the 6<sup>th</sup> 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.

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.

## Table of Contents

1. INTRODUCTION .....	1
1.1. Solid phase air pollutants .....	1
1.2. Manganese: toxicity, neurotoxicity, mechanism of action .....	4
1.3. Structures and functions of the rat nervous system involved in the study.....	5
1.4. Aims of the study .....	9
2. METHODS .....	10
2.1. Animals and materials .....	10
2.2. Treatment .....	11
2.3. General toxicological investigations.....	12
2.4. Behavioural test: open field .....	14
2.5. Electrophysiological investigations .....	14
2.6. Statistical analysis.....	16
3. RESULTS .....	17
3.1. Experiment I .....	17
3.2. Experiment II .....	17
3.3. Experiment III.....	25
3.4. Experiment IV.....	31
3.5. Tissue manganese measurements .....	42
4. DISCUSSION.....	44
5. REFERENCES .....	48
6. ACKNOWLEDGEMENT.....	54
7. APPENDIX .....	55

## Abbreviations

AUD	auditory
<i>Con</i>	control
ECoG	electrocorticogram
EP	evoked potential
HD	high dose (Experiment III and IV)
<i>LD</i>	low dose (Experiment III and IV)
<i>n1v1x</i>	low dose, once daily (Experiment II)
<i>n1v2x</i>	low dose, twice daily (Experiment II)
<i>n2v1x</i>	high dose, once daily (Experiment II)
<i>n2v2x</i>	high dose, twice daily (Experiment II)
NP	nanoparticle
SS	somatosensory
UFP	ultrafine particle
VIS	visual
<i>W</i>	vehicle treated (Experiment IV)

## 1. INTRODUCTION

### 1.1. Solid phase air pollutants

#### 1.1.1. Origin and properties of airborne particles

The state of the environment and human health are in close connection. Environmental pollution, mainly due to human activity, causes the presence of several toxicants in the air, water and soil; and affects the population in return.

Among these media, air is the most sensitive to pollution. While water and particularly soil have greater self purification capacity, air can hardly neutralize or degrade pollutants. In addition, soil can store and withhold hazardous materials (for example, under natural conditions, heavy metals are immobile in the soil because they strongly attach to the surface of the soil colloids), but air is obviously the most mobile carrier medium, able to quickly transport the particles far away from the place of emission. Thus, air pollution concerns the biggest part of the human population.

Entrance of materials into the air can occur from natural (e. g. volcanism, wind) and artificial (e. g. energy production, chemical industry, transportation, disasters) sources. The fate of these substances in the air depends on physical (volatility, particle size etc.) and chemical (solubility, dissociation, stability, propensity for complex formation etc.) factors. From these factors, particle size is one of the most determinative properties.

Particulate pollutants can, by their size, be grouped as dusts ( $>10\ \mu\text{m}$ , quick sedimentation), fine dusts or fumes (100 nm-10  $\mu\text{m}$ ) and ultrafine particles (UFPs,  $<100\ \text{nm}$ ). 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 heavy metals, emitted into the air by industrial processes, transportation and utilization of agrochemicals, and from household articles. These metals are known to be toxic. They are able to form stable complexes with proteins, thus blocking the centre of several enzymes, leading to deterioration of various organ systems. 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.

### **1.1.2. Nanoparticles: origin, characteristics, and effects on living organisms**

Ambient fine particles consist of ultrafine particles (UFPs, <100 nm; “nanoparticles” in the strict sense are only artificial, engineered ones) and accumulation-mode particles (approximately 100 to 1000 nm: Oberdörster, 2000a). Normal background level of UFPs in the urban atmosphere is in the range of  $1-4 \times 10^4/\text{cm}^3$ ; however, their mass concentration is normally not greater than  $2 \text{ mg}/\text{m}^3$  (Oberdörster, 2000a). Naturally UFPs are formed during gas-to-particle conversion or forest fires (Li et al., 2003), but recently the exposure to airborne UFPs has increased intensively due to the appearance of several anthropogenic sources (internal combustion engines, power plants, nanotechnology etc.). In workplace atmospheres, UFPs occur regularly in metal fumes and polymer fumes; both of which can induce acute inflammatory responses in the lung upon inhalation (Oberdörster et al., 2000b).

Manufactured nanomaterials are likely to enter the environment from several sources. They are being used in personal care products (e.g. cosmetics, sunscreens), electronics, tires, fuel cells, and many other consumers’ goods (Oberdörster et al., 2005). In case of soil remediation, dozens of sites have already been injected with various nanomaterials (e.g. nano-iron) in order to remove pollutants. Due to their small size, high number concentration, and large specific surface area, UFPs have greater biological activity per given mass than larger particles (Oberdörster, 2000a; Oberdörster et al., 2005), including oxidative stress induction, increased adsorption of organic molecules, and enhanced ability to penetrate cellular targets in the lung and systemic circulation (Li et al., 2003, Nemmar et al., 2002). In the recent years, studies have shown that UFPs can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs (Oberdörster et al., 2005). Several lines of epidemiological evidence have associated the enhanced level of ambient UFPs with adverse respiratory and cardiovascular effects, and demonstrated increasing morbidity and mortality in susceptible parts of the population (Li et al., 2003; Nemmar et al., 2002; Oberdörster et al., 2005; Stone et al., 2007).

In vivo and in vitro toxicological studies confirmed that low solubility and low toxicity materials (such as  $\text{TiO}_2$ , carbon black, or polystyrene beads) are more toxic and

inflammogenic in ultrafine than in fine particle form (Long et al., 2006; Stone et al., 2007). Nanoparticles (NPs) generate reactive oxygen species more intensely than larger particles, leading to increased synthesis of pro-inflammatory mediators via intracellular signalling pathways (Stone et al., 2007). In addition to causing respiratory injury, exposure to particles has been shown to modify blood indices such as C-reactive protein, fibrinogen, factor VII, as well as red blood cell, neutrophil and platelet counts (Nemmar et al., 2002).

For investigating the toxicology of NPs, it is important to determine their fate after inhalation. The 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 (Oberdörster et al., 2005), and deposition is most likely either in the nasopharynx or in the alveoli (ICRP, 1994). 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 (Oberdörster et al., 2005). The extrapulmonary effects of NPs depend on several factors including particle solubility, particle or aggregate size, the site of deposition, and the integrity of the epithelial lining (Elder et al., 2006). 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 (Crossgrove and Yokel, 2005).

In the olfactory epithelium the primary olfactory neurons are in contact with the environment and via the axonal projections they are also connected to the olfactory bulbs of the brain. Therefore, the primary olfactory neurons provide a direct pathway by which foreign materials may gain access to the brain (Calderon-Garciduenas, 2002; Tjalve et al., 1996). Several studies have proved that the olfactory nerve and olfactory bulbs are portals of entry to the central nervous system (CNS) for intranasally instilled NPs (Gianutsos et al., 1997; Henriksson and Tjalve, 1999; Henriksson et al., 2000). Elder et al. (2006) found that inhaled manganese NPs, once they reached the olfactory bulb, spread to the striatum, midbrain, frontal cortex and cerebellum.

## **1.2. Manganese: toxicity, neurotoxicity, mechanism of action**

Manganese (Mn) is an essential trace element, required for the development and the normal working of the brain (Elder et al., 2006). Within the organism, Mn is mainly found in tissues rich in mitochondria (liver, muscles, brain etc.), where it forms stable complex with ATP and inorganic phosphate. It functions as a constituent of metalloenzymes, and as an activator of several other enzymes. For example, Mn is a necessary component of glutamine synthetase, superoxide dismutase (SOD) and pyruvate carboxylase (Erikson and Aschner, 2003; Normandin and Hazell, 2002). In the central nervous system, glutamine synthetase (a glia-specific Mn metalloprotein) catalyzes the conversion of glutamic acid to glutamine, thereby removing the transmitter. This enzyme requires Mn but is inhibited by its excess (Normandin and Hazell, 2002). SOD detoxifies superoxide radicals by catalysing their conversion to hydrogen peroxide, and pyruvate carboxylase works as a rate-limiting enzyme in gluconeogenesis (Takeda et al., 1997a).

The neurotoxic effects of Mn were described first 150 years ago, when miners, working with manganese-oxide, showed unsteady gait and muscular weakness. Since that time, many publications have demonstrated the occupational health risk of Mn exposure in mining, welding, smelting etc. (Roels et al., 1997). There is also environmental exposure to this metal. The agriculture applies Mn in fungicides (maneb, mancozeb: Ferraz et al., 1988), some countries use methylcyclopentadienyl manganese tricarbonyl (MMT) as anti-knock petrol additive to improve the octane rating (Normandin et al., 2004), and the communal waste also can contain Mn (e.g. in used batteries) eventually released in waste incineration. In certain geographical locations, drinking water was high in Mn (in Greece, 50mg/L in the drinking water was associated with neurological effects; Kondakis et al., 1989).

Although overexposure to Mn has been found to trigger extrapyramidal disorders (tremor, postural instability, muscular dystonia) akin to those in Parkinson's disease (Crossgrove and Yokel, 2005; Fechter et al., 2002; Takser et al., 2003; Yu et al., 2003), manganism (chronic manganese neurotoxic damage) does not directly involve the nigrostriatal pathway, but interferes with the output pathways downstream from the nigrostriatal dopaminergic pathway by causing loss of neurons in the globus pallidus (Erikson and Aschner, 2003). The similarities with Parkinson's disease are the presence of generalized bradykinesia and

widespread rigidity; and the dissimilarities, less-frequent resting tremor, more frequent dystonia, a particular propensity to fall backward, and failure to achieve a prolonged therapeutic response to levodopa (Takeda, 2002).

The most important cellular effect of Mn is the impairment of energy metabolism, resulting from mitochondrial disorder and free radical production. The disturbed energy metabolism can alter excitatory transmission by the abnormal release of glutamate, by blocking the glutamate reuptake, and by increasing postsynaptic responses to glutamate receptor activation (Centonze et al., 2001; Normandin and Hazell, 2002). Inhibited mitochondrial functions probably block tyrosine hydroxylation, a crucial step of dopamine synthesis, thus decreasing brain dopamine level (Parenti, 1988). Dopamine depletion can also happen by Mn-induced autooxidation of dopamine. In addition,  $Mn^{2+}$  was found to interfere with Ca-channels of neurons and presynaptic endings (Takeda, 2002), resulting in generally abnormal conduction and transmission of excitation.

The uptake and elimination patterns of manganese following ingestion have been well studied. Excretion of Mn occurs mainly through liver uptake and biliary excretion (Elder et al, 2006; Fechter et al., 2002). The amounts not excreted can, however, pass the blood-brain barrier in transferrin-bound form (Takeda et al., 1997b), because transferrin is a plasma carrier protein and transferrin receptors are present on the surface of the brain capillary endothelial cells; and as free  $Mn^{2+}$  ion via a cation transporter (Aschner et al., 1999), and finally can deposit in the brain. Less is known about inhalation exposure, by which Mn is likely to reach directly cerebral target sites before hepatic clearance (especially in case of very fine particles, see above). This direct path from the respiratory tract to the brain is most probably the main reason for the differential toxicity of inhaled and ingested Mn (Henriksson and Tjalve, 2000; Roels et al., 1997).

### **1.3. Structures and functions of the rat nervous system involved in the study**

#### **1.3.1. Electrophysiological phenomena and the underlying structures**

Sensory systems receive information from the environment through receptors at the periphery of the body and transmit this information to the central nervous system. The latter processes the input and initiates adequate motor and other responses. The cerebral cortex, serving the

highest level processing of information, is uninterruptedly active throughout the whole life of the organism. This is reflected, due to the electrochemical elements of neuronal activity, 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 (and EEG) can be defined as a stream of more or less irregular electrical deflections, and characterized by its spectral composition, that is, by the presence of waves of various frequencies as components. It is the result of spatiotemporal summation of postsynaptic potentials of the cortical pyramidal cells. 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 (uppermost) terminals of the sensory pathways. Within these, the barrel cortex is that part of the somatosensory cortex of rodents where sensory inputs from the whiskers on the contralateral side of the body are represented. The infraorbital branch of the trigeminal nerve innervates the whisker pad, and runs to the trigeminal ganglion (nu. principalis) containing the cell bodies of incoming sensory nerve fibres. From there, a single large sensory root enters the brainstem at the level of the pons, containing axon to the ventral posteromedial nucleus of the thalamus. Inputs from the thalamus terminate principally in the primary somatosensory cortex. The cortical area occupied by the vibrissal afferents is large, corresponding to the importance of the whiskers as sensory organs. The complete pathway is highly somatotopic which is expressed in the existence of “barrels”, the localization of neurons receiving afferent input from a single vibrissa in a cylinder-like shape.

The innervation of the tail includes a pair of dorsolateral and ventrolateral mixed nerve bundles which enter the spinal cord between the 3<sup>rd</sup> sacral and 3<sup>rd</sup> 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 three subcortical brain areas: the pretectal region and the superior colliculus of the midbrain, and – most importantly – the lateral geniculate nucleus of the thalamus. Finally the afferents from the lateral geniculate nucleus enter the primary visual cortex (Kandel and Schwartz, 1985).

Sounds reaching the ear are mechanically transmitted to the sensory receptor cells of the inner ear, the hair cells contained within the organ of Corti. The hair cells are innervated by bipolar neurons of the spiral ganglion in the modiolus of the cochlea. The central process of the spiral ganglion cells make up the auditory nerve. Auditory fibres in the eighth nerve terminate in the cochlear nucleus. The central auditory pathways extend from the cochlear nucleus to the primary auditory cortex (Kandel and Schwartz, 1985).

In the present work, the function of the pathways described above was examined by eliciting so-called 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 (Kandel and Schwartz, 1985). 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.

All electrical phenomena of the nervous system result from the activity of neurons and depend highly on synaptic transmission. Electrophysiological analysis of the synaptic transmission can provide detailed information on the mechanisms by which a neurotoxic compound may disrupt nerve function (Chang and Slikker, 1995). The process begins with the invasion of an action potential into the presynaptic terminal, resulting in activation of clusters of voltage-sensitive  $\text{Ca}^{2+}$  channels located very near the release sites (active zone) of the nerve ending. Opening  $\text{Ca}^{2+}$  channels let  $\text{Ca}^{2+}$  flow into the nerve terminal, driven by their electrochemical gradient.  $\text{Ca}^{2+}$  binds to proteins which triggers prepackaged neurotransmitter vesicles positioned close to the terminal membrane to fuse with the membrane and release their contents into the synaptic cleft. The transmitter diffuses across the cleft and interacts with receptors located on the postsynaptic cell to initiate an electrical or biochemical response. Persistent action of the transmitter is prevented by its rapid elimination from the synaptic cleft by enzymatic inactivation or by reuptake into the presynaptic terminal or surrounding glial cells (Chang and Slikker, 1995). Sufficient (suprathreshold) depolarization of the postsynaptic neuron by an excitatory synapse initiates an action potential, travelling down the axon and activating the next synapse. These events require a lot of energy, and depend on the intact state and function of specific protein structures like ion channels and postsynaptic receptors.

Neurotoxicants can, in theory, attack any of them. How Mn can affect the nervous system was described in 1.2.

### **1.3.2. Behavioural phenomena and the underlying structures**

Behavioural tests have long been applied in assessing the effects of pharmaceutical and medicinal compounds acting on the central nervous system (Chang and Slikker, 1995). Recently more and more studies investigate the environmental chemicals as potential sources of the behavioural alterations. In contrast to cellular and molecular mechanisms, behaviour is an integrated output of a vast array of chemical and electrophysiological changes in the nervous system (Paul et al., 1997).

Open field test are widely used to study locomotor and other activities that comprise exploratory behaviour of the rat (Clark et al., 2005; Nemati and Whishaw, 2007; Whishaw et al., 2006). There are several theories about what motivates locomotor behaviour in animals that are not deprived of food or water. According to one, exploration is motivated by fear and the information acquired during exploration serves to reduce fear. Others stated that exploration is only a response to unpredicted stimulation and functions to gather information about that stimulation; and still others that exploration is not a manifestation of a single motivation but a product of the combined influences of curiosity and fear (Whishaw et al., 2006).

When rats are placed in a novel environment, they alternate between progression and stopping. When they move, they stop briefly in many places, and try to move close to objects. The place where they spend the longest cumulative time is termed home base. In this place the occasions of grooming and of rearing are often the highest (Eilam and Golani, 1989). The home base serves as centre for excursions, thus the number of visits to it is the most frequent. From the home base the animals make slow outward progressions with periodic stops and more rapid and direct homeward returns (Nemati and Whishaw, 2007).

