PhD Thesis

NERVOUS SYSTEM EFFECTS AND OXIDATIVE STRESS IN RATS TREATED WITH METAL OXIDE NANOPARTICLES

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The Applicant's Relevant Publications

I. *Oszlánczi, G.*, Vezér, T., Sárközi, L., Horváth, E., Szabó, A., Horváth, E., Kónya, Z., Papp, A.: Metal deposition and functional neurotoxicity in rats after 3 to 6 weeks nasal exposure by two physicochemical forms of manganese.
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IV. *Oszlánczi, G.,* Papp, A., Szabó, A., Nagymajtényi, L., Sápi, A., Kónya, Z., Paulik, E., Vezér, T.: Nervous system effects in rats on subacute exposure by lead-containing nanoparticles via the airways.
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Abstracts:

Sárközi, L, Szabó, K., Hornyik, T., Horváth, E., *Oszlánczi, G.*: Neurotoxic effects in rats following subacute manganese exposure in various forms. *IBRO International Workshop; Debrecen, 2008.*
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Oszlánczi, G., Horváth, E., Nagymajtényi, L., Papp, A., Vezér, T.: Functional alterations and metal deposition in the CNS of rats following subacute manganese exposure through the airways. *FENS Forum; Amsterdam, 2010.*
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Oszlánczi, G., Horváth, E., Horváth, E., Sápi, A., Kozma, G., Kónya, Z., Papp, A.: Intratracheal Exposure By Metal Oxide Nanoparticles: General And Neurotoxic Effects. *12th Danube-Kris-Mures-Tisa Euroregion Conference on Food, Environment and Health, Novi Sad; 2010.* **Book of Abstracts on CD (ISBN 798-86-80995-84-7) p. 53 (2010)**

Oszlánczi, G., Hajdú, A., Szabó, A., Berczi, S., Szalay, B., Tombácz, E.: Acute Distribution Of Magnetic Fluids Stabilized By Different Ways Studied In Rats. *11th International Symposium Interdisciplinary Regional Research; Szeged, 2010.*
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SUMMARY

Inhaled air can cause direct exposure to harmful pollutants of both natural and artificial origin, which are highly variable in chemical composition, particle size etc. Airborne particles can be classified by size as sedimenting dust (>10 μm), suspended or fine dust (100 nm-10 μm) and ultrafine dust or nanoparticles (NPs, <100 nm). Inhaled NPs are either deposited in the nasopharynx or get down to the alveoli. Then, they translocate to other body parts by transcytosis through cell layers, and/or substances dissolved from their surface reach the bloodstream. Sensory nerves endings provide a direct pathway for NPs to the brain. Due to their small size, high number concentration, and large specific surface area, NPs have greater biological activity per given mass than larger particles, and can have different toxicological properties than the same compound in more conventional states. For this thesis, NPs consisting of lead, cadmium and manganese oxide (PbO, CdO_2, MnO_2) were chosen; based on practical importance and on previous experiences of the Department.

Lead (Pb) has been a ubiquitous environmental pollutant, and is toxic even in low doses. Primary production and reprocessing of Pb is based on smelting, with substantial emission of metal fumes. Inhaled airborne Pb causes significant internal exposure both in humans and in experimental animals. Its nervous systems effects can manifest in brain damage, diminished learning ability and behavioral problems in children; and peripheral neuropathy. In adults occupationally exposed to Pb, alterations of various forms of central and peripheral evoked activity were described.

Cadmium, used for industrial purposes (electroplating, batteries, pigments, alloys etc.) is one of the most toxic environmental pollutants; damaging the lungs, liver, kidney, testis, brain etc. Significant inhalation of Cd can occur from tobacco smoke and in occupational settings. Amyotrophic lateral sclerosis, optic nerve damage, striatal damage and peripheral polyneuropathy were observed as long term neurotoxic consequences. In children, a straight relationship between hair Cd and altered visual or auditory evoked potential parameters was found.

Manganese (Mn) is, in contrast to lead and cadmium, an essential micronutrient, e.g. as cofactor in metallo-enzymes. It is used in many important alloys, so welding fumes and similar industrial emissions are a source of Mn-containing NPs. Chronic inhalation of manganese compounds causes severe neurologic disorders; starting with apathy, asthenia and headache, and ending in a Parkinson-like syndrome. Disorders with electrophysiological signs after Mn exposure include e.g. myoclonus in welders and epileptic activity in an accidentally exposed child.

In earlier works of the Department, recording and analysis of electrophysiological signals from the brain and from a peripheral nerve proved sufficiently sensitive to detect the effects of orally applied Mn, Pb and Cd on the nervous system of rats. In the present experiments, a new model, with metal oxide NPs instilled into the trachea, was introduced; and the electrophysiology was supplemented with behavioral and chemical measurements. The questions, to be answered on the basis of the results of this work, were as follows:

- Is intratracheal instillation of suspension of NPs containing lead, cadmium and manganese a usable technique to model the effect of airborne metals?
- Can significant internal exposure be induced this way?
- Does this internal metal exposure induce functional alterations in the rats' nervous system, to be detected by electrophysiological recording and by open field test?
- Can oxidative stress be detected in the treated rats?
- Is there any correlation between internal exposure, and the neuro-functional and biochemical changes, which may underline a causal relationship?