All these events involve the motor systems of the rats. Motor systems comprise the cortex, especially the precentral gyrus, subcortical structures like the basal ganglia, the cerebellum, the spinal cord and the peripheral nerves, and the neuromuscular junction (Chang and Slikker, 1995). The basal ganglia (substantia nigra, caudate, putamen and globus pallidus) have been found to contribute to the initiation and execution of movements, sequencing of movements,

automatic execution of routine movements, inhibition of competing motor programs, motor learning and reward mechanisms (Hauber, 1998; Herrero et al., 2002). 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. Akinesia and bradykinesia (observed among the symptoms of both Parkinson's disease and manganism), related to slower ongoing motor execution, are the major impairments associated with basal ganglia dysfunctions (Hauber, 1998).

#### **1.4. Aims of the study**

NPs have several potential portals of entry (skin, gastrointestinal tract, respiratory tract, olfactory bulbs) and target tissues; and proven or suspected negative effects on human health have been reported in the literature. 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. According to the literature, lung and brain are principal target organs for such NPs. Therefore, investigating their neurotoxic effects is of high importance. In our experiments we tested the behavioural, electrophysiological and general toxicological consequences of Mn-containing NPs, in different doses and ways of administration.

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

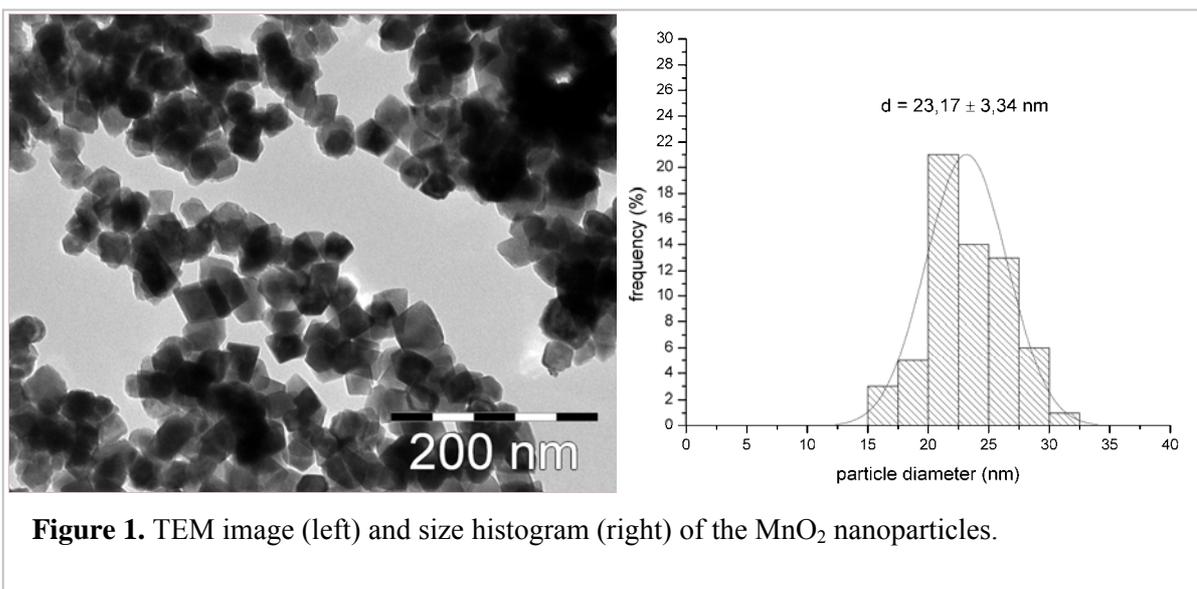
- Does subacute application (up to 9 weeks) of Mn-containing NPs to rats cause any neurotoxic and general toxic effect?
- Does it cause changes in the spontaneous and stimulus-evoked cortical electrical activity?
- Does it cause changes in the rats' open field exploratory behaviour?
- If yes, how do 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?

## 2. METHODS

### 2.1. Animals and materials

Adult male Wistar rats (200-250 g body weight) were obtained at the University's breeding centre and were housed in an air-conditioned room maintained at 22 °C with a 12-h light/dark cycle (light on at 06:00), and free access to tap water and standard pellet.

The Mn-containing nanosuspension, used for the treatment of the rats, consisted of MnO<sub>2</sub> 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. An appropriate amount of aqueous KMnO<sub>4</sub> solution was mixed with ethylene glycol and sonicated with a Hielscher UIP1000 ultrasound device. The resulting dark suspension was loaded to a Teflon-lined stainless steel autoclave. The autoclave was heated at 200°C for 16 h in an oven and then allowed to cool to room temperature naturally. The brownish precipitate formed was filtered and washed with 80°C preheated distilled water to remove any unreacted starting material and the soluble byproducts formed during the reaction. The precipitate was dried at 100°C for 1 h. Its chemical purity was checked by X-ray diffraction, and its particle size, by X-ray diffraction and transmission electron microscopy (TEM).



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). The viscous medium was received from the Department of Pharmaceutical Technology, University of Szeged Faculty of Pharmacy, and contained 1 % Na hyaluronate, 10 % polyetoxylated 40 hydrogenated castor oil, and 89 % distilled water. Before and during administration, the nanosuspension was sonicated in an Elmasonic E-15 H ultrasound water bath to counteract aggregation.

Manganese chloride ( $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$ , min. 99% purity), used in the experiments I and II, was obtained from REANAL, and was dissolved in the mentioned viscous medium.

## 2.2. Treatment

In experiments I and II, intranasal instillation was applied. The rats were anesthetized with diethyl ether in a glass jar with air-tight lid. When the anaesthesia was complete, the rat was laid on its back, and the material was administered into the left nasal cavity by means of a pipette tip, pulled out to ca. 0.2 mm diameter over a flame, and attached to a 100  $\mu\text{l}$  Hamilton syringe. The role of the viscous medium was to keep the substances long on the mucosa.

For another model of exposure by airborne NPs, intratracheal instillation performed in experiments III and IV, the animals were anesthetized in the same way before, and were then suspended on an oblique board, standing at  $60^\circ$  to horizontal, with their upper incisors hung in a wire loop to hold the animal in place and keep its mouth open (Oka et al., 2006). The trachea was illuminated transdermally by means of a fibre optic light guide brought into direct contact with the animal's neck. The tongue was pulled forward with a pair of 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. Before taking up the materials, an equal quantity of air was taken up into the syringe, and was pushed out after the suspension to assure that the whole amount was emptied from the syringe and tube and delivered into the trachea. Treatment was performed under an exhaust hood to remove ether vapours.

The doses listed in Table 1 were based, initially, on literature data (Henriksson and Tjalve, 2000; Henriksson et al., 1999) showing that ca. 1000  $\mu\text{g}$  of Mn, given intranasally, can cause

histological alterations. The final dose in Experiment I was determined by the technically available concentration of the nanosuspension and the limited volume that could be instilled in the nasal cavity (40  $\mu$ l). Later, this dose (and its multiples) were retained so that the results of different experiments could be compared more directly.

### **2.3. General toxicological investigations**

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.

In the same experiments, following electrophysiological recordings, the rats were sacrificed with an overdose of urethane. They were dissected, organs were removed and weighed, and the relative organ weight of the brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals, related to 1/100 of body weight or to the brain weight, was calculated. During dissection, blood samples were collected from the vena cava by means of a heparinized syringe into 5 ml plastic tubes. These blood samples were stored at -22°C along with the brain, lung and liver samples.

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

Application method	INTRANASAL						INTRATRACHEAL							
	I			II			III				IV			
<b>Experiments</b>														
<b>Groups</b>	<i>Nano</i>	<i>Solute</i>	<i>Con</i>	<i>Nano</i>	<i>Solute</i>	<i>Con</i>	<i>n1v2x</i>	<i>n2v1x</i>	<i>n1v1x</i>	<i>Con</i>	<i>HD</i>	<i>LD</i>	<i>W</i>	<i>Con</i>
<b>Materials</b>	MnO <sub>2</sub>	MnCl <sub>2</sub>	VM	MnO <sub>2</sub>	MnCl <sub>2</sub>	VM	MnO <sub>2</sub>		DW		MnO <sub>2</sub>		DW -	
<b>Duration of treatment</b>	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.)			
<b>Total (cumulative) dose (mg Mn/rat)</b>	2.53			3 weeks: 37.9 6 weeks: 75.9			n1v1x: 9.86 n2v1x: 17.75 n1v2x: 17.75 Details: see Table 4.				3w: 19.72 3w: 9.86 6w: 39.44 6w: 19.72 9w: 59.16 9w: 29.58 Details: see Table 6.			
<b>Investigations at the end of treatment</b>	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)

## **2.4. Behavioural test: open field**

The rats' spontaneous locomotor activity was measured in an open field box of 48x48x40 cm size, equipped with two arrays of infrared movement detectors at floor level and in 12 cm height (Conducta 1.0 System, Experimetria Ltd, Budapest, Hungary). The test was performed in experiments I, III and IV, once, after the last treatment as shown in Table 1, between 8 and 11 hours in the morning. Just before the test, the animals were allowed to get acclimatized in the dimly lit 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. More than 40 mm shift in the location of interrupted beams at the floor level during a time unit of 1 s was interpreted as horizontal activity, less shift, as local activity, and no shift at all, as immobility. Rearing was recorded if beams at floor level and at the higher level were interrupted simultaneously. The previous neurotoxicological works of the Department (Vezér et al., 2005, 2007) proved that this behavioural test was usable for investigating the impairment of higher nervous functions caused by heavy metals.

## **2.5. Electrophysiological investigations**

### **2.5.1. Preparation of the rat**

The animals were prepared for electrophysiological recording on the day indicated in Table 1. In urethane anaesthesia (1000 mg/kg b.w ip.; Field et al., 1993), 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. The stereotaxic atlas of Zilles (1982) was used to locate the sensory areas on the cortical surface, and the electrodes were shifted a bit on the surface if this was needed for optimal recording. A stainless steel clip

was fixed to the cut skin as an indifferent electrode. During the measurement, the animal's body temperature was stabilized by a thermostated base plate.

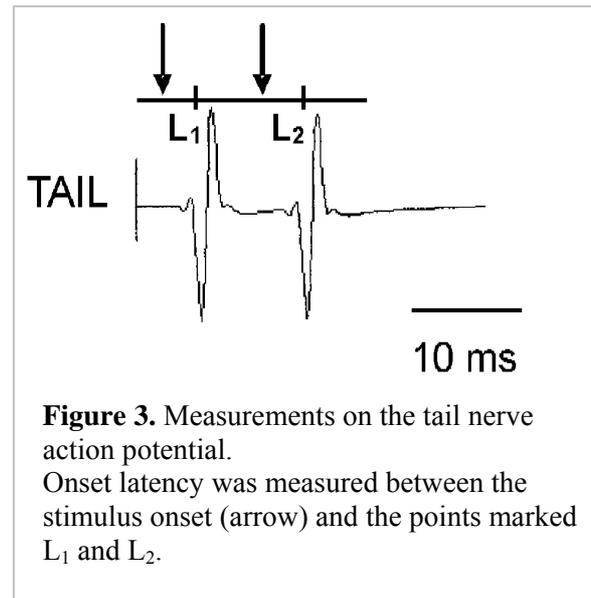
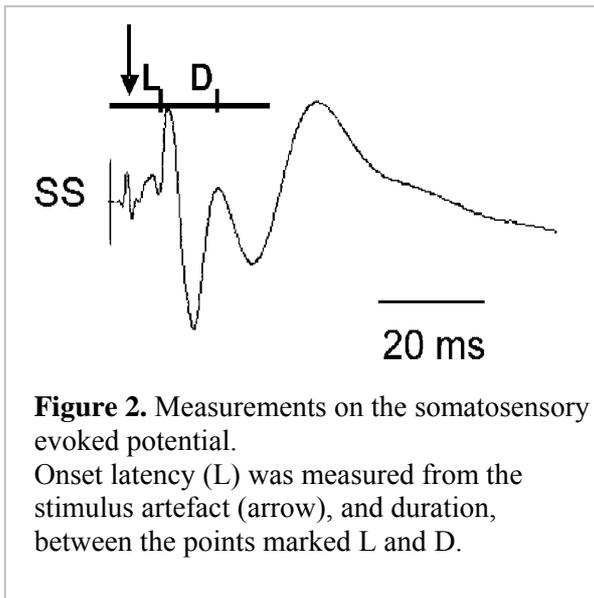
### **2.5.2 Recording procedure**

The recording sequence started with 2 x 3 minutes of ECoG recorded 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 from 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 the latency and amplitude. Then, double stimuli with different inter-stimulus intervals were given for refractory period calculation (see below). All electrophysiological recording and analysis was done by means of the Neurosys 1.11 software (Experimetria Ltd, Budapest, Hungary).

### **2.5.3. Evaluation**

From the ECoG records, the software automatically calculated the relative band activities (band powers) according to the classical human EEG bands (delta, theta, alpha, beta1, beta2, and gamma: Kandel and Schwartz, 1985). The records of the cortical EPs and tail nerve potential were automatically averaged, and latency and duration of the EP was measured manually by means of screen cursors of the software. How latency and duration was determined is shown in Fig. 2. In case of the tail nerve action potential, the amplitude was measured peak-to-peak and the latency in the same way as for cortical EPs (Fig.3). From the latency data, conduction velocity of the tail nerve was obtained (using the distance of the electrodes). From the data with double stimulation, refractory periods were calculated. By plotting the ratio of the second/first latency against the inter-stimulus time, a hyperbolic curve

can be obtained, the horizontal and vertical asymptotes of which correspond to the relative (latency of the second response longer than that of the first) and absolute (latency of the second response infinitely long) refractory period, respectively. The exact values were determined by linear transformation of the curve (Anda et al., 1984; Dési and Nagymajtényi, 1999).



## 2.6. Statistical analysis

From the general toxicological, electrophysiological and behavioural data, group means ( $\pm$ 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.

### 3. RESULTS

#### 3.1. Experiment I

In this experiment the animals received a single intranasal dose of Mn, and the investigations were performed 5 days after the treatment. There were 3 groups of 10 rats each: the control (*Con*) group got the previously mentioned viscous medium, the *Nano* group was treated with nanosuspension MnO<sub>2</sub>, and the *Solute* group was given MnCl<sub>2</sub>. The total dose was 2.53 mg Mn/rat in both treated groups. The investigation comprised the open field behavioural test and the electrophysiological measurements.

In this experiment no explicit changes were observed, thus no figures are presented in the thesis. In the OF test, ambulation distance, ambulation time, and rearing count and time decreased slightly, while count and time of both local activity and immobility increased a little. This was seen in all groups – probably due to aging and to repetition of OF test within short time (6 days) – but was more pronounced in the treated rats.

The changes of the ECoG bands had not even a clear trend and were fully inconclusive. The duration of the SS EPs was diminished in both treated groups; moreover this change was significant in the *Nano* group at 2 Hz stimulation rate. On the VIS and AUD EPs, decrease of the latency was observed in both treated groups. This alteration was opposite to the results of longer treatments and was more similar to the acute effect of Mn.

#### 3.2. Experiment II

In this experiment, the same substances as in experiment I were administered intranasally to the rats, once every weekday for 3 and 6 weeks. The groups (*Con*, *Nano*, *Solute*) were started with 16 rats each, of which 8 were processed after 3 weeks, and the remaining 8 after 6 weeks. The total doses were 37.9 mg Mn/rat in 3 weeks, and 75.9 mg Mn/rat in 6 weeks. The investigations (open field test, electrophysiological measurements, dissection and organ weighing) were done on the day following the last administration.

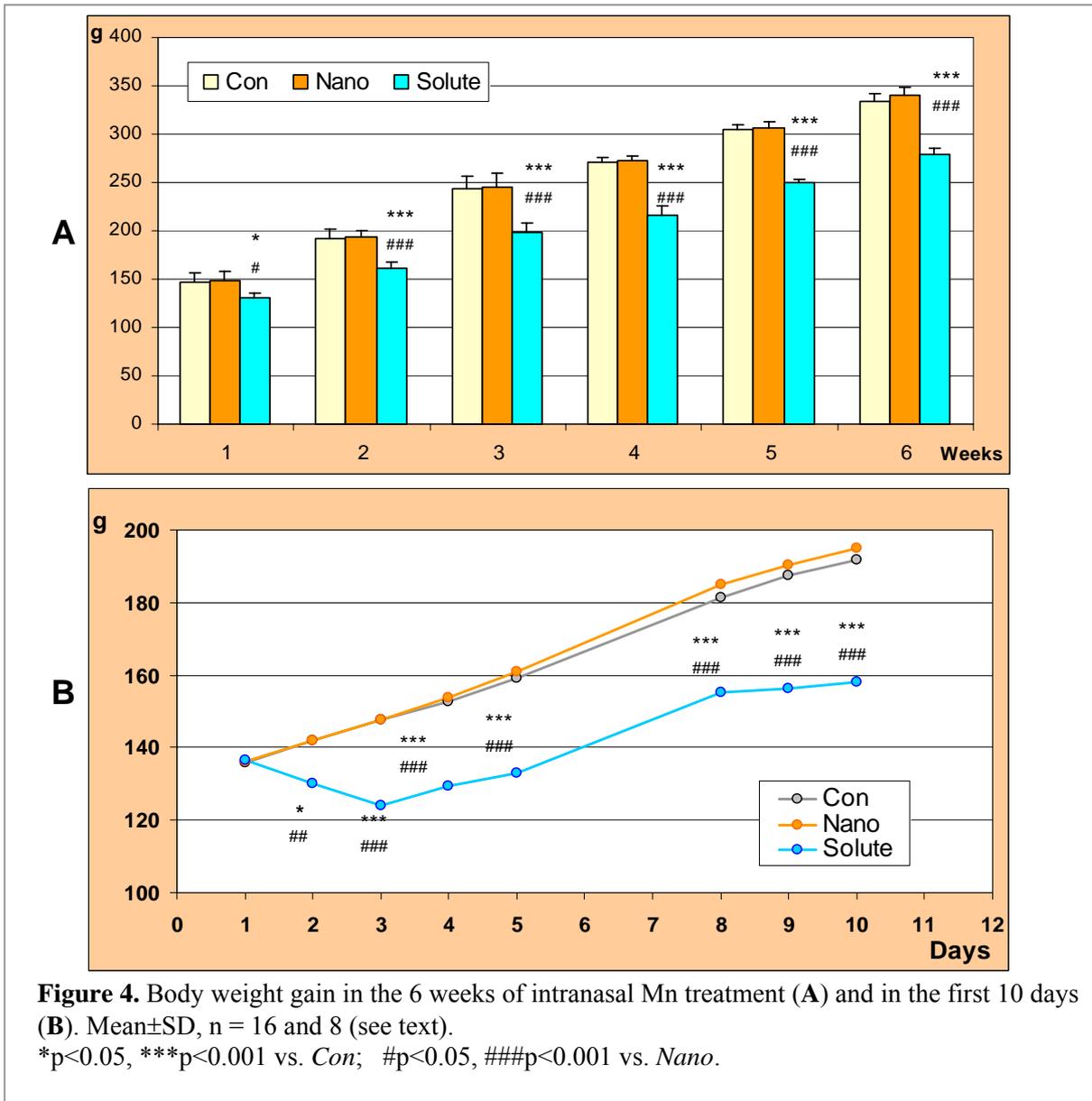
### 3.2.1. Body and organ weights

MnCl<sub>2</sub> caused markedly slowed increase of the body weight, from the first week on, compared to the *Con* and *Nano* groups (Fig. 4A). MnO<sub>2</sub> NPs had no effect on the body weight gain. Fig. 4B shows that the effect of MnCl<sub>2</sub> started immediately. The changes of the relative organ weight, another indicator of the general toxicity of the substances, were calculated both on the basis of body weight and weight of the brain, due to the body weight effect of the treatment (Table 2). After 6 weeks, body-relative kidney weight significantly decreased in both treated groups. Diminished body-relative heart and spleen weight was also observed in the *Nano* group. Relative thymus weight decreased, and adrenal weight increased, significantly in the *Solute* group. When compared to brain weight, most of these changes disappeared or lost significance, only the diminished thymus weight remained. After only 3 weeks, slight changes in the same directions were seen.

**Table 2.** Relative organ weights after 6 weeks treatment in Experiment II. Mean±SD, n=8.

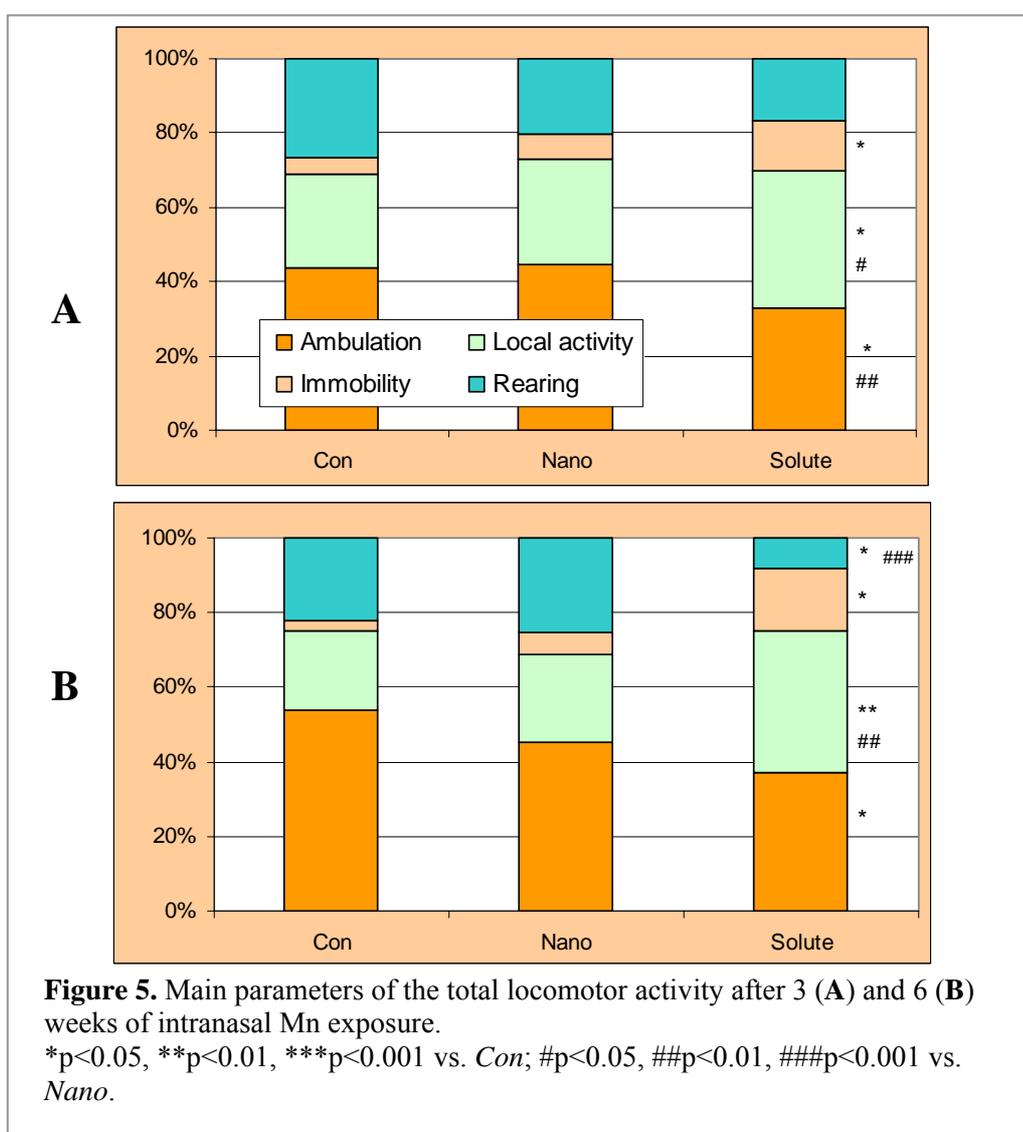
Groups	Related to 1/100 body weight			Related to brain weight		
	<i>Con</i>	<i>Nano</i>	<i>Solute</i>	<i>Con</i>	<i>Nano</i>	<i>Solute</i>
Heart	0.310±0.037	0.272±0.024*	0.290±0.012	0.498±0.032	0.484±0.042	0.459±0.046
Spleen	0.202±0.013	0.188±0.021	0.192±0.029	0.328±0.035	0.333±0.039	0.305±0.061
Thymus	0.153±0.035	0.146±0.037	0.107±0.024* <sup>#</sup>	0.246±0.057	0.262±0.081	0.169±0.037* <sup>#</sup>
Adrenals	0.016±0.003	0.015±0.004	0.019±0.002 <sup>#</sup>	0.026±0.004	0.026±0.006	0.029±0.004
Liver	3.282±0.328	3.236±0.214	3.296±0.548	5.303±0.489	5.748±0.417	5.265±1.300
Kidney	0.752±0.102	0.639±0.041*	0.669±0.038*	1.215±0.166	1.136±0.086	1.062±0.130
Lungs	0.377±0.017	0.428±0.101	0.426±0.052	0.610±0.047	0.760±0.177	0.670±0.063
Brain	0.620±0.048	0.565±0.043* <sup>#</sup>	0.638±0.073	-	-	-

\*p<0.05 vs. *Con*; <sup>#</sup>p<0.05 vs. *Nano*.



### 3.2.2. Open field activity

After 3 weeks of treatment, the rat's horizontal and vertical activity was decreased, and their local activity and immobility was increased (Fig. 5A). Ambulation time was significantly diminished in the *Solute* group, but changed hardly in the *Nano* group. Time spent with local activity was also significantly enhanced only by  $MnCl_2$ . Increased immobility and decreased rearing was observed in both treated groups. In the 6 weeks treatment, the above trend was continued and the changes were more expressed in also the *Nano* group, however these were still non-significant (Fig. 6B).



The complete set of OF data (Table 3) shows that not only the summed time but also the distance covered by ambulation decreased in the treated groups. In other forms of activity, the change of counts was parallel with that of time, indicating that the treated animals commenced, e.g., more immobility and less rearing periods.

**Table 3.** Open field data in Experiment II. Mean±SD, n=16 or 8.

<i>Treatment time</i>	<i>3 weeks</i>			<i>6 weeks</i>		
<b>Groups</b>	<i>Con</i>	<i>Nano</i>	<i>Solute</i>	<i>Con</i>	<i>Nano</i>	<i>Solute</i>
Ambulation distance (cm)	216.44±33.41	222.83±28.97	153.82±29.45** <sup>##</sup>	293.41±37.67	240.85±58.83	168.31±50.43** <sup>##</sup>
Ambulation time (s)	261.80±35.56	266.86±27.41	196.63±45.65** <sup>##</sup>	324.00±24.48	272.00±53.31	222.88±63.32*
Ambulation count	29.80±3.83	32.29±6.18	27.13±9.17	26.00±3.81	27.75±6.16	20.75±5.39
Local activity time (s)	153.60±42.37	171.00±23.57	222.00±38.43 <sup>#</sup>	126.80±22.52	142.25±41.62	228.38±41.66** <sup>##</sup>
Local activity count	61.20±12.34	64.29±5.38	82.00±11.60** <sup>##</sup>	59.40±7.30	61.50±13.97	82.13±9.09** <sup>##</sup>
Immobility time (s)	25.80±20.96	42.57±19.41	83.38±53.26*	18.20±3.96	35.38±45.42	100.00±72.57*
Immobility count	16.40±13.48	23.86±6.69	38.63±19.81	12.00±3.96	16.88±12.54	43.50±15.09** <sup>##</sup>
Rearing time (s)	159.80±55.87	120.57±40.43	99.00±62.63	132.00±38.50	151.38±52.91	49.75±27.69** <sup>##</sup>
Rearing count	59.80±8.41	50.00±12.99	45.38±16.03	61.00±12.59	76.75±17.91	32.63±15.38** <sup>##</sup>

\*p<0.01, \*\*p<0.01 vs. *Con*; <sup>#</sup>p<0.05, <sup>##</sup>p<0.01 vs. *Nano*.

### 3.2.3. Spontaneous cortical activity

After 3 weeks treatment, the spontaneous cortical activity in the SS area showed increased gamma and beta2 band activity in both treated groups, and this effect was significant for beta2 in the *Solute* group (Fig. 6A). Delta band was significantly diminished in the *Solute* group but in the *Nano* group the changes, although of similar direction, were not significant. After 6 weeks, similar but stronger effects were observed, again mainly in the *Solute* group.

In the VIS area (Fig. 6B) ECoG showed significantly increased gamma and beta2 activity in the *Solute* group after 3 weeks treatment. The same could also be observed after 6 weeks, but more pronounced. This longer treatment period had also other (non-significant) effects, like the diminishing of the alpha band in the *Nano* group, and delta in *Solute*.

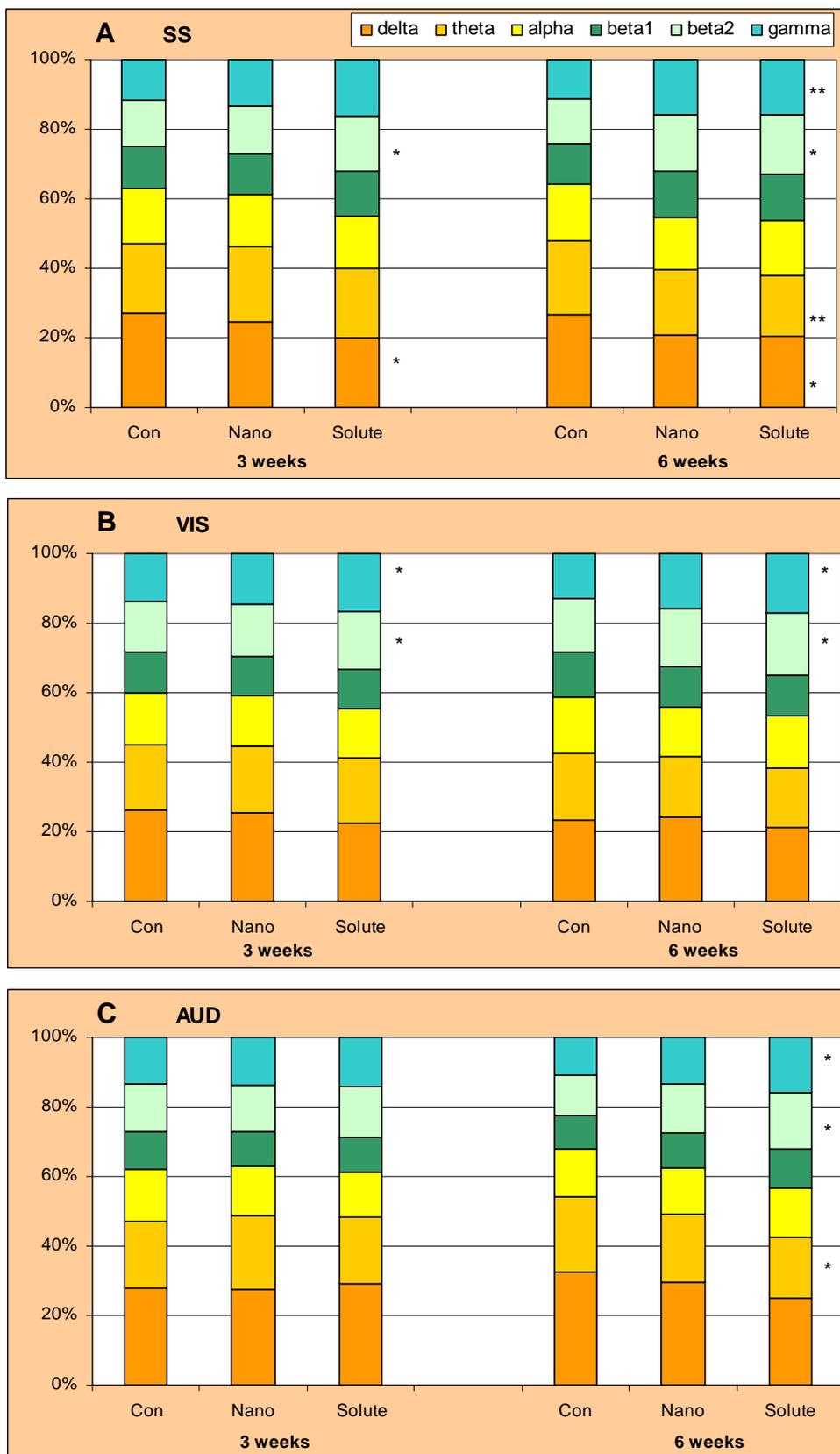
ECoG in the AUD area (Fig. 6C) had visible alterations only after 6 weeks treatment. Similarly to the SS and VIS activity, the gamma and beta2 bands increased in both groups, but only in the *Solute* group was this significant. The alpha band was decreased by nano-MnO<sub>2</sub>, and delta band, by MnCl<sub>2</sub>.

The general trend was alike in each cortical area. The proportion of the slow frequency bands (delta, theta, alpha) decreased, and of the fast bands (beta1, beta2, gamma) increased. These effects were more clear, and became significant, with longer treatment time, that is, with increasing summed dose. In the *Nano* group, the changes were not significant, but the direction of the shift was the same as with MnCl<sub>2</sub>.