For the experiments, young adult male Wistar rats were used in groups of 8-10 animals per treatment dose and time. The metal nanoparticles were synthesized at the Department of Applied Chemistry, University of Szeged. The mean diameter of MnO_2 particles was ca. 23 nm, of PbO was ca. 19 nm, of CdO_2 was ca. 20 nm. For administration to the rats, the nanoparticles were suspended in distilled water. The suspension was sonicated to prevent aggregation, and was instilled into the rats' trachea. The treatment scheme was as follows:

Group	**Code**	**Substance**	**Dose (mg metal / kg b.w.)**	**Duration of the treatment**
Untreated control	*Con*	---	---	3, 6, 9 weeks
Vehicle control	*W*	Distilled water	---	3, 6, 9 weeks
Manganese low dose	*Mn-LD*	MnO_2 nanosuspension	2.63	3, 6, 9 weeks
Manganese high dose	*Mn-HD*		5.26	
Cadmium low dose	*Cd-LD*	CdO_2 nanosuspension	0.04	3, 6 weeks
Cadmium high dose	*Cd-HD*		0.40	
Lead low dose	*Pb-LD*	PbO nanosuspension	2.00	3, 6 weeks
Lead high dose	*Pb-HD*		4.00	

For intratracheal instillation the animals were briefly anesthetized with diethyl ether, then suspended on an oblique board. The tongue was pulled forward with a pair on non-traumatic forceps, and the nanosuspension (or distilled water for the controls) was instilled into the trachea. The animals were under continuous clinical observation during the experiment and

abnormal reactions were noticed. Body weight, as a general indicator of the rats' health state, was measured once weekly.

At the end of the treatment period the rats' spontaneous locomotor activity was measured in an open field (OF) box. The instrument recorded their horizontal and vertical movements (in one 10 min session per rat). Counts, time and run length of the activity forms (ambulation, local activity, immobility, rearing) were automatically calculated.
The next day, the animals were anaesthetized by intraperitoneal injection of 1000 mg/kg b.w. urethane. The left hemisphere was exposed, and ball-tipped silver recording electrodes were positioned on the dura over the primary somatosensory (SS) area (projection of the whisker pad), and over the primary visual (VIS) and auditory (AUD) area. SS stimulation was done by a pair of needles inserted into the whisker pad, delivering square electric pulses. VIS stimulation was performed by flashes, and AUD stimulation by sound clicks. Compound action potential of the tail nerve was evoked by means of a pair of stimulating needle electrodes inserted at the base of tail, and recorded by another pair of needles 50 mm distally.
One session consisted of six minutes recording of spontaneous activity (electrocorticogram, ECoG) first, from the three sensory cortical areas simultaneously. Then evoked potentials (EPs) from the same cortical areas were recorded, and finally the compound action potential of the tail nerve.

From the ECoG records, the relative spectral power of the frequency band was determined by the software automatically, and the so-called "ECoG index" was calculated with the formula: ([delta]+[theta])/([beta1]+[beta2]). The recorded evoked responses were automatically averaged off-line, then onset latency and duration were measured manually.
Right after finishing the electrophysiological investigation, the animals were sacrificed by an overdose of urethane, and 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. Blood, brain, lung and liver samples were collected and stored at -22°C.
From the samples, protein content, SOD and Mn-SOD activity, and GSH content was determined biochemically. Statistical evaluation of all data was done by one-way ANOVA after Kolmogorov-Smirnov normality check. The relationship between the bodily metal load and the measured neuro- and general toxicological parameters was tested by linear regression

Instillation of MnO_2 NPs significantly reduced the rats' body weight. From the 6^{th} week on, there was no weight gain in the *Mn-LD* group, and the rats in *Mn-HD* showed even some

weight loss. The relative weight of the lungs strongly increased by the 9th week in both treated groups, while the relative weight of the liver decreased. The general trend of Mn NP treated rats in the open field was dose- and time-dependently decreasing motility. Not only the time of ambulation but also the distance covered, and the speed of walking, were reduced, and the rats were apparently reluctant to change to ambulation once they were only locally active or not active at all. In the spontaneous cortical activity after 9 weeks, both the decrease of delta, and increase of beta and gamma activity was significant, in the *Mn-HD* and partly the *Mn-LD* group. Onset latency of the cortical EPs was significantly lengthened on Mn NP exposure. Also the frequency-dependent latency increase was more expressed in the treated groups. The conduction velocity of the tail nerve was significantly decreased after 9 weeks. After 9 weeks, significant Mn deposition was detected in the treated rats' brains. In the total SOD and Mn-SOD activity of the brain samples, the Mn NP exposure caused an increase, but only the increase of total SOD was significant. The level of reduced glutathione was moderately decreased. The correlation of the ECoG index and the nerve conduction velocity with the brain Mn level was significant, and also the relationship of ECoG index and brain GSH level.

Intratracheal treatment by Pb NPs also caused significant retardation in the rats' body weight gain, from the 2nd treatment week on, and the lungs showed significantly increased relative weight after 6 weeks treatment. Decrease in the relative weight of the liver, and increase in that of the kidneys remained below significance. After 6 weeks exposure, *Pb-LD* and *Pb-HD* rats spent significantly more time with ambulation than the controls. The decrease in rearing, and even more the increase in local activity, was significant only vs. *Con*. In the ECoG activity, a weak trend of decrease in the low and increase in the high frequency bands was seen after 6 weeks Pb NP exposure but this was significant mostly in the *Pb-HD* group only, and not in every recorded area. The latency of the SS EP significantly increased in the *Pb-HD* group, and the frequency-dependent increase was also more pronounced. The VIS and AUD EP also had increased latency in the *Pb-HD* group. The conduction velocity of the tail nerve was reduced in both treated groups. In the treated rats' organs, massive increase of the Pb content was measured. Pb NP treatment diminished the total SOD, but not Mn-SOD, activity in the tested organs GSH level significantly increased in liver and lung, but not brain samples.