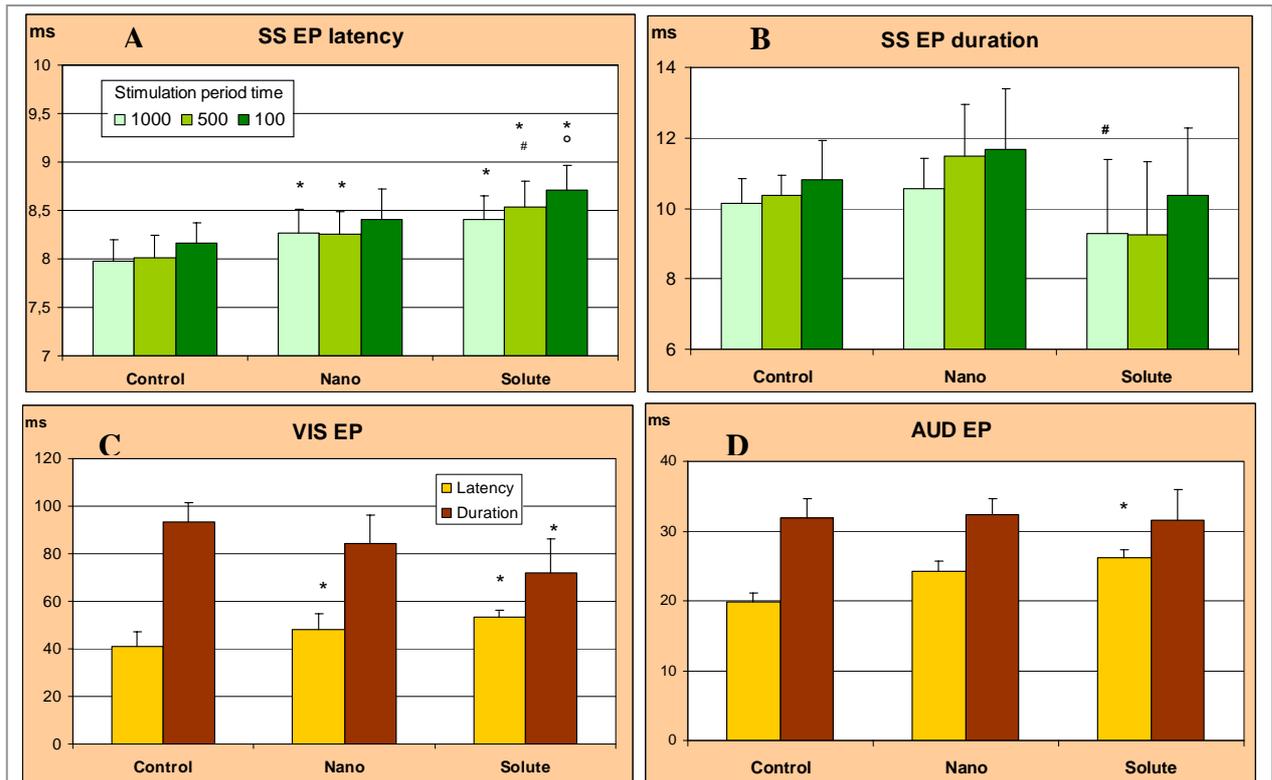
### 3.2.4. Evoked activities

As judged from the ECoG changes, it was reasonable to analyse the recorded EPs only after 6 weeks treatment. The latency of the SS EP was lengthened. This was significant at 1000 and 500 ms stimulation period time (corresponding to 1 and 2 Hz rate, respectively) in the *Nano* group; and at 1000, 500 and 100 ms period in the *Solute* group (Fig. 7A). The duration of the SS EP was only decreased by MnCl<sub>2</sub>, (significantly at 1000 ms period; Fig. 7B). In the *Solute* group, the frequency dependence of the latency was also significantly altered.

NP treatment caused significant increase in the latency of the VIS EP (Fig. 7C). This manganese form reduced here also the duration. With MnCl<sub>2</sub>, these effects were similar, and the duration was also significantly diminished. Among the parameters of the AUD EP, only the latency was significantly altered and only in the *Solute* group (Fig. 7D).



**Figure 6.** Band spectra of the spontaneous cortical activity (ECoG). (SS, somatosensory; VIS, visual; AUD, auditory).  
 \* $p < 0.05$ , \*\* $p < 0.01$  vs. *Con*.

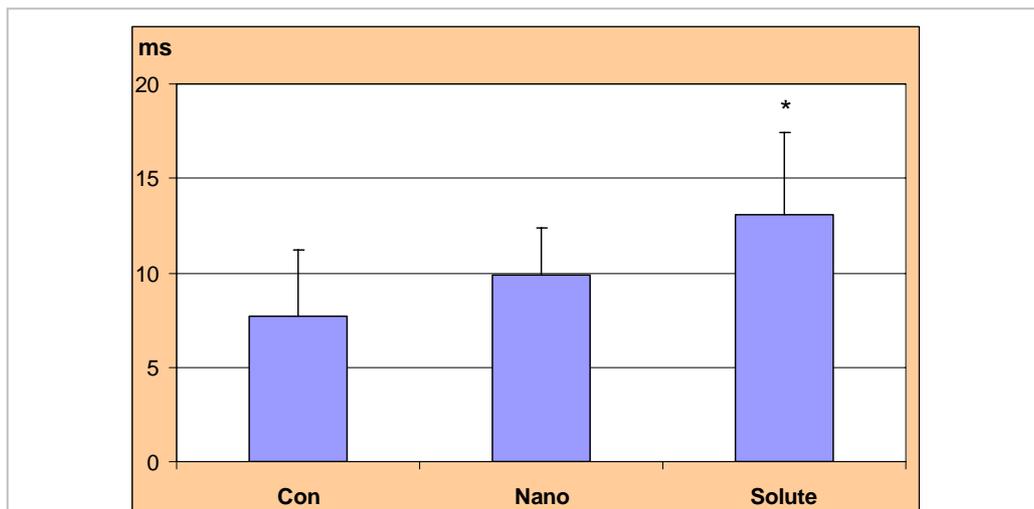


**Figure 7.** Changes of the latency and duration of the cortical evoked potentials after 6 weeks.

Mean $\pm$ SD, n=8.

\*p<0.05 vs. Con; #p<0.05 vs. Nano; ° p<0.05 vs. 1000 ms stimulus period time.

In the tail nerve, the increase of relative refractory period was significant in the *Solute* group, but the trend was also observed in the *Nano* group (Fig. 8). Other tail nerve parameters showed no clear effect.



**Figure 8.** Changes of the relative refractory period of the tail nerve (calculated from action potentials obtained with double stimuli).

Mean $\pm$ SD, n=8. \*p<0.05 vs. Con.

### 3.3 Experiment III

In this experiment the animals were given only the NP form of manganese (nano-MnO<sub>2</sub>), but another way of application, intratracheal instillation, was introduced. There were 5 groups of 8 rats each; the control group was instilled with distilled water, and the treated groups (*n1v1x*, *n2v1x*, *n1v2x*, *n2v2x*) were given the nanosuspension every weekday, for 3 weeks. The daily and total doses are presented in Table 4. The investigations (electrophysiological recording, dissection and organ weighing) were done on the next day after the last instillation.

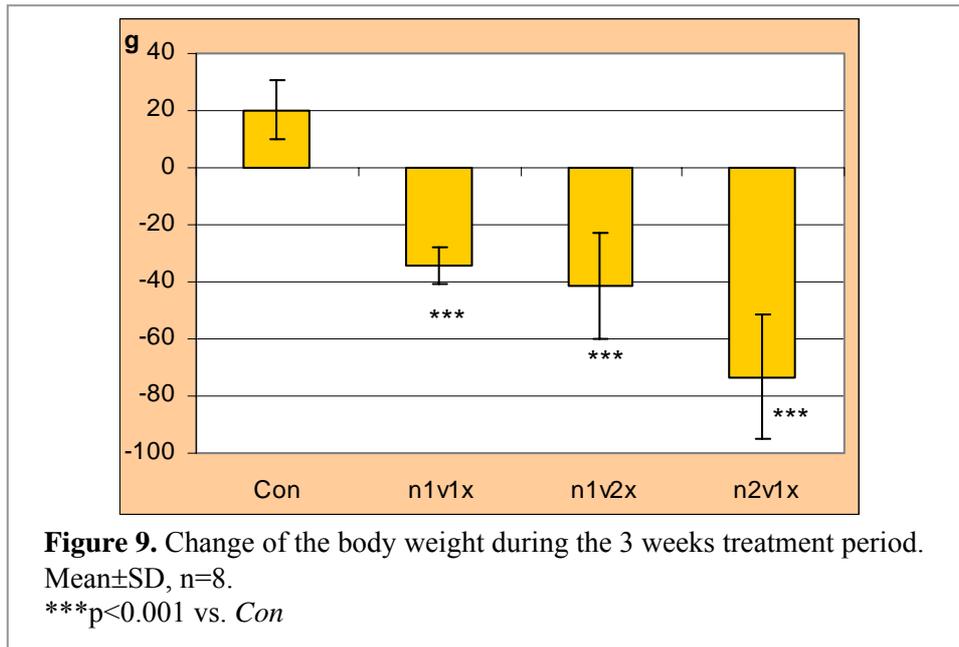
**Table 4.** Detailed time scheme of Experiment III.

Group Code	<i>Con</i>	<i>n1v1x</i>	<i>n1v2x</i>	<i>n2v1x</i>	<i>n2v2x</i>
Duration of treatment	3 weeks, 5 days per week				
Treatments per day	1	1	2	1	2
Dose given per treatment (mg Mn/kg b. w.)	-	2.63	2.63	5.26	5.26
Total dose* (mg Mn/rat)	-	9.86	17.75	17.75	35.5

\*Calculated on the basis of group average body weights.

#### 3.3.1 Body and organ weights

Intratracheal instillation of MnO<sub>2</sub> NPs had dramatic effect on the rats' body weight. While there was a normal body weight gain in the control rats during the treatment period, the body weights of the treated animals decreased massively by the end of the 3rd week (Fig. 9 shows the start-to-end weight change, the full time course of body weight was less interesting due to the short treatment period). The general toxicity indicated by the weight loss was so strong that in the highest dose group, *n2v2x*, the majority of the rats perished during the treatment period. This group was, hence, excluded from further evaluation.



Among the relative organ weights related to 1/100 body weight, the weight of the lungs increased strongly with ascending summed dose. In all treated groups this change was significant vs. control. In case of *n2v1x*, the increase was also significant vs. *n1v1x* (Table 5). In this group (and in the animals of the *n2v2x* group perished during the treatment period) the excised lungs were also severely emphysematic. A slight increase of the relative brain weight was present in every treated group, significantly in *n2v1x*. Dose-dependently enhanced heart weight (significant in the *n2v1x* group) might be secondary, due to compensatory hypertrophy; and a similar weight change of the adrenals, to stress. The relative kidney weights were slightly increased by the treatments. Considering the massive effect of NP treatment on the body weight, the relative organ weights were also calculated on the base of brain weight. Doing so, the increase of the lung, adrenal, and spleen weights remained significant (Table 5), indicating that these were real effects.

**Table 5.** Relative organ weights in Experiment III, related to 1/100 body weight and to brain weight. Mean $\pm$ SD, n=8

Groups	Con	<i>n1v1x</i>	<i>n1v2x</i>	<i>n2v1x</i>	Con	<i>n1v1x</i>	<i>n1v2x</i>	<i>n2v1x</i>
Organs	Related to 1/100 body weight				Related to brain weight			
Heart	0.2732 $\pm 0.0200$	0.3089 $\pm 0.0395$	0.3394 $\pm 0.0534$	0.3573 $\pm$ 0.0528**	0.5451 $\pm 0.0517$	0.5551 $\pm 0.0927$	0.5958 $\pm 0.0121$	0.5564 $\pm 0.0818$
Spleen	0.1655 $\pm 0.0148$	0.22.10 $\pm 0.0274^{**}$	0.1848 $\pm 0.0376$	0.1774 $\pm 0.0240^{*}$	0.3296 $\pm 0.0277$	0.3943 $\pm 0.0438^{**}$	0.3238 $\pm 0.0308$	0.2766 $\pm 0.0403^{**}$
Thymus	0.1136 $\pm 0.0155$	0.1370 $\pm 0.0238$	0.1314 $\pm 0.0639$	0.1278 $\pm 0.0184$	0.2258 $\pm 0.0252$	0.2451 $\pm 0.0466$	0.2266 $\pm 0.0840$	0.1997 $\pm 0.0338$
Adrenals	0.0173 $\pm 0.0027$	0.0228 $\pm 0.0038$	0.0229 $\pm 0.0030$	0.0312 $\pm 0.0071^{***}$	0.0344 $\pm 0.0049$	0.0408 $\pm 0.0074^{*}$	0.0413 $\pm 0.0099^{*}$	0.0479 $\pm 0.0071^{**}$
Liver	3.0268 $\pm 0.2545$	3.3906 $\pm 0.1844$	3.1970 $\pm 0.3821$	3.1153 $\pm 0.2330$	6.0233 $\pm 0.3805$	6.0751 $\pm 0.5566$	5.6723 $\pm 0.8138$	4.8881 $\pm 0.7653^{***}$
Kidney	0.6729 $\pm 0.0627$	0.7413 $\pm 0.0613$	0.6776 $\pm 0.0293^{**}$	0.7841 $\pm 0.0494$	1.3383 $\pm 0.0945$	1.3347 $\pm 0.2043$	1.2045 $\pm 0.1338$	1.2277 $\pm 0.1641$
Lungs	0.4572 $\pm 0.0382$	1.0126 $\pm 0.1097^{**}$	1.3775 $\pm 0.3640^{***}$	1.7247 $\pm 0.4862^{***\#}$	0.9125 $\pm 0.0950$	1.8045 $\pm 0.1272^{***}$	2.3940 $\pm 0.3663^{***}$	2.6387 $\pm 0.5243^{***}$
Brain	0.5025 $\pm$ 0.0270	0.5619 $\pm 0.0564$	0.5686 $\pm 0.0780$	0.6460 $\pm 0.0730^{**}$	-	-	-	-

\*p<0.05, \*\*p<0.01, \*\*\* p<0.001 vs. *Con*; # p<0.05 vs. *n1v1x*.

### 3.3.2. Spontaneous cortical activity

There were no significant changes in the power spectrum of the ECoG after the 3 weeks of treatment, and the visible trends were not uniform. In the SS area, the proportion of the theta band slightly decreased, whereas the beta2 band was faintly enhanced in the *n1v2x* and *n2v1x* group. Gamma band was also slightly stronger but only in the *n2v1x* group.

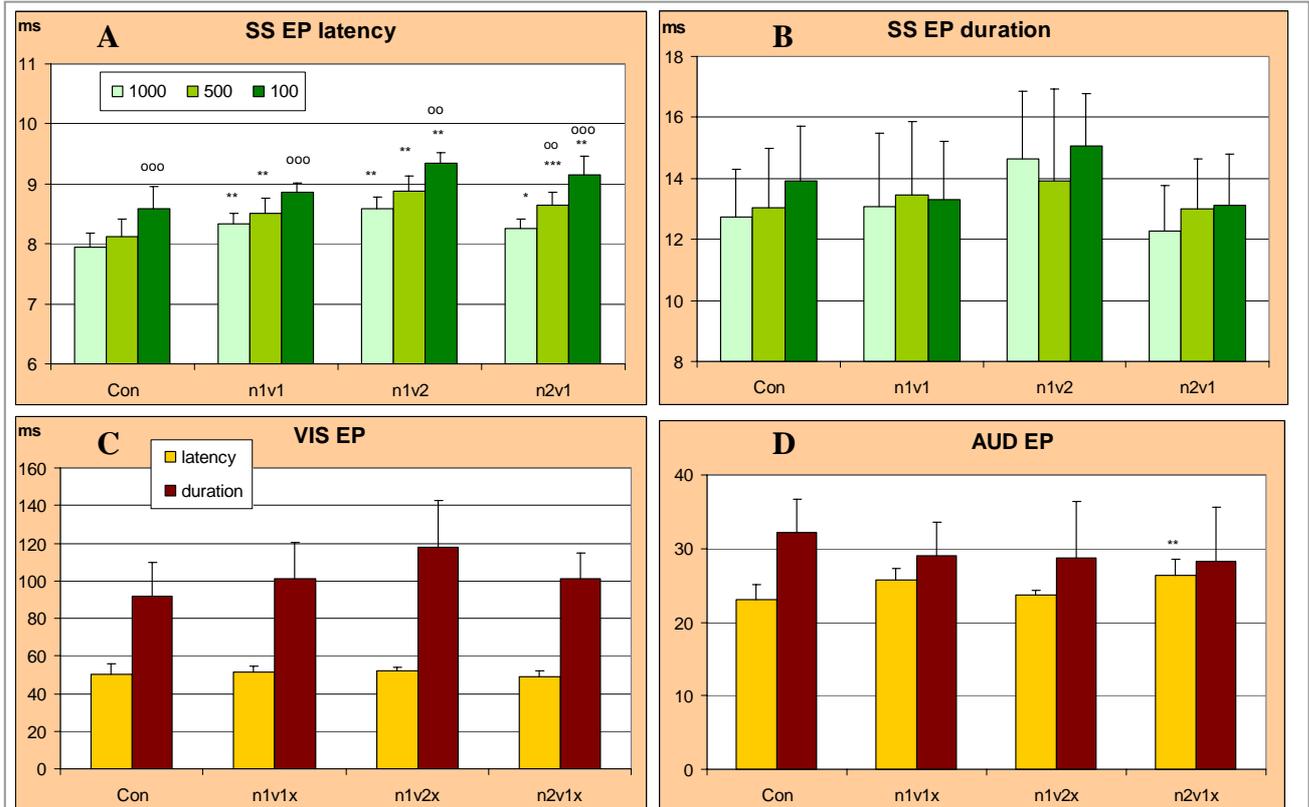
In case of ECoG from the VIS and AUD area, no such changes were visible. VIS ECoG was not altered at all, and in the AUD area some increase of the slow and decrease of fast bands was observable.

### 3.3.3. Evoked activities

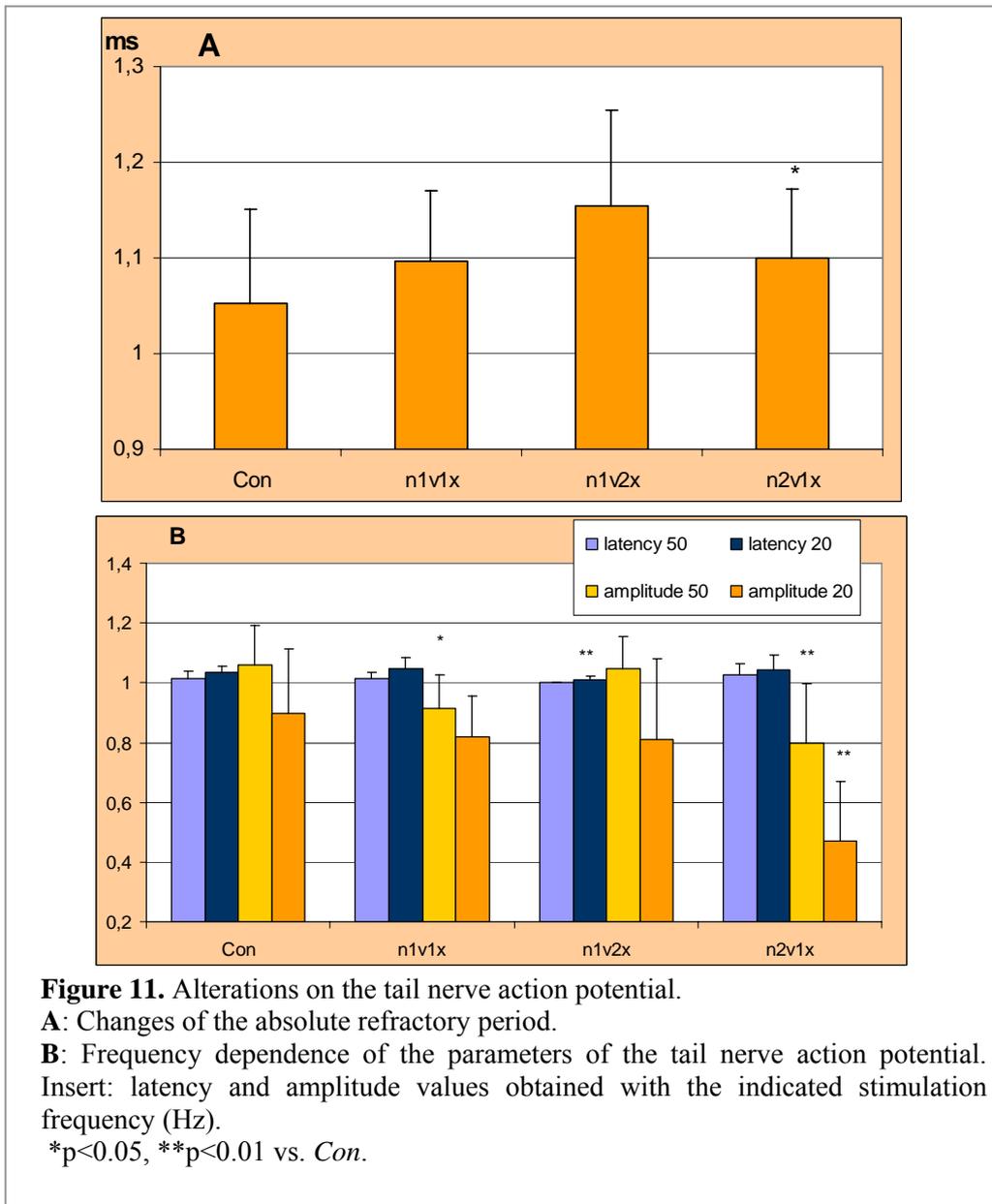
Among the parameters of evoked activity, the latency of SS EPs was the most prominent (Fig. 10A). In all treated groups and at all stimulation frequencies, the latency was significantly longer than in *Con*. In *n2v1x*, the frequency-dependent increase of the latency was also significantly stronger than in the control. The duration of the SS EPs slightly decreased in the *n2v1x* group (Fig. 10B).

Latency lengthening was observed also on the AUD EPs, in case the *n1v1x* and *n2v1x* groups, however, it was only significant in the latter group. The changes of AUD EP duration, and all changes of the VIS EP, remained below significance (Fig. 10C,D).

Concerning the tail nerve, the parameter showing clear dose-dependent change was the absolute refractory period (Fig. 11A). This parameter was lengthened in each treated group, significantly in *n2v1x*. 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 SS EP (Fig. 11B, cf. Fig. 10A).



**Figure 10.** Changes of the latency and duration of the cortical evoked potentials. Displayed as in Fig. 9.



### 3.4. Experiment IV

Experiment III indicated that instillation is a usable way of NP application but showed also that the dosage had to be changed because the heavy general toxicity may have masked some specific effects. So, in Experiment IV, the treatment was prolonged for 3, 6 and 9 weeks and the most extreme doses (those requiring two applications daily) were omitted. The same nanosuspension as in the previous experiment was administered to the rats; intratracheally once a day, 5 days per week, in two different doses. To see if the manipulation itself had any effect on the studied neuro-functional and general toxicological parameters, two control groups were used: the animals in the *W* group got the ether anaesthesia and instillation with distilled water (vehicle control), while the *Con* group had no treatment at all. Groups and doses are summarized in Table 6. Each group consisted of 24 rats at start, of which 8 were processed after 3, another 8 after 6, and the remaining 8 after 9 weeks exposure. The investigations (open field test, electrophysiological measurements, dissection and organ weighing) were done on the next day after the treatment period.

**Table 6.** Groups and doses in Experiment IV.

Group	Code	Treatment and daily dose	Duration	Total dose (mg Mn/rat)*
Untreated control	<i>Con</i>	---		---
Vehicle control	<i>W</i>	Distilled water 1 ml/kg b.w.		---
Low dose	<i>LD</i>	MnO <sub>2</sub> nanosuspension, 2.63 mg Mn/kg b.w.; 1 ml/kg b.w.	3, 6 and 9 weeks	3 weeks: 9.86 6 weeks: 19.72 9 weeks: 29.58
High dose	<i>HD</i>	MnO <sub>2</sub> nanosuspension, 5.26 mg Mn/kg b.w.; 2 ml/kg b.w.		3 weeks: 19.72 6 weeks: 39.44 9 weeks: 59.16

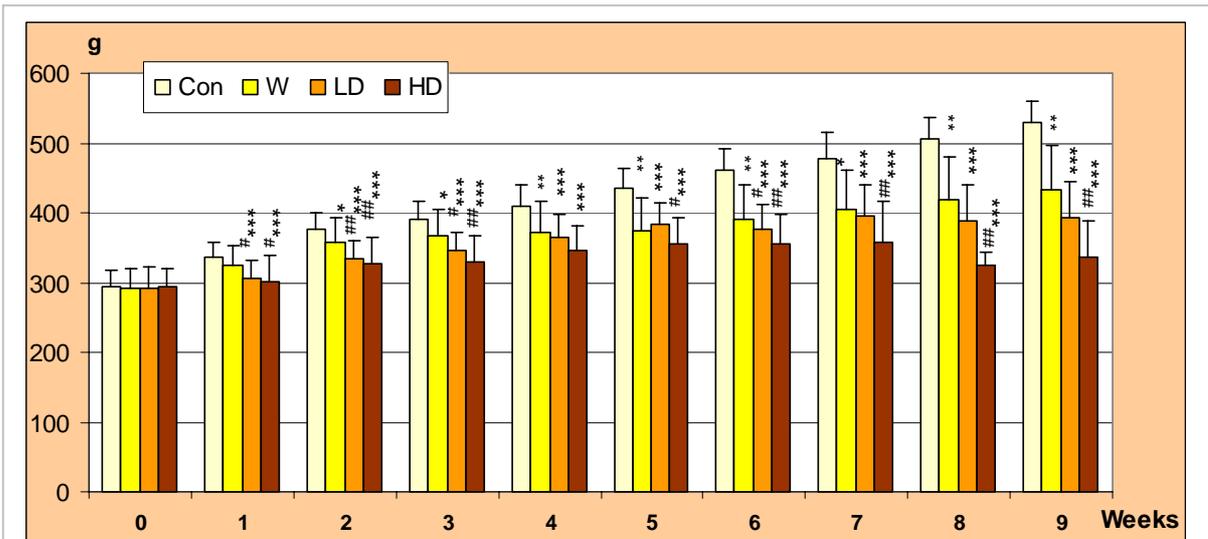
\*Calculated on the basis of group average body weights.

### 3.4.1. Body and organ weights

Intratracheal instillation had a clear-cut effect on the rats' body weight. There was normal body weight gain in the control (*Con*) rats during the treatment period. Compared to that, the *W* group had a lower but still uninterrupted weight gain – indicating that the manipulation (anaesthesia and instillation) alone was not responsible for the alterations observed. In both NP treated groups (*HD*, *LD*), however, body weight ceased to increase in the 6<sup>th</sup> week (Fig. 12).

When calculated on the basis of body weight, (Table 7A) the relative weight of the lungs increased strongly with increasing summed dose, and the organs also had a more and more emphysematic exterior (like in Experiment III). It was noteworthy; however, that distilled water instillation alone (group *W*) had no effect on the lung weight. By the 9<sup>th</sup> week, significant decrease of the liver relative weight developed also. The relative kidney weight increased by the end of the 9<sup>th</sup> week (significantly in the *HD* group). Dose- and time-dependently increased weight of the heart and adrenals was also seen (although without significance); which was probably secondary, due to compensatory hypertrophy and to stress, respectively.

The effects seen on the body weight-related data, even if significant, could be questionable due to the effect of the treatment on the body weight itself, although it was not as severe as in Experiment III. The absolute brain weight data were less variable so that brain weight was also used as calculation basis (Table 7B). With this calculation, the increase of relative lung and decrease of liver weight retained significance (and the difference between relative liver weight decrease of the *W* vs. *LD* and *HD* groups was clearer, arguing against an artefact caused by the procedure). By the 9<sup>th</sup> week, the dose- and time-dependently increased weight of the adrenals (probably due to stress, see above) also became significant.



**Figure 12.** Body weight gain of the control and treated rats in Experiment IV. Insert: groups.

Mean±SD, n=24, 16 or 8 (see text).

\*p<0.05, \*\*p<0.1, \*\*\*p<0.001 vs. Con; #p<0.05, ##p<0.01 vs. W.

**Table 7.** Relative organ weights, related to 1/100 body weight (**A**) and to brain weight (**B**) after 3, 6 and 9 weeks exposure to Mn nanoparticles in Experiment IV.

Mean±SD, n=24, 16 or 8 (see text).

A	Groups	Con	W	LD	HD
<b>3 weeks</b>					
	Lung	0.656±0.255	0.529±0.128	1.076±0.364 <sup>#</sup>	1.012±0.363 <sup>#</sup>
	Liver	3.065±0.276	3.147±0.131	3.044±0.361	3.065±0.179
	Brain	0.481±0.023	0.493±0.031	0.532±0.038*	0.530±0.030*
	Kidney	0.651±0.054	0.638±0.053	0.668±0.057	0.663±0.027
	Heart	0.288±0.018	0.280±0.016	0.300±0.032	0.283±0.028
	Spleen	0.175±0.019	0.185±0.029	0.210±0.016	0.209±0.032
	Thymus	0.118±0.029	0.115±0.034	0.118±0.027	0.121±0.031
	Adrenals	0.013±0.002	0.012±0.003	0.019±0.003* <sup>#</sup>	0.018±0.004
<b>6 weeks</b>					
	Lung	0.357±0.027	0.375±0.056	0.958±0.205*** <sup>###</sup>	0.989±0.207*** <sup>###</sup>
	Liver	3.003±0.171	3.106±0.170	3.014±0.193	3.016±0.203
	Brain	0.422±0.024	0.444±0.098	0.495±0.048	0.505±0.047
	Kidney	0.604±0.062	0.632±0.034	0.635±0.065	0.642±0.043
	Heart	0.264±0.028	0.262±0.015	0.282±0.030	0.287±0.028
	Spleen	0.163±0.015	0.167±0.033	0.182±0.032	0.201±0.031
	Thymus	0.100±0.033	0.079±0.016	0.114±0.029	0.124±0.029
	Adrenals	0.012±0.002	0.013±0.004	0.016±0.006	0.018±0.004
<b>9 weeks</b>					
	Lung	0.321±0.011	0.370±0.024	0.841±0.249** <sup>###</sup>	1.112±0.336*** <sup>###</sup>
	Liver	3.037±0.228	2.804±0.206*	2.902±0.243**	2.729±0.283***
	Brain	0.404±0.020	0.470±0.060	0.490±0.048	0.538±0.086
	Kidney	0.600±0.022	0.619±0.036	0.640±0.107	0.658±0.100**
	Heart	0.253±0.011	0.269±0.030	0.296±0.068	0.329±0.050
	Spleen	0.157±0.017	0.173±0.018	0.173±0.034	0.170±0.034
	Thymus	0.077±0.019	0.068±0.019	0.112±0.023	0.109±0.029
	Adrenals	0.010±0.002	0.014±0.003	0.016±0.006	0.022±0.007

\*p<0.05, \*\*p<0.01, \*\*\* p<0.001 vs. Con; <sup>#</sup>p<0.05, <sup>##</sup>p<0.01, <sup>###</sup>p<0.001 vs. W.