In the rat receiving high dose Cd NP exposure, body weight gain was practically halted in the first two weeks of treatment and the difference vs. *Con* remained high during the whole treatment period. Increase of lung and thymus weight, and decrease of spleen weight was observed. The Cd NP exposure applied in the experiment caused no noteworthy change in the rats' open field behavior. After 6 weeks Cd NP exposure, a shift to higher frequencies was

seen on the ECoG spectrum, which was significant in the delta, beta2 and gamma bands in the *Cd-HD* group. The latency of the SS EP was significantly lengthened only with the high dose group The VIS and AUD EPs also had lengthened latency, in VIS in both treated groups vs. *Con*. In the tail nerve the Cd NP exposure had no significant effect. Cd was detected in lung and liver, but not brain and blood, samples. Mn-SOD levels decreased in the samples from the Cd-treated rats but mostly only moderately. GSH increased in the brain and liver, but decreased in the lungs.

Investigation of the nervous system effects of NPs in rats using subacute intratracheal instillation and a set of neuro-functional tests (supplemented with chemical ones) seems to be a model of human exposure and its consequences to which no direct parallel was found in the literature. In terms of internal exposure, the model proved adequate, as significant increase of the Mn, Pb and Cd level in the tissue samples was achieved. The qualitative similarity of the majority of neuro-functional alterations seen in rats treated with the three different metal NPs suggest that common mechanisms of action exist. These can be first of all oxidative stress, interference with Ca-dependent phenomena, and effects on transmitter systems. The ability of Mn-containing welding fumes to induce oxidative stress is known. The turnover of the main excitatory transmitter of the CNS, glutamate, is also disturbed. Uptake of glutamate into the astrocytes is inhibited by each metal involved in the experiments, and Mn inhibits also the transformation to glutamine. Excess glutamate may desensitize the postsynaptic receptors leading to weaker and/or slower postsynaptic excitation, and can also contribute to the observed increase of cortical activation (shift of the ECoG to higher frequencies). The changes in open field motor behavior showed effects first of all on the dopaminergic system. Dopaminergic neurons are especially vulnerable to oxidative stress, and the tendency of all three metals to induce oxidative stress is another common point in their effects on the nervous system.

The results of this work can be summarized, and the questions listed above answered, as follows:

- Intratracheal instillation of the suspension of metal (manganese, lead and cadmium) containing NPs proved technically possible.
- Significant internal exposure developed after 6 or 9 weeks of instillation, indicated first of all by the metal level of the treated rats' brains; although the Cd content of the NPs was detected only in the lungs and liver, but not the brain of the animals.

- Functional alterations in the rats' nervous system were in fact observed. The cortical evoked potentials were the most sensitive, altered significantly after Mn, Pb and Cd exposure. Spontaneous cortical activity, peripheral nerve conduction velocity as well as open field behavior was significantly altered by Mn and Pb, but not by Cd.
- Oxidative stress could be detected in the treated rats, although the changes in superoxide dismutase activity and reduced glutathione level were moderate.
- In the Mn- and Pb-treated rats – where significantly elevated brain and blood metal levels were measured – correlation with neuro-functional alterations could be shown, and it was in several cases significant. The measured oxidative stress parameters were, however, much less strongly associated to the metal levels, so that no direct relationship of oxidative state and functional alterations could be shown, except for the significant correlation of ECoG index and brain GSH level in the Mn-treated rats. Cd application failed to cause measurable metal load in the brain and blood, so the correlation could not be studied, although some neurophysiological and biochemical effects were clearly present.

TABLE OF CONTENTS

Abbreviations

AUD	auditory
Con	control
ECoG	electrocorticogram
EP	evoked potential
GSH	glutathione, reduced
HD	high dose
LD	low dose
NP	nanoparticle
SOD	superoxide dismutase
SS	somatosensory
VIS	visual
W	vehicle treated

1. INTRODUCTION

1.1. Nanoparticles in the environment

1.1.1. Air as an element of the environment and as a health determinant

Environmental conditions constitute one of the four major determinants of human health (the other three being genetic disposition, health care, and lifestyle). The presence of several toxicants in the air, water and soil – in our times, mostly due to man-made pollution – can affect health at population level.

The environmental medium able to cause the most direct exposure to harmful pollutants is air. Air has low self-purification capacity (although the physical and chemical transformation of primary pollutants can be of importance) and can quickly transport the emitted substances far away from the site of emission. Hence, air pollution concerns the great majority of the human population, and the typical way of exposure is inhalation.

Foreign materials in the air can be of both natural (e.g. volcanism, wind) and artificial (e.g. energy production, chemical industry, transportation, disasters) origin; and can be highly variable in chemical composition and properties such as physical state, volatility, particle size etc. From these factors, particle size is one of the most determinative properties, as concerns environmental fate and health effects.

Airborne particles can be classified by their size as sedimenting dust (>10 μm), suspended or fine dust (100 nm-10 μm; often called PM10) and ultrafine dust or nanoparticles (NPs, <100 nm). The origin, environmental presence and health effects of NPs gained much attention in the last ca. 20 years due partly to substantial developments in methodology and instrumentation.

1.1.2. Origins and properties of environmental nanoparticles

Particles in the atmosphere can be either primary or secondary. Primary particles are emitted to the atmosphere directly (that is, in pre-formed state). Other environmental NPs are secondary, being formed in the atmosphere by gas-to-particle conversion which can be a physical or chemical process (e.g., the conversion $NH_3 + SO_3 \rightarrow (NH_4)_2SO_4$ is a chemical process while the aggregation of Na^+ and Cl^- ions in sea spray is simple crystallization).