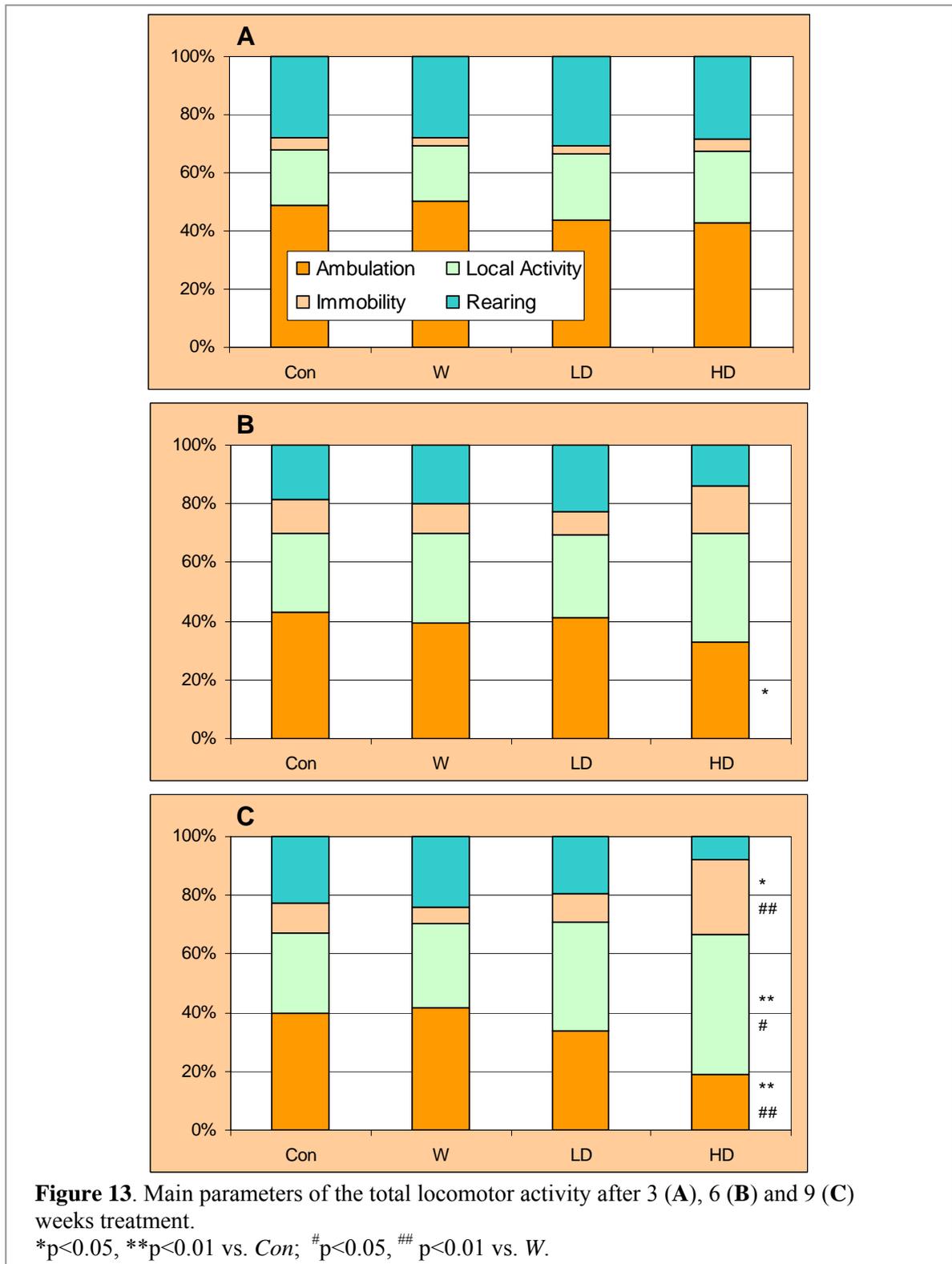
<b>B</b>	<b>Groups</b>	<i>Con</i>	<i>W</i>	<i>LD</i>	<i>HD</i>
	<b>3 weeks</b>				
	Lung	1.596±0.479	1.111±0.259	1.955±0.566 <sup>#</sup>	1.911±0.682 <sup>#</sup>
	Liver	6.288±0.805	6.431±0.598	5.737±0.684	6.037±0.539
	Kidney	1.314±0.107	1.318±0.114	1.260±0.132	1.254±0.094
	Heart	0.596±0.040	0.568±0.021	0.565±0.054	0.534±0.058
	Spleen	0.366±0.049	0.365±0.055	0.398±0.047	0.396±0.067
	Thymus	0.244±0.039	0.246±0.049	0.222±0.049	0.227±0.051
	Adrenals	0.027±0.005	0.026±0.005	0.035±0.005	0.034±0.008
	<b>6 weeks</b>				
	Lung	0.845±0.051	0.928±0.461	1.932±0.335 <sup>***###</sup>	1.959±0.363 <sup>***###</sup>
	Liver	7.121±0.397	7.489±2.670	6.129±0.610	5.986±0.250
	Kidney	1.431±0.138	1.508±0.457	1.287±1.117	1.283±0.161
	Heart	0.625±0.065	0.628±0.212	0.574±0.089	0.569±0.040
	Spleen	0.386±0.036	0.409±0.198	0.369±0.066	0.404±0.089
	Thymus	0.239±0.082	0.193±0.079	0.231±0.067	0.248±0.062
	Adrenals	0.029±0.004	0.034±0.020	0.032±0.011	0.036±0.008
	<b>9 weeks</b>				
	Lung	0.797±0.055	0.797±0.093	1.614±0.468 <sup>***###</sup>	2.047±0.426 <sup>***###</sup>
	Liver	7.528±0.665	6.080±1.104 <sup>**</sup>	5.979±0.803 <sup>**</sup>	5.134±0.546 <sup>***#</sup>
	Kidney	1.488±0.073	1.332±0.160	1.309±0.263	1.227±0.087 <sup>**</sup>
	Heart	0.626±0.034	0.575±0.032	0.597±0.086	0.619±0.091
	Spleen	0.390±0.057	0.371±0.048	0.358±0.094	0.317±0.041
	Thymus	0.192±0.049	0.148±0.050	0.235±0.060 <sup>##</sup>	0.202±0.044
	Adrenals	0.024±0.005	0.029±0.006	0.033±0.011	0.041±0.011 <sup>**#</sup>

\*p<0.05, \*\*p<0.01, \*\*\* p<0.001 vs. *Con*; #p<0.05, ## p<0.01, ###p<0.001 vs. *W*.

### 3.4.2 Open field activity

The treated rats' open field activity showed a shift to less and less motility (Fig. 13). Ambulation time decreased already after 3 weeks. This change became significant by the end of the 6<sup>th</sup> week in the *HD* group. Local activity and immobility began to increase in the *HD* group by the end of the 6<sup>th</sup> week, and further increased by the 9<sup>th</sup> week in both treated groups (significantly only in *HD*). There was some decrease of ambulation and increase of local activity and immobility also in the two controls (*Con* and *W*) during the 9 weeks, which was most probably due to increasing age of the rats as judged from earlier experiences. Dose-dependent decrease of rearing time could be observed in the rats treated for 6 and 9 weeks.

The complete set of OF data from Experiment IV (Table 8) shows that ambulation distance decreased more markedly with increasing age of the rats, but the treatment-dependent decrease of ambulation distance and time was more or less in parallel in the treated groups (vs. *Con* and *W*). Ambulation count changed little (except *HD* after 9 weeks) meaning that treated rats initiated typically as many ambulations as controls but covered smaller distances. For local activity and immobility, time and count changed together, indicating that the average rate of switching activities was not much altered. After 9 weeks treatment, however, the average time of one event of immobility and local activity increased in the *HD* group, and the average length of one rearing, decreased.



**Table 8.** Complete open field data after 3, 6 and 9 weeks exposure to Mn nanoparticles in Experiment IV. Mean±SD, n=24, 16 or 8 (see text).

	Groups	Con	W	LD	HD
3 weeks	Ambulation distance (cm)	2527.65±340.02	2676.33±391.19	2067.03±269.69 <sup>#</sup>	1940.80±332.75 <sup>*##</sup>
	Ambulation time (s)	292.50±39.63	301.17±29.45	263.50±24.37	257.83±31.78
	Ambulation count	32.17±8.70	30.67±6.62	31.38±5.37	30.67±4.37
	Local activity time (s)	116.50±28.54	116.00±20.42	135.00±33.70	148.33±43.98
	Local activity count	54.00±10.92	50.50±3.39	63.88±9.85	63.67±11.38
	Immobility time (s)	24.67±20.47	15.83±60.5	18.00±12.17	23.50±16.28
	Immobility count	13.67±9.37	10.17±3.82	12.13±8.32	14.50±10.71
	Rearing time (s)	167.33±31.37	168.00±30.98	184.50±51.90	171.33±36.18
	Rearing count	73.17±9.54	72.67±8.33	73.63±14.48	68.17±10.38
	6 weeks	Ambulation distance (cm)	211.46±37.12	182.74±12.73	208.72±39.65
Ambulation time (s)		260.00±27.05	236.67±17.87	249.00±28.47	197.17±44.20 <sup>*</sup>
Ambulation count		28.20±2.28	28.50±4.76	32.00±1.58	26.83±5.27
Local activity time (s)		161.00±42.26	183.17±34.03	168.00±58.66	222.67±44.96
Local activity count		71.40±8.68	70.50±7.09	64.40±14.66	83.67±11.64
Immobility time (s)		69.20±47.91	61.00±28.51	47.60±33.51	96.50±54.61
Immobility count		28.40±11.55	29.33±9.83	24.00±15.75	42.33±17.60
Rearing time (s)		110.80±44.12	120.17±49.26	136.40±78.39	84.67±78.21
Rearing count		45.60±19.27	47.67±17.13	50.60±25.66	33.83±26.42
9 weeks		Ambulation distance (cm)	195.35±45.14	185.05±51.89	155.14±26.17
	Ambulation time (s)	240.60±55.29	250.20±63.03	204.50±28.18	113.83±50.70 <sup>**##°</sup>
	Ambulation count	29.80±6.98	29.00±7.35	29.00±4.20	20.00±5.44
	Local activity time (s)	162.20±38.75	171.60±63.01	220.00±61.06	288.00±38.11 <sup>**#</sup>
	Local activity count	68.80±15.83	71.60±12.56	81.17±13.23	95.17±5.27 <sup>#</sup>
	Immobility time (s)	61.60±50.16	35.80±19.68	60.17±32.11	150.50±69.38 <sup>*##°</sup>
	Immobility count	31.20±16.96	22.60±11.39	32.17±16.77	65.67±16.74 <sup>*##°</sup>
	Rearing time (s)	136.60±42.89	143.40±64.89	116.33±81.29	48.67±31.09
	Rearing count	51.60±8.32	54.60±18.82	42.67±21.88	25.67±18.34

\*p<0.05, \*\*p<0.01 vs. Con; <sup>#</sup>p<0.05, <sup>##</sup>p<0.01 vs. W; <sup>°</sup>p<0.05 HD vs. LD.

### 3.4.3. Spontaneous cortical activity

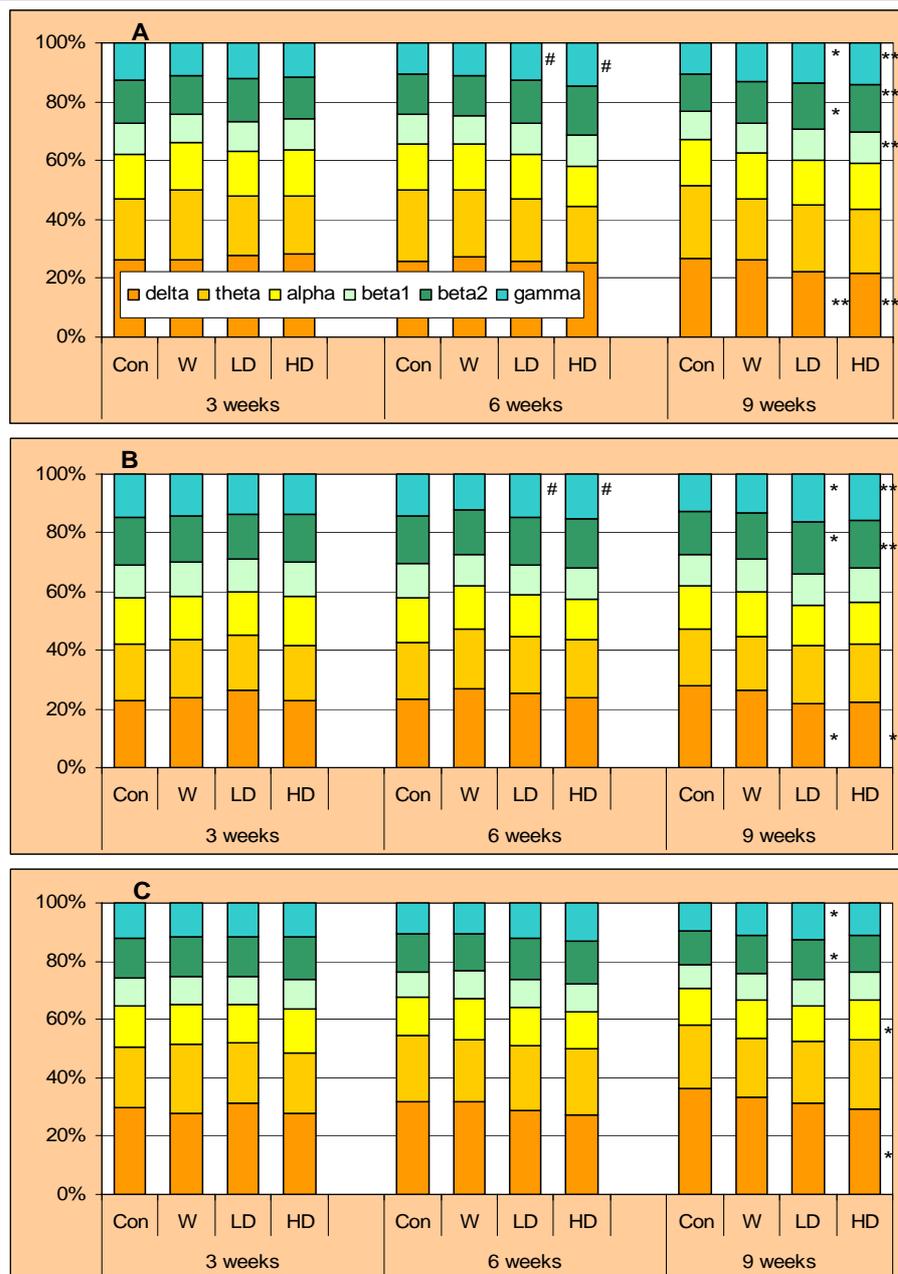
In the SS spontaneous cortical activity, after 3 weeks, there could be seen a slight decrease of the theta frequency band in both treated groups, but in case the fast frequency bands the treatment did not generate any visible alterations (Fig. 14A). By the 6<sup>th</sup> week the theta band continued its diminishing; and the alpha band was decreased significantly in the *HD* group. In addition, the fast frequency bands began to enhance; the beta1 and beta2 bands showed this change dose-dependently, and in case of gamma band, the high dose had significant increase. After 9 weeks, the last mentioned changes were strengthened; the delta band was decreased, the gamma and beta2 bands were increased significantly in both treated groups, while the beta1 band was only enhanced in the *HD* group in significant way.

In the visual cortical activity (Fig. 14B), there were no remarkable alterations after 3 weeks. By the 6<sup>th</sup> week, the gamma band was significantly increased in both treated groups, but in case the slow frequency bands there were still no changes. After 9 weeks treatment, the gamma and beta2 bands were significantly enhanced, whereas the delta band was significantly diminished in both treated groups.

In the auditory cortical activity (Fig. 14C), the delta band was slightly decreased at the end of the 3<sup>rd</sup> week in the treated group, which was strengthened by the 6<sup>th</sup> week; finally, by the end of the 9<sup>th</sup> week this alteration became significant in the *HD* group. From the fast frequency bands, the gamma and beta2 bands were increased by the 6<sup>th</sup> week in both treated groups, which developed to significant effect by the end of the 9<sup>th</sup> week in *LD* (in *HD*, alpha band changed significantly).

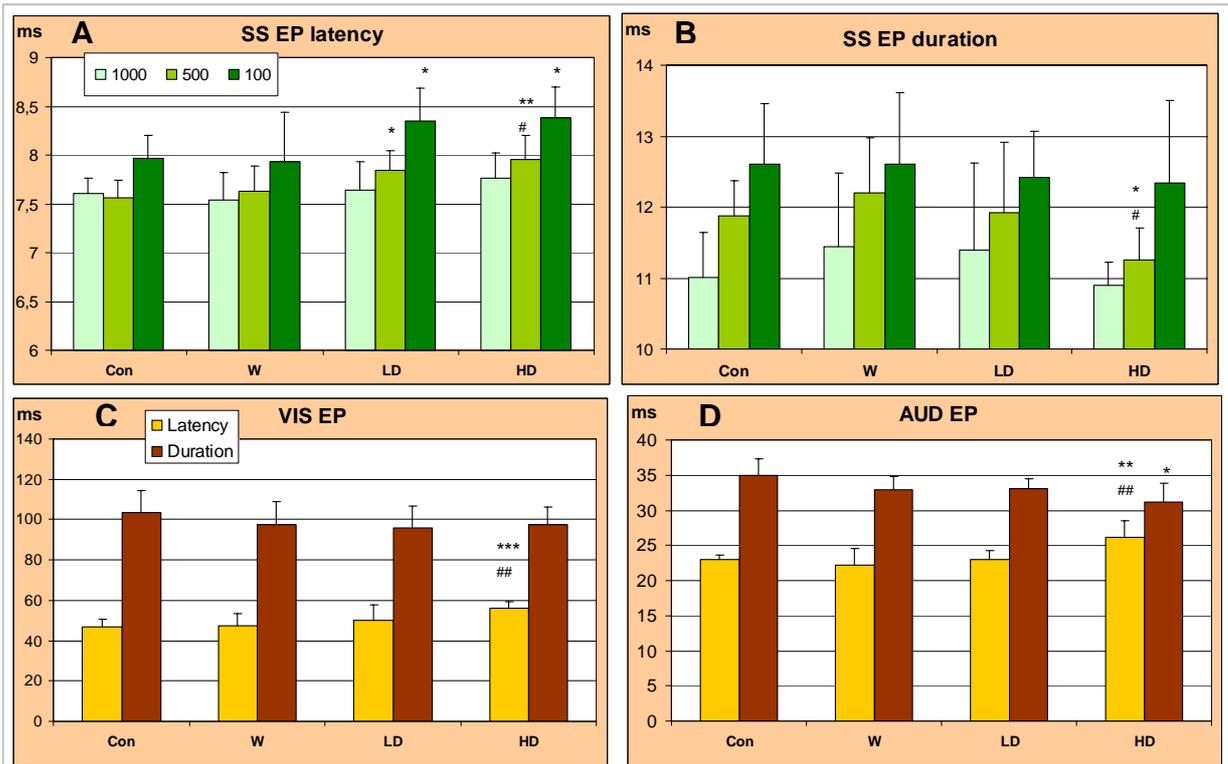
### 3.4.4. Evoked activities

Among the parameters of evoked activity, lengthening of the latency was the most prominent, developing in a dose- and time-dependent manner (gradually increasing changes in the 3 and 6 weeks data, not shown) to significant changes after 9 weeks. In case of the SS EP (Fig. 15A,B) latency increase was significant in both treated group vs. *Con*, and also in the *HD* group vs. *W*, but only at more frequent stimulation (2 and 10 Hz instead of 1). This, possibly, indicated the increased fatigability of the cortex in the treated rats. The changes in the duration were not as clear-cut; the only significant change was the decrease in the *HD* group at 2 Hz stimulation frequency.

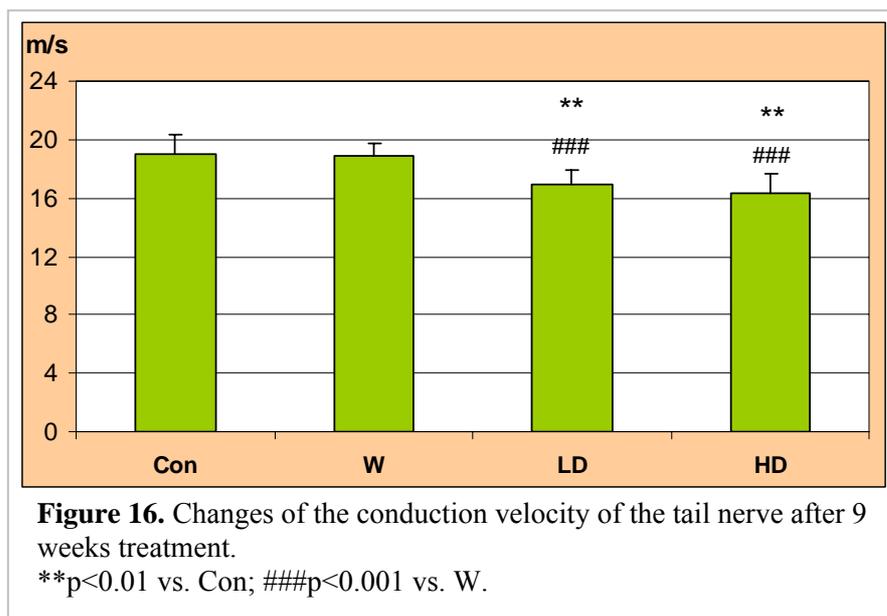


**Figure 14.** Change of spontaneous auditory activity after 3, 6 and 9 weeks intratracheal exposure to MnO<sub>2</sub> nanoparticles. **A**, Somatosensory; **B**, visual; **C**, auditory area. \* $p < 0.05$ , \*\* $p < 0.01$  vs. Con; # $p < 0.05$  vs. W

In the VIS EP (Fig. 15C) the most marked effect was also the latency lengthening in *HD*. The durations were slightly decreased in both treated groups. The AUD EP (Fig. 15D) showed similar latency increase as VIS EP, and the duration decrease was also significant in *HD* vs. *Con*. The mostly negligible difference between the values of the *Con* and *W* group showed again that the procedure alone had in itself no influence on the cortical activity.



In the tail nerve (Fig. 16), conduction velocity was significantly decreased by the end of the 9 weeks treatment in both treated groups. The increase of the refractory periods was below significance.

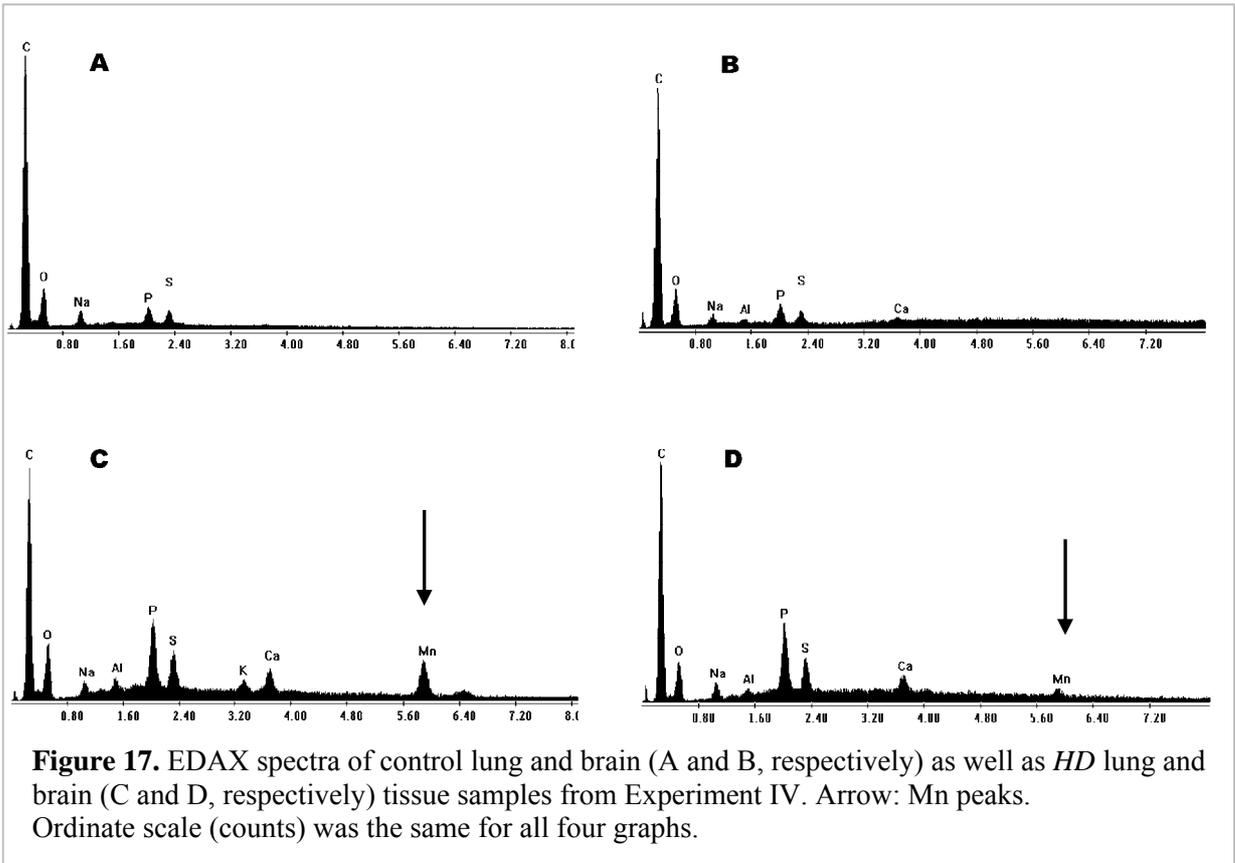


### 3.5. Tissue manganese measurements

The tissue samples mentioned in 2.3. were primarily taken for Mn level determination. These investigations have not yet been completed but the 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.

In cooperation with the National Institute of Environmental Health (Budapest), scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM-EDAX) was performed on brain and lung samples (fixed in 7% formalin) of three randomly chosen rats per group from Experiment IV. The microscope was operated in “environmental” mode (water vapor atmosphere and moderate vacuum, 0.1 - 20 torr, in the chamber). SEM observations were carried out at various magnifications with 25 kV electron beam energy and ca. 10 mm working distance. An energy dispersive X-ray spectrum was collected from the selected areas of the tissue in the 0 - 10 keV range. The elements observed were C, O, Na, Al, P, S, K, Ca and Mn, with detection limit >1wt%. The relative elemental composition of the tissue samples was computed directly with the EDAX software.

Fig. 17 shows typical EDAX spectra of lung and brain samples from *Con* and *HD* rats. Mn content (wt%) was zero in *Con*, but gave a clear-cut peak in the *HD* samples (on the spectra shown, 15.56% and 4.76% in the lung and brain sample, respectively). The results of elemental analysis by ICP-MS, on two brain samples of the *Con* and *HD* group, were in accordance.



## 4. DISCUSSION

Excess Mn is known to cause a multitude of functional abnormalities in humans (Mergler et al., 1999). In case studies on effects of airways exposure by welding fumes, which mainly consist of NPs (McNeilly et al., 2004) and always contain Mn (Antonini et al., 2006), parkinsonism (Bowler et al., 2006) and epileptic syndrome (Hernandez et al., 2003) was mentioned. In the same exposure group, altered EEG and event-related potentials were reported (Sinczuk-Walczak et al., 2001; Sjögren et al., 1996). Previous works of our laboratory revealed cortical electrophysiological changes in rats following acute (Pecze et al., 2003, 2005) and subchronic (Vezér et al., 2005, 2007) exposure to Mn in form of orally applied MnCl<sub>2</sub> solution. The latter study also demonstrated significant behavioural alterations and an increase in the blood, cortex and hippocampus Mn levels. As continuation and development of these experiments, we applied here two novel administration methods (intranasally and intratracheally) and a physicochemical form of Mn (oxide NPs) by which airborne occupational and environmental Mn exposure can be modelled more properly.

Traditionally it has been supposed that the target organ for effects of inhaled solid particles is the respiratory tract. However, more recent evidence from human epidemiological and controlled clinical, and also from animal, studies with ambient particulate air pollutants showed that extrapulmonary organs are also affected (Elder et al., 2006). It has been hypothesized that inhaled UFPs accumulate and cause effects in the cardiovascular system and the CNS (Oberdörster et al., 2005) because of their propensity to translocate across epithelial barriers. The deposited Mn can be translocated to extrapulmonary organs from the respiratory tract in two main ways. The indirect way involves transcytosis across epithelia of the respiratory tract into the interstitium and access to the blood circulation via lymphatics, resulting in distribution throughout the body (Oberdörster et al., 2005). In the direct way, sensory nerve endings embedded in the epithelia of the airways take up Mn (Tjalve et al., 1996). In our study, the intratracheal administration was performed as a model of indirect absorption route of Mn particles; while with intranasal instillation, the effects of the directly absorbed NP Mn were investigated.

The olfactory translocation route has been well demonstrated for soluble Mn compounds (Dorman et al., 2004; Tjalve and Henriksson, 1999), and it has been suggested that solubility

is an important determinant of efficiency of this process (Dorman et al., 2001). Tjalve et al. (1996) revealed that intranasally administered  $\text{MnCl}_2$  in rats resulted in Mn being taken up by the olfactory bulbs via primary olfactory neurons and then migrating via secondary and tertiary olfactory pathways to most parts of the brain. Afterwards Elder et al. (2006) demonstrated that dissolution is not a prerequisite for neuronal uptake and translocation of solid Mn UFPs. On comparison of uptake of soluble  $\text{MnCl}_2$  and non soluble  $\text{MnO}_2$  NPs (diameter: approximately 30 nm, similar to our NPs) from the nasal mucosa it was found that although  $\text{MnCl}_2$  could be taken up to higher degree,  $\text{MnO}_2$  also appeared in the brain. The axons of olfactory neurons narrow to a diameter of approximately 200 nm and become tightly packed where they pass through the cribriform plate pores. Thus, in order to be transported through the cribriform plate, solid particles deposited on the olfactory mucosa should be <200 nm in size. Our results (experiment I and II) supported Elder et al.'s (2006) conclusions with the evidence that the administered Mn caused decreased locomotor activity and lengthened evoked potential latencies in both treated groups ( $\text{MnCl}_2$  and nano- $\text{MnO}_2$ ), but the solute form had stronger effects. The mechanisms of absorption can be complicated by the dissolution of Mn-containing NPs.  $\text{MnO}_2$  is, in bulk, water-insoluble, but in acidic environment (such as in the phagolysosomes of alveolar macrophages; Lundborg et al., 1985) a significant amount of Mn may be dissolved from the surface of the particles. Ionic Mn then reaches the CNS by cation transport through the blood-brain barrier (Aschner et al., 1999). Dissolving NPs, beyond being sources of systemic exposure, can generate cytotoxic metal concentrations locally. The qualitative similarity of effects in Experiment II was in line with the possibly dual – particulate and dissolved – absorption of Mn after NP exposure.

At cellular level, the most important effect of Mn exposure is the impairment of energy metabolism, resulting from mitochondrial disorder and free radical production. Mitochondrial complex II (Malecki, 2001) and complex III (Zhang et al., 2003) were found to be inhibited by the presence of Mn, leading to insufficiency in energy demanding processes of neurons like ion pumps (to regain normal resting potential), or to disturbed synthesis, release, reuptake, and postsynaptic reaction to glutamate (Centonze et al., 2001), dopamine (Shinotoh et al., 1997; Yamada et al., 1986) and other transmitters. Tyrosine hydroxylation, a crucial step of dopamine synthesis, was blocked by Mn in vitro (Hirata et al., 2001) - possibly by a mechanism depending on inhibition of mitochondrial function.

In rodents exposed experimentally to Mn, morphological changes resembling those seen in Parkinson's disease patients were found (Ponzoni et al., 2000), which underpins the relevance of our animal model, and indicates the involvement of dopaminergic structures in the observed functional alterations. Motivation, and hence open field locomotor activity, is regulated by mesolimbic/mesocortical dopaminergic structures (Alexander et al., 1990). Normandin et al. (2004) have demonstrated that long term inhalation exposure to Mn sulphate and Mn phosphate caused a loss of dopamine level in the brain, and the neuronal cell damage was manifested by a decrease in the motor activity – similarly to our results. In chronic manganese exposure, liver damage (indicated by significantly decreased organ weight in Experiment III and IV) can affect the substrate supply for the synthesis of the monoamine neurotransmitters (Verity, 1999). Beyond that, hyperammonemia due to the liver damage may have lead to ammonia-dependent elevation of extracellular glutamate (and further to NMDA-mediated neurotoxicity: Butterworth, 1998).

Mn-dependent inhibition of astrocytic glutamine synthetase (Aschner et al., 1999) caused likely elevated extracellular glutamate level, possibly leading to enhanced synaptic transmission in the cortex (Hazell and Norenberg, 1997). This may result acutely in increased EP amplitudes, as reported by Pecze et al. (2005), and also may explain the epileptogenic effect described by Hernandez et al. (2003); and subchronically in increased EP latency as observed in Experiment II, III and IV (in a manner apparently depending on the summed dose), and reported previously by Vezér et al. (2005). The changes of ECoG band activity; decrease of slow (delta, theta) and increase of fast (beta1, beta2, gamma) bands; were similar to that obtained with another mitochondrial toxin, 3-nitropropionic acid, in subacute exposure (Szabó et al., 2005) and may have resulted from increased collateral input of the (glutamatergic) specific afferents to the ascending reticular activation. As, however, Mn is known to inhibit choline acetyltransferase (Lai et al., 1981), increased reticular activation may not be the only explanation of faster cortical spontaneous activity in the treated rats.

Pulmonary insufficiency and tissue hypoxia did, most probably, develop in the treated rats, as suggested by the severe state of the lungs observed on dissection. This, however, is not a likely explanation of the ECoG changes as experimental hypoxia in humans (van der Post et al., 2002) and mitochondrial dysfunction (Smith and Harding, 1993) typically cause slowed EEG.

Oxidative stress most probably contributed to the alterations observed on the dissected organs. Pro-inflammatory effect of (Mn-containing) welding fumes follows from induction of oxidative stress (McNeilly et al., 2004). 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). This is, most likely not a Mn-specific effect, as it was also observed in rats exposed intratracheally by oxide NPs of other metals namely cadmium and lead (Papp and Sárközi, 2008, 2009). The surface chemistry of various oxide NPs is capable of inducing oxidative stress (Oberdörster et al., 2005).

Similarly to what was observed in our treated animals, accumulation of Mn in the brain was observed in rats exposed to welding fumes for 2 months (Erikson and Aschner, 2003), together with biochemical indicators of oxidative stress. Hyperactivity of the Mn-dependent superoxide dismutase may be one factor leading to oxidative stress due to overproduction of hydrogen peroxide (Weisiger and Fridovich 1973).

Investigations towards the environmental presence and health effects of NPs has become technically feasible 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. As functional tests seem suitable for studying nervous system effects of environmental or occupational origin (Papp et al., 2005) studies like that presented above can give a better understanding of the health consequences.

The questions pointed out as aims of the study (1.4.) 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.