Immediately following gas-to-particle conversion (called nucleation) the secondary particles are very small (1–10 nm), and gain size by coagulation or are condensed onto existing submicron particles. Nucleation may occur in hot combustion gases and in metallurgical processes, including welding.

Another aspect differentiates the NPs as being either natural or man-made. Within the latter, emission of NPs as pollutants, and synthesis of NPs as nanomaterials for novel technologies, are to be distinguished.

Natural sources of NPs include forest and bush fires, volcanoes, sea spray, erosion of rocks and soil, etc. The main anthropogenic sources of NPs are combustion (including lighting, heating, operation of motor vehicles, energy production, waste disposal, etc.) other high temperature processes (metallurgy, casting, welding, etc.: Antonini et al., 2003) and working on solid materials (although the latter, and mechanical effects generally, do not give many particles much below the 100 nm limit). In the outdoor environment, one can find ca. 10^6-10^8 NPs per litre air. In rural areas, these originate mostly from the oxidation of various volatile compounds of biogenic or anthropogenic origin. In urban areas, the primary sources of NPs are vehicle engines (first of all diesels), but photo-oxidation processes produce significant amount of NPs also in urban areas. The vehicle exhaust aerosol contains mostly particles of less than 50 nm diameter. The highest particle number concentrations and smallest particle sizes are associated with high-speed road traffic, when the exhaust stream is hot and strong, and is not much cooled before leaving the tailpipe and being diluted by air. Aerosol concentration (which necessarily includes NPs) is today an important measure of ambient air quality; with limits set by national authorities (Decree No. 14/2001). At European Union level, the air quality directive (Directive 2008/1/EC) requires member states to limit the exposure of citizens to suspended dust by setting an annual limit for PM10 of 40 $\mu g/m^3$ and a daily limit of 50 $\mu g/m^3$ that must not be exceeded more than 35 times per year.

Nanotechnology, the purposeful production and application of nano-sized particles and structures, is another potential source of emission of NPs – although it is noteworthy that the production of industrial materials unintentionally containing NPs, and the application of such materials (e.g. in pigments, resins and cosmetics), has been existing for decades. Intentionally manufactured nanomaterials contain at least one component that has at least one dimension in the 1 to 100 nm range. Products of nanotechnology may add to the load of primary NPs in the

atmosphere (and the subsequent agglomeration of emitted NPs will add to the load of atmospheric PM10).

Manufactured nanomaterials are used in personal care products, electronics, tires, fuel cells, and many other consumers' goods (Oberdörster et al., 2005). Application of NPs and nanofibres in consumers' products means that routes of uptake such as ingestion and dermal absorption, must be considered in addition to inhalation (see 1.2.2).

A major problem about nanotechnology and its direct and indirect health effects is the lack of toxicity data for most manufactured NPs. Even in case of materials which have been manufactured for tens of years in tons of volume, a lack of data exists because substances in NP form can show profoundly different properties than in more conventional states (an example is TiO_2 used as white pigment, see 1.2.2).

As mentioned above, high temperature procedures of the metal technology emit a lot of particles in the local environment. Exposure to metal-containing airborne NPs is thus primarily an occupational hygienic problem, recognized relatively recently. For this thesis, three metals were chosen; based on the one hand on practical importance (that is, the metal in question is in fact found in engineering materials and in workplace settings, and working with them includes procedures generating NPs) and on the other hand, on previous experiences at the Department (Nagymajtényi et al., 1997; Vezér et al., 2000, 2005; Institóris et al., 2002; Papp et al., 2003; Sárközi et al., 2008). Finally, manganese, cadmium and lead were chosen, the general properties and toxic effects of which are described in sections 1.3. to 1.5.

1.2. Interactions of nanoparticles and living organisms

1.2.1. Absorption and transport

To act on any living thing, the NPs first have to come in contact with it. As NPs of natural or artificial origin occur most likely in the (indoor or outdoor) air, the most significant route of exposure is – for higher animals and humans – inhalation. Particles in the inhaled air are deposited at different sites within the airways, determined first of all by their size. NPs are either deposited in the nasopharynx or get down to the alveoli (ICRP, 1994).

Once deposited, NPs translocate readily to other body parts and reach other target organs by different transfer routes and mechanisms. This involves transcytosis (by caveola formation)

across epithelia of the respiratory tract into the interstitium (Oberdörster et al., 2005). Caveolae are 50-100 nm sized invaginations of the cell membrane, serving endo- and transcytosis of a number of molecules and microstructures (Razani and Lisanti, 2001). Crossing the alveolar and capillary membrane, NPs can reach the blood circulation directly or via the lymph drainage, and will be distributed 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., 2007). Metal ions dissolved from the surface of metal-containing NPs in the acidic microenvironment of phagosomes – after the NPs had been phagocyted by alveolar macrophages (Lundborg et al., 1985) – also must be considered among the mechanisms of action. It is for example possible that the blood-brain barrier, weakened by the toxic metal ions, will be more permeable for NPs.

In the olfactory epithelium, the primary olfactory neurons are in contact both with the environment and with the central nervous system (CNS) via the olfactory nerve fibres and the olfactory bulb. These neurons thus provide a direct pathway by which foreign materials may gain access to the brain (Calderon-Garciduenas et al., 2002). Elder et al. (2006) found that inhaled manganese NPs, once they reached the olfactory bulb, spread to the striatum, midbrain, frontal cortex and cerebellum. The presence of Mn after intranasal application was also seen in an own study (Oszlánczi et al., 2010).