## 5. REFERENCES

1. Alexander G.E., Crutcher M.D., DeLong M.R., 1990. Basal ganglia-thalamocortical circuits: parallel substrates for motor, oculomotor, “prefrontal” and “limbic” functions. *Prog. Brain Res.* 85, 119-146.
2. Anda, E., Dura, Gy., Lőriunczi, I. 1984. Effects of carbon monoxide on the peripheral nerves. *Egészségtudomány* 28, 270-277.
3. Aschner, M., Vrana, K.E., Zheng, W., 1999. Manganese uptake and distribution in the central nervous system (CNS). *NeuroToxicol.* 20, 173-180.
4. Bowler, R. Koller, W., Schultz, P.E. 2006. Parkinsonism due to manganism in a welder: Neurological and neuropsychological sequelae. *NeuroToxicol.* 27, 327-332.
5. Butterworth, R.F. 1998. Pathogenesis of acute hepatic encephalopathy. *Digest.* 59, 6-21.
6. Calderon-Garciduenas, L., Azzarelli, B., Acuna, H., Garcia, R., Gambling, T. M., Osnaya N., Monroy, S., Rizapantzi, M. D. R., Carson, L. J., Villarreal-Calderon, A., Rewcastle, B. 2002. Air pollution and brain damage. *Toxicol. Pathol.* 3, 373-389.
7. Centonze, D., Gubellini, P., Bernardi, G., Calabresi, P. 2001. Impaired excitatory transmission in the striatum of rats chronically intoxicated with manganese. *Exp. Neurol.* 172, 469-476.
8. Chang, L. W. and Slikker, W. 1995. *Neurotoxicology: approaches and methods.* Academic Press. New York, pp. 133-221.
9. Clark, B. J., Hines, D. J., Hamilton, D. A., Whishaw, I. Q. 2005. Movements of exploration intact in rats with hippocampal lesions. *Behav. Brain Res.* 16, 91-99.
10. Crossgrove, J. S., Yokel, R. A. 2005. Manganese distribution across the blood-brain barrier IV. evidence for brain influx through store-operated calcium channels. *NeuroToxicol.* 26, 297-307.
11. Dési, I., Nagymajtényi, L., 1999. Electrophysiological biomarkers of an organophosphorous pesticide, dichlorvos. *Toxicol. Lett.* 107, 55-64.
12. Dorman, DC., McManus, BE., Parkinson, CU., Manuel, CA., McElveen, AM., Everitt, JI. 2004. Nasal toxicity of manganese sulphate and manganese phosphate in young male rats following subchronic (13 week) inhalation exposure. *Inhal Toxicol* 16, 481-488.
13. Dorman, DC., Struve, MF., James, RA., Marshall, MW., Parkinson, CU, Wong, BA. 2001. Influence of particle solubility on the delivery of inhaled manganese to the rat brain: manganese sulphate and manganese tetroxide pharmacokinetics following repeated (14-day) exposure. *Toxicol. Appl. Pharmacol.* 170, 79-87.
14. Eilam, D., Golani, I. 1989. Home base behavior of rats (*Rattus norvegicus*) exploring a novel environment. *Behav. Brain Res.* 3, 199-211.
15. Elder, A., Gelein, R., Silva, V., Feikert, T., Opanashuk, L., Carter, J., Potter, R., Maynard, A., Ito, Y., Finkelstein, J., Oberdörster, G. 2006. Translocation of ultrafine

- manganese oxide particles to the central nervous system. *Environ. Health Persp.* 114, 1172-1178.
16. Erikson, K.M., Aschner, M., 2003. Manganese neurotoxicity and glutamate-GABA interaction. *Neurochem. Int.* 43, 475-480.
  17. Fechter, L. D., Johnson, D. L., Lynch, R. A. 2002. The relationship of particle size to olfactory nerve uptake of a non-soluble form of manganese into brain. *NeuroToxicol.* 23, 177-183.
  18. Ferraz, HB., Bertolucci, PH., Pereira, JS., Lima, JG., Andrade, LA. 1988. Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology* 38, 550-553.
  19. Field, K.J., White, W.J., Lang, C.M., 1993. Anesthetic effects of chloral hydrate, pentobarbitone and urethane in adult male rats. *Lab. Anim.* 27, 258-269.
  20. Gianutsos, G., Morrow, GR., Morris, JB. 1997. Accumulation of manganese in rat brain following intranasal administration. *Fundam. Appl. Toxicol.* 37, 102-105.
  21. Hauber, W. 1998. Involvement of basal ganglia transmitter systems in movement initiation. *Progr. Neurobiol.* 56, 507-540.
  22. Henriksson, J., Tallkvist, J., Tjalve, H. 1999. Transport of manganese via the olfactory pathway in rats: dosage dependency of the uptake and subcellular distribution of the metal in the olfactory epithelium and the brain. *Toxicol. Appl. Pharmacol.* 156, 119-120.
  23. Henriksson, J., Tjalve, H. 2000. Manganese taken up into the CNS via olfactory pathway in rats affects astrocytes. *Toxicol. Sci.* 55, 392-398.
  24. Hernandez, E.H., Discalzi, G., Dassi, P., Jarre, L., Pira, E., 2003. Manganese intoxication: The cause of an inexplicable epileptic syndrome in a 3 year old child. *NeuroToxicol.* 24, 633-639.
  25. Herrero, M., Barcia, C., Navarro, J. 2002. Functional anatomy of thalamus and basal ganglia. *Child's Nerv. Syst.* 18, 386-404..
  26. Hirata, Y., Kiuchi, K., Nagatsu, T. 2001. Manganese mimics the action of 1-methylphenylpyridinium ion, a dopaminergic neurotoxin, in rat striatal tissue slices. *Neurosci. Lett.* 311, 53-57.
  27. International Commission on Radiation Protection. 1994. Human respiratory tract model for radiological protection. A report of a task group of the ICRP. *Annals of the ICRP*, ICRP Publication 66, Pergamon Press, Oxford.
  28. Kandel, E. R., Schwartz, J. H. 1985. *Principles of Neural Science*. Elsevier, New York, pp. 643-644.
  29. Kondakis, X.G., Makris, N., Leotsinidis, M., Papapetropoulos, T., 1989. Possible health effects of high manganese concentration in drinking water. *Arch. Environ. Health.* 44, 175-178.
  30. Lai, J.C., Leung, T.K., Kim, L. 1981. Brain regional distribution of glutamic acid decarboxylase, choline acetyltransferase, and acetylcholinesterase in the rat Effects of

- chronic manganese chloride administration after two years. *J. Neurochem.* 36, 1143-1148.
31. Li, N., Sioutas, C., Cho, A., Schmitz, D., Misra, C., Sempf, J., Wang, M., Oberley, T., Froines, J., Nel, A. 2003. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environ. Health Persp.* 4, 455-460.
  32. Long, T.C., Saleh, N., Tilton, R.D., Lowry, G.V., Veronesi, Bellina 2006. Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): Implications for nanoparticle neurotoxicity. *Environ. Sci. Technol.* 14, 4346-4352.
  33. Lundborg, M., Eklund, A., Lind, D.B., Camner, P. 1985. Dissolution of metals by human and rabbit alveolar macrophages. *Brit. J. Ind. Med.* 42, 642-645.
  34. Malecki, E.A., 2001. Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons. *Brain Res. Bull.* 55, 225-228.
  35. McNeilly, J.D., Heal, M.R., Beverland, I.J., Howe, A., Gibson, M.D., Hibbs, L.R., MacNee, W., 2004. Soluble transition metals cause the pro-inflammatory effects of welding fumes *in vitro*. *Toxicol. Appl. Pharmacol.* 196, 95-107.
  36. Mergler, D., Baldwin, M., Belanger, S., Larribe, F., Beuter, A., Bowler, R., Panisset, M., Edwards, R., de Geoffroy, A., Sassine, M-P., Hudnell, K., 1999. Manganese neurotoxicity, a continuum of dysfunction: Results from a community based study. *NeuroToxicol.* 20, 327-342.
  37. Nemati, F., Whishaw, I. Q. 2007. The point of entry contributes to the organization of exploratory behavior of rats on an open field: an example of spontaneous episodic memory. *Behav. Brain Res.* 182, 119-128.
  38. Nemmar, A., Hoylaerts, M. F., Hoet, P.H.M., Vermeylen, J., Nemery, B. 2002. Size effect of intratracheally instilled particles on pulmonary inflammation and vascular thrombosis. *Toxicol. Appl. Pharmacol.* 186, 38-45.
  39. Normandin, L., Beaupré, L. A., Salehi, F., St.-Pierre, A., Kennedy, G., Mergler, D., Butterworth, R. F., Philippe, S., Zayed J. 2004. Manganese distribution in the brain and neurobehavioral changes following inhalation exposure of rats to three chemical forms of manganese. *NeuroToxicol* 25, 433-441.
  40. Normandin, L., Hazell, A. S. 2002. Manganese neurotoxicity: an update of pathophysiological mechanisms. *Metab. Brain Dis.* 17, 375-387.
  41. Oberdörster, G. 2000b. Toxicology of ultrafine particles: *in vivo* studies. *Phil. Trans. Royal Soc. London, Series A-Mathematical Physical and Engineering Sciences* 1775, 2719-2739.
  42. Oberdörster, G., Finkelstein, J.N., Johnston, C., Gelein, R., Cox, C., Baggs, R., Elder, A.C. 2000a. Acute pulmonary effects of ultrafine particles in rats and mice. *Res. Rep. Health Eff. Inst.* 96, 75-86.
  43. Oberdörster, G., Oberdörster, E., Oberdörster, J. 2005. Nanotoxicology: An Emerging discipline evolving from studies of ultrafine particles. *Environ. Health Persp.* 7, 823-839.

44. Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W., Cox, C. 2004. Translocation of inhaled ultrafine particles to the brain. *Inhal. Toxicol.* 16, 437-445.
45. Oka, Y., Mitsui, M., Kitahashi, T., Sakamoto, A., Kusuoka, O., Tsunoda, T., Mori, T., Tsutsumi, M. 2006. A reliable method for intratracheal instillation of materials to the entire lung in rats. *J. Toxicol. Pathol.* 19, 107-109.
46. Papp, A., Szabó, A., Lengyel, Zs., Nagymajtényi, L. 2005. Biomarkers of certain environmental neurotoxicants: an overview. *Centr. Eur. J. Occup. Environ. Med.* 11, 135-143.
47. Parenti, M., Rusconi, L., Cappabianca, V., Parati, E.A., Gropetti, A. 1988. Role of dopamine in manganese neurotoxicity. *Brain. Res.* 473, 236-240.
48. Paul, C., Beltz, B., Berger-Sweeney, J. 1997. Observational methods used to assess rat behavior: behavioral patterns. *The Experimental Basis of Neuroscience*. CSHL Press, Cold Spring Harbor, NY, USA.
49. Pecze, L., Papp, A., Nagymajtényi, L. 2003. Changes in the spontaneous and stimulus-evoked activity in the somatosensory cortex of rats on acute manganese administration. *Toxicol. Lett.* 148, 125-131.
50. Pecze, L., Papp, A., Nagymajtényi, L., Dési, I. 2005. Effect of acute administration of certain heavy metals and their combinations on the spontaneous and evoked cortical activity in rats. *Environ. Toxicol. Pharmacol.* 19, 775-784.
51. Ponzoni, S., Guimaraes, F.S., Del Bel, E.A., Garcia-Cairasco, N., 2000. Behavioral effects of intra-nigral microinjections of manganese chloride: interaction with nitric oxide. *Prog. Neuro-Psychopharmacol. Biol. Psych.* 24, 307-325.
52. Roels, H., Meiers, G., Delos, M., Ortega, I., Lauwerys R., Buchet, J.P., Lison, D. 1997. Influence of the route of administration and the chemical form (MnCl<sub>2</sub>, MnO<sub>2</sub>) on the absorption and cerebral distribution of manganese in rats. *Am. J. Ind. Med.* 11, 307-327.
53. Shinotoh, H., Snow, B.J., Chu, N.S., Huang, C.C., Lu, C.S., Lee, C., Takahashi, H., Calne, D.B., 1997. Presynaptic and postsynaptic striatal dopaminergic function in patients with manganese intoxication: a positron emission tomography study. *Neurology* 48, 1053-1056.
54. Sinczuk-Walczak, H., Jakubowski, M., Matczak, W., 2001. Neurological and neurophysiological examinations of workers occupationally exposed to manganese. *Int. J. Occup. Med. Environ. Health.* 14, 329-337.
55. Sjögren, B., Iregren, A., Frech, W., Hagman, M., Johansson, L., Tesarz, M., Wennberg, A., 1996. Effects of the nervous system among welders exposed to aluminium and manganese. *Occup. Environ. Med.* 53, 32-40.
56. Smith, S.J., Harding, A.E., 1993. EEG and evoked potential findings in mitochondrial myopathies. *J. Neurol.* 240, 367-372.

57. Stone, V., Johnston, H., Clift, MJ. 2007. Air pollution, ultrafine and nanoparticle toxicology: cellular and molecular interactions. *IEEE Trans. Nanobioscience* 6, 331-340.
58. Szabó, A., Papp, A., Nagymajtényi, L. 2005. Effects of 3-nitropropionic acid in rats: general toxicity and functional neurotoxicity. *Arh Hig Rada Toksikol*; 56, 297-302.
59. Takeda, A. 2002. Manganese action in brain function. *Brain Res. Rev.* 41, 79-87.
60. Takeda, A., Kodama, Y., Ishiwatari, S., Okada, S. 1997a. Manganese transport in the neural circuit of rat CNS. *Brain Res. Bull.* 45, 149-152.
61. Takeda, A., Sawashita, J., Okada, S. 1997b. Manganese concentration in rat brain: manganese transport from the peripheral tissues. *Neurosci. Lett.* 242, 45-48.
62. Takser, L., Mergler, D., Hellier, G., Sahuquillo, J., Huel, G. 2003. Manganese, monoamine metabolite levels at birth, and child psychomotor development. *NeuroToxicol.* 24, 667-674.
63. Tjalve, H., Henriksson, J. 1999. Uptake of metals in the brain via olfactory pathways. *NeuroToxicol.* 20, 329-337.
64. Tjalve, H., Henriksson, J., Tallkvist, J., Larsson B.S., Lindquist, N.G. 1996. Uptake of manganese and cadmium from the nasal mucosa into the central nervous system via olfactory pathways in rats. *Pharmacol. Toxicol.* 79, 347-356.
65. Van der Post, J., Noordzij, L.A., de Kam, M.L., Blauw, G.J., Cohen, A.F., van Gerven, J.M., 2002. Evaluation of tests of central nervous system performance after hypoxemia for a model for cognitive impairment. *J. Psychopharmacol.* 16, 337-343.
66. Verity, M.A. 1999. Manganese neurotoxicity: a mechanistic hypothesis. *NeuroToxicol.* 20, 489-497.
67. Vezér, T., Kurunczi, A., Náray, M., Papp, A., Nagymajtényi L, 2007. Behavioral effects of subchronic inorganic manganese exposure in rats. *Amer. J. Ind. Med.* 50, 841-852.
68. Vezér, T., Papp, A., Hoyk, Zs., Varga, Cs., Náray, M., Nagymajtényi, L. 2005. Behavioral and neurotoxicological effects of subchronic manganese exposure in rats 19, 797-810.
69. Weisiger, R.A., Fridovich, I.. 1973. Superoxide dismutase. Organelle specificity. *J. Biol. Chem.* 248, 3582-3592.
70. Whishaw, I. Q., Gharbawie, O. A., Clark, B. J., Lehmann, H. 2006. The exploratory behavior of rats in an open environment optimizes security. *Behav. Brain Res.* 171, 230-239.
71. Yamada, M., Ohno, S., Okayasu, I., Okeda, R., Hatakeyama, S., Watanabene, H., Ushio, K., Tsukagoshi, H., 1986. Chronic manganese poisoning: a neuropathological study with determination of manganese distribution. *Acta Neuropathol.* 70, 273-278.
72. Yu, J., Park, J. D., Park, E. S., Song, K. S., Han, K. T., Han, J. H., Chung, Y. H., Choi, B. S., Chung, K.H., Cho, M. H. 2003. Manganese distribution in brains of Sprague-Dawley rats after 60 days of stainless steel welding-fume exposure. *NeuroToxicol.* 24, 777-785.

73. Zhang, J., Fitsanakis, V. A., Gu, G., Jing, D., Ao, M., Amarnath, V., Montine, T.J. 2003. Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *J. Neurochem.* 84, 336-346.
74. Zilles, K., 1982. *The cortex of the rat. A stereotaxic atlas.* Springer Verlag, Berlin-Heidelberg-New York-Tokyo.

## 6. ACKNOWLEDGEMENT

I would like to thank to prof. dr. László Nagymajtényi, Head of the Department of Public Health, that he accepted me as PhD student for an unconventionally short period and gave the opportunity to perform experimental work.

I am especially grateful to dr. András Papp, head of the neurotoxicological laboratory, for his scientific guidance, introduction to techniques and his all-time help; to dr. Tünde Vezér for making me familiar with the open field method; and to dr. Andrea Szabó for always giving a hand in electrophysiology.

I am thankful to dr. Tibor Hornyik, dr. Edina Horváth and dr. Gábor Oszlanczi for their contribution to recording and dissection work.

The contribution of prof. dr. Imre Kiricsi, dr. Zoltán Kónya, and Endre Horváth from the Department of Applied Chemistry was crucial by providing the manganese nanoparticles.

Brigitta Szalay (National Institute of Environmental health, Budapest, Hungary) deserved mentioning by performing the electron microscopic work.

I also thank to the technical staff (animal house: Németh Mihályné, Kiss Gyuláné; documentation and IT: Imre Gera, Szalay Lászlóné) for their valuable cooperation.

## 7. APPENDIX

*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 14<sup>th</sup> 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 8<sup>th</sup> 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.

*Sárközi, L., Horváth, E., Szabó, A., Horváth, E., Sági, 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 10<sup>th</sup> International Symposium of Interdisciplinary Regional Research**, Vajdahunyad, 2009.