Gastrointestinal and dermal absorption of NPs is, compared to the above mechanisms, less important, but it is possible that all peripheral nerve endings can serve as starting point for NPs in their axonal transport to distant parts of the body (Oberdörster et al., 2005).

1.2.2. Health effects

Due to their small size, high number concentration, and large specific surface area, NPs have greater biological activity per given mass than larger particles (Oberdörster, 2000; Oberdörster et al., 2005), including oxidative stress induction (Li et al., 2003) or increased adsorption of organic molecules. In vivo and in vitro toxicological studies confirmed that even relatively inert materials are more toxic and inflammatogenic in NP form than in more coarse particles. TiO_2 is, e.g., a generally used white pigment (now fully replacing the overtly toxic lead white, $PbCO_3$) and is harmless enough to be applied in the coating of tablets. It was

found, however, that nanosized TiO_2 (ca. 20 nm diameter) caused more severe inflammation than the same compound in "pigment grade" (ca. 250 nm) grains (Oberdörster, 2000).

NPs generate reactive oxygen species more intensely than larger particles, leading to increased synthesis of pro-inflammatory mediators via intracellular signalling pathways (Long et al., 2006; Stone et al., 2007). NPs can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs (Oberdörster et al., 2005). A healthy blood-brain barrier should prevent foreign particles from entering the brain; NPs of various composition were, however, detected in the brain of rats after application through the airways (Kreyling et al., 2006).

Epidemiological evidence associates enhanced level of ambient NPs with adverse respiratory and cardiovascular effects, which means increased 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). The ill effects of NPs indicate that their level in the ambient air should be limited. In the European Union, Directive 2008/50/EC defines the target value of 25 $\mu g/m^3$ for the yearly average of fine articles (PM2.5, an aerosol fraction including NPs). Separate monitoring of ambient NPs – although theoretically of interest in terms of health – is not done routinely because the technology required is novel, expensive and not yet standardized.

1.3. Lead: properties and applications

Lead (Pb) has been used for thousands of years and during this time it has become an ubiquitous environmental pollutant. It is a heavy metal with no known biological functions, and is toxic even in low doses (ATSDR, 1999).

Pb in metal and inorganic forms occurs in lead-acid batteries, paints, piping, solders, etc. Tetraethyl lead as petrol additive was used worldwide (and is still in use in several countries). It is oxidized during the combustion in car engines so the exhaust contains Pb mostly as fine and ultrafine particles of PbO_2. This used to be the single largest source of lead exposure in urban areas and along main roads. In Hungary, the ban of leaded petrol in 1999 brought about a tenfold decrease in atmospheric and soil Pb levels (Kertész et al., 2001). Airborne Pb causes exposure primarily by inhalation, leading to significant internal exposure both in humans and

in experimental animals (Griffin et al., 1975a, b). Pb in humans is absorbed from the alveoli to ca. 50 %, and from the intestines to 10-15 %, but in children to 50% (Järup, 2003).

Other applications of Pb (past or present) involve the metal and its alloys or compounds mostly in solid form. However, both the primary production from ore and the reprocessing of Pb waste (batteries etc.) is based on smelting, a high-temperature process with substantial emission of metal fumes. Beyond lead metallurgy, further jobs with potential exposure to airborne Pb (where the presence of NPs is absolutely likely) include the renovation of old residential buildings (painted originally with lead white) and steel structures (painted originally with red lead oxide rust-proofing paint, also know as minium). Such cases from the renewal works on Margit-híd in Budapest were depicted in online media (ATV.hu, 2010; STOP.hu, 2010).

1.3.1. Toxicity and neurotoxicity of lead

Lead can cause disruption of the biosynthesis of haemoglobin and hence anaemia, rise in blood pressure, kidney damage, miscarriages and subtle abortions, declined fertility of men through sperm damage, etc. Its nervous systems effects can manifest in brain damage; diminished learning ability and behavioral problems (aggression, impulsive behavior and hyperactivity) in children; and peripheral neuropathy (Feldman, 1999). Pb can also enter to the foetus through the placenta and cause serious damage to the nervous system before birth. Pb is accumulated in the central nervous system, first of all in the cortex and hippocampus (Grandjean, 1978), and produces encephalopathy at blood Pb levels of 1000-1200 ppb in adults and 800-1000 ppb in children (Chisolm, 1965). Exposure to low levels of Pb has been associated with behavioral abnormalities, learning impairment, decreased hearing, and impaired cognitive functions in humans and in experimental animals (Shannon and Graef 1992; Ruff et al., 1996). Some estimates suggest that every 100 ppb increase in blood Pb level is associated with a 1-5 point decrease in the IQ of exposed children (Goyer, 1996). IQ differences in Hungarian schoolchildren, attributable to airborne Pb, were described by Füzesi (1997).

In adults occupationally exposed to Pb, alterations of various forms of central and peripheral evoked activity, like sensory evoked potentials and nerve conduction velocity, were described (Araki et al., 2000; Goodman et al., 2002). Pb treatment induced EEG disorders and learning

disability in young rats (Kumar and Desiraju, 1992). In earlier studies of our Laboratory, Pb given orally in to rats altered cortical electrical activity (Nagymajtényi et al., 1997) and memory performance (Vezér et al., 2000).

Absorption of lead (first of all in Pb^{2+} form) and its distribution within the organism is interconnected with that of Ca^{2+} at several points due to chemical similarity, resulting finally in interference with a number of regulatory processes.

At presynaptic endings, Pb^{2+} blocks the voltage-gated Ca-channels but is partly permeated to the intracellular space, where it acts as false activator of various Ca-dependent processes including transmitter release (Suszkiw et al., 1984). Alterations in the dopaminergic, cholinergic and glutamatergic control of behaviour were observed in Pb-treated animals (Cory-Schlechta, 1995; Minnema and Micheaelson, 1986; Minnema et al., 1988). Pb may induce oxidative stress which in turn may be involved in toxic effects of Pb such as neurodegeneration and cognitive problems (Ahamed and Siddiqui, 2007). The level of lipid peroxidation was directly proportional to Pb concentrations in various brain regions (Flora et al., 2008). GSH, one of the most important compounds both in the detoxification and excretion of heavy metals and in the elimination of oxidative free radicals (Gayathri et al., 2007) was found decreased in erythrocytes from workers exposed to Pb (Sugawara et al., 1991). Literature on the influence of Pb on SOD activity is divergent (Adonaylo et al., 1999; Sandhir and Gill, 1995). In workers of lead-acid battery production, strongly elevated blood Pb was concomitant with increased GHS level and decreased SOD activity (Gayathri et al., 2007).

1.4. Cadmium: properties and applications

The natural background level of cadmium (Cd) in the environment results from the gradual erosion and abrasion of rocks and soils, and from single events such as forest fires and volcanic eruptions, and is the source of the low Cd content of food and drinking water in areas not affected by man-made pollution.

Cadmium is produced as a by-product from the extraction, smelting and refining of zinc, lead and copper, and has been used for industrial purposes since the 19th century. The most significant application of Cd used to be in corrosion protective electroplating layers on steel.

Beside that, metallic Cd and its compounds are used as pigments, UV-resistance stabilizers in plastics, coatings, specialty alloys, Cd-based semiconductors, and on large scale in rechargeable Ni-Cd batteries. Due to its various toxic effects, the applications of Cd have been generally decreasing in the last two decades. The European Commission included Cd in the RoHS Directive (Directive 2002/95/EC on the restriction of the use of certain hazardous substances in electrical and electronic equipment) with the exception of its use in Ni-Cd batteries, CdTe (cadmium telluride) solar panels, and in LED display screens. CdTe is found in the so-called quantum dots, a recent development of nanotechnology. Their size (2-100 nm) and chemical composition imparts the quantum dots special toxicological properties (Rzigalinski and Strobl, 2009).

1.4.1. Toxicity and neurotoxicity of cadmium

Cd is one of the most toxic environmental and industrial pollutants due to its ability to damage, among other, the lungs, liver, kidney, testis and the placenta (ATSDR, 2008a) and the CNS (Méndez-Armenta, 2007). International Agency for Research on Cancer classified cadmium as a human carcinogen (Group I) while the European Commission has classified some cadmium compounds as possibly carcinogenic (Carcinogen Category 2; Directive 67/548/EEC, 2006).

Cd accumulates easily via the food chain. Important cultivated plants, first of all cereals, tend to accumulate Cd from the soil (Järup, 1998). Cd levels can reach 10 μg/kg in fruits, vegetables and meat 100-1000 μg/kg in kidney and liver, and 200-2000 μg/kg in shellfish (Galal-Gorchev, 1991). In the surroundings of industrial plants with high Cd emission, house dust can also be an important source of human exposure (Hogervorst et al., 2007). Elsewhere, airborne Cd contributes only to a few percent of the total absorbed dose of Cd in the body (Vahter et al., 1992).

Significant inhalation of Cd can occur, however, from tobacco smoke and in occupational settings. Tobacco leaves accumulate this metal so that one cigarette may contain 1–2 μg Cd. Of that, ca. 10% is inhaled, and approximately 50% of that is absorbed in the lungs (Elinder et al., 1983). A person smoking 20 cigarettes per day will absorb about 1 μg cadmium daily. Occupational Cd exposure is due to metal dusts and fumes, or to paint spray, originating from the applications mentioned above. Measured airborne levels were ca. 30 $\mu g/m^3$ (indoors in car

body repair shops; Vitayavirasuk et al., 2005) or 1-19 μg/m^3 (outdoors, in bridge maintenance; Conroy et al., 1995). Depending on the size of the particles, airborne Cd is absorbed from the respiratory tract in 2-50% (Chaney et al., 2004). In a case reported by Okuda et al. (1997) acute Cd poisoning with respiratory signs developed over 3-6 months to a Parkinson-like state (stiffness of the limbs, bradykinesia, muscle rigidity) that did not improve on antiparkinsonian medication. Amyotrophic lateral sclerosis, optic nerve damage, striatal damage and peripheral polyneuropathy were also observed as long term neurotoxic consequences of Cd (Bar-Sela et al., 2001; Fern et al., 1996; O'Callaghan and Miller, 1986; Viaene et al., 1999). In children, a straight relationship between hair Cd and altered visual or auditory evoked potential parameters was found (Thatcher et al., 1982), and school behavioral problems were reported (Marlowe et al., 1985). Similar effects were observed in rats (Agar et al., 1999). Our previous works on Cd neurotoxicity revealed altered electrocorticogram (ECoG) power spectrum, and effects on cortical evoked potentials and peripheral nerve action potentials (Papp et al., 2003; Institóris et al., 2002).

As to mechanism of neurotoxicity, Cd^{2+} can block the influx of Ca^{2+} in the presynaptic terminal, which may result in altered transmitter release (Antonio et al., 1998). Excitatory neurotransmitters (glutamate and aspartate) were found decreased, while the inhibitory neurotransmitters (glycine and GABA) were increased in the amygdala of Cd-exposed animals, suggesting that Cd affects the balance of excitation/inhibition in synaptic transmission (Minami et al., 2001). Contents of dopamine, serotonin and norepinephrine in adult male rats were found decreased in all brain regions after a 24 hrs exposure to Cd (Lafuente et al., 2003). Cd is also potent inhibitor of the brain (Na^+/K^+)-ATPase (Antonio et al., 2002), and inhibits choline transport in synaptosomes (Chandra et al., 1994).

Cadmium intoxication significantly increased malondialdehyde level and glutathione peroxidase activity in rats and mice. In acute application, Cd increased the activity of antioxidant defense enzymes (Cu-Zn-SOD, glutathione peroxidase, glutathione reductase and glutathione-S-transferase but caused oxidative stress indirectly by displacing Fe and Cu from metalloenzymes (Flora et al., 2008).

Notwithstanding all toxicity of Cd, the marine diatome *Thalassiosira weissflogii* apparently has a carboanhydrase 2 enzyme with Cd in the active centre instead of Zn (Lane et al., 2005).

1.5. Manganese: properties, biological roles and applications

Manganese (Mn) is, in contrast to lead and cadmium, an essential micronutrient for humans and for all living organisms. The human body contains about 10 mg Mn, stored mainly in the liver and kidneys. The daily demand is 2-3 mg (ATSDR, 2008b). Mn is required for the development and the normal function of the CNS (Elder et al., 2006). Within the organism, it is mainly found in tissues rich in mitochondria (liver, muscles, brain etc.), where it forms stable complex with ATP and inorganic phosphate.

Mn is cofactor in several classes of metallo-enzymes, including oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, lectins and integrins. Mn-containing superoxide dismutase (Mn-SOD: Law et al., 1998) is the type of SOD present in eukaryotic mitochondria and in most bacteria. Glutamine synthetase (a glia-specific Mn metalloprotein) catalyzes in the CNS the conversion of glutamic acid to glutamine, thereby inactivating the transmitter. This enzyme requires Mn, but is inhibited by its excess which is of importance in the neurotoxic mechanisms of Mn (Normandin and Hazell, 2001).

For humans, the main source of Mn is food (and, less importantly, drinking water). Groundwater used for drinking (well water) can contain, depending on geological conditions, concentrations of Mn which are toxic on chronic exposure (50mg/L; Kondakis et al., 1989). Cases of foodborne overexposure by Mn in babies, fed on cow milk- or soybean-based formulas, were reported (Marlowe and Bliss, 1993). Iwami et al. (1994) described the neurotoxicity of high Mn plus low Mg intake. Long term parenteral nutrition has occasionally also led to hypermanganesaemia (Crook, 2001). Except for that, overexposure to Mn has traditionally been an occupational risk factor in, e.g., mining and the metal industry. “Manganese madness” caused by Mn containing mineral dust was first reported ca. 170 years ago (Couper, 1837).

Manganese is used in many important alloys. In steel, manganese improves hardness, strength, and wear resistance. Welding rods are frequently coated with a Mn-containing layer to reduce the oxidation of the steel parts to be jointed at the temperature of welding. There are different data in the literature on the relationship between airborne Mn and internal exposure. In a smelter, 0.1-3 mg/m^3 airborne Mn resulted in 12.5 ppb Mn in the blood (Myers et al., 2003). In alloy production workers, 0.89 mg/m^3 Mn in the total and 0.04 mg/m^3 Mn in the

respirable dust (that is, PM10) for about 10 years resulted in ca. 10 ppb whole blood Mn level (Mergler et al., 1994); but in another study, 0.014-11.48 mg/m^3 total dust and 0.001-1.273 mg/m^3 respirable dust Mn resulted in 11.3 ppb whole blood level (Bouchard et al., 2005). Neuro-functional damage was observed above 7.5 (Mergler, 1999) or 10 (Lucchini et al., 1999) ppb blood Mn. Although these studies did not separately discuss the effect of nano-sized particles, such particles were obviously present and exerted their effect, regarding the composition of welding fumes and similar industrial emissions (reviewed by Antonini, 2003).
Further uses of Mn involving the risk of environmental or occupational exposure include the agricultural application of certain fungicides (Prochloraz manganese, Maneb, Mancozeb; Ferraz et al., 1988). Mn compounds are used in the production of dry cell batteries and in paints, bleaching agents, and disinfectants (such as $KMnO_4$ solution). Manganese is also used as a coloring agent in glass and ceramics industry. Methylcyclopentadienyl manganese tricarbonyl (MMT) was used as an anti-knock petrol additive in Canada between 1976 and 1998, approved for use in several other countries (Australia, New Zealand, Russia, etc.; Davis, 1998). A novel use of Mn is in the production of semiconductor nanocrystals (Yang et al., 2005) and $ZnS:Mn^{2+}$ nanoflowers (synthetic nanostructures growing in a flower- or tree-like shape and gaining application e.g. in supercapacitors; Chen et al., 2005).

1.5.1. Toxicity and neurotoxicity of manganese

Mn is a potential environmental neurotoxicant, mainly after chronic exposure to higher doses. Inhalation of aerosol is the primary route of occupational exposure for welders and industrial workers. Acute exposure may produce metal fume fever (Piscator, 1976) or manganese pneumonitis.
Chronic inhalation of manganese compounds is known to cause severe neurologic disorders. The illness, called manganism, progresses in three stages (Saric et al., 1977; Calne et al., 1994). The first stage is marked by nonspecific symptoms like apathy, anorexia, asthenia, headache, hypersomnia, spasms, arthralgia, weakness of the legs, and irritability. In the second stage, psychomotor and psychic disturbances dominate, such as dysarthria, excess salivation, and difficulty in walking. The third stage represents a Parkinson-like syndrome with its associated symptoms. Manganese-induced Parkinsonism was also observed in patients undergoing maintenance hemodialysis (Ohtake et al., 2005) or in inadvertent

overdosing due to long-term ingestion of a health supplement containing high levels of Mn. In spite of the similar symptoms, the site of damage in manganism and in Parkinson's disease is different, viz. striatal neurons, rather than mesencephalic dopaminergic neurons, are the target of manganese toxicity (Erikson and Aschner, 2003).

Disorders with electrophysiological signs after Mn exposure include myoclonus in welders (Ono et al., 2002) and epileptic activity in an accidentally exposed child (Hernandez et al., 2003). In young shipyard workers, EEG and visual evoked potential alterations were observed and blood Mn levels up to 14 ppb were measured (Halatek et al., 2005). In reference groups, blood Mn is ca. 5–7 ppb (Bader et al., 1999). EEG and evoked potential disturbances following occupational Mn exposure were also reported by Sinczuk-Walczak et al. (2001) and Sjögren et al. (1996).

Oxidative stress is apparently a major feature of Mn neurotoxicity. This includes mitochondrial toxicity (inhibition of complex II and III) as well as decreased activity of Mn-SOD and glutathion peroxidase (Hamai and Bondy, 2004). In rats after one month of oral Mn exposure, increased lipid peroxidation was found and was linked to decreased motility (Avila et al., 2008). In other studies, however, ROS generation was not found to play significant role in the neurotoxicity of Mn (Taylor et al., 2006).

1.6. Aims

As seen from the previous sections, exposure to airborne metal particles is an important issue in occupational, and possibly environmental, hygiene, among others because of the effects of the metals on the nervous system. In previous experiments of the Department it was found that recording and analysis of electrophysiological signals from the brain and from a peripheral nerve is a sufficiently sensitive method to detect the effects of lead, cadmium and manganese on the nervous system of rats in dissolved form, following acute exposure (Papp et al., 2006) or repeated application for 4-12 weeks (Nagymajtényi et al., 1997; Papp et al., 2003; Vezér et al., 2005).

In the present work, a new model with more realistic chemical form of the metals (oxide NPs) and more realistic way of application (instillation into the trachea) was introduced and the electrophysiological measurements were supplemented with behavioral and chemical ones.

The questions, to be answered on the basis of the results of this work, are as follows:

- Is intratracheal instillation of suspension of NPs containing lead, cadmium and manganese a usable technique to model the effect of airborne metals?
- Can significant internal exposure be induced this way?
- Does this internal metal exposure induce functional alterations in the rats' nervous system, to be detected by electrophysiological recording and by open field test?
- Can oxidative stress be detected in the treated rats?
- Is there any correlation between internal exposure, and the neuro-functional and biochemical changes, which may underline a causal relationship?

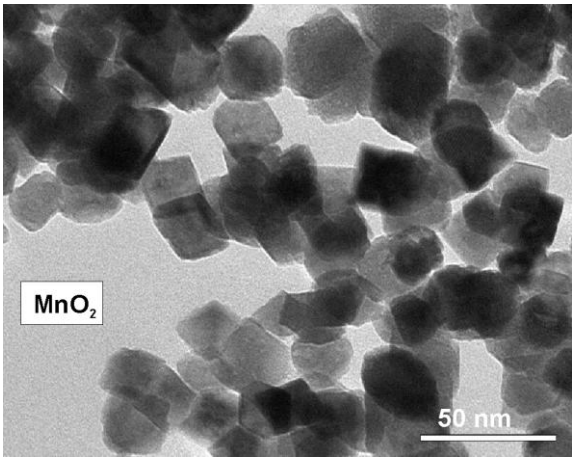
2. MATERIALS AND METHODS

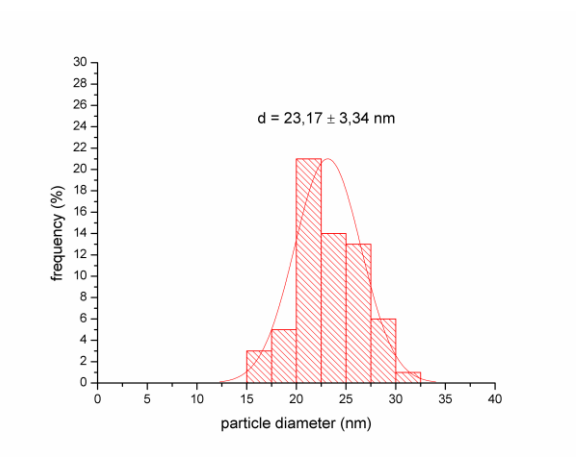
2.1. Experimental animals, housing and chemicals

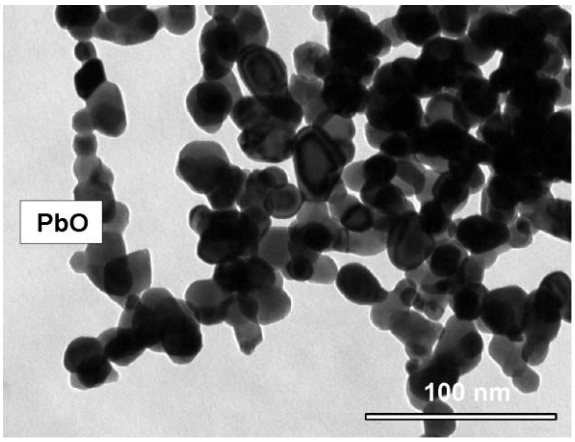
Young adult male Wistar rats (280–350 g body weight at start) obtained from the Breeding Centre of the University, were used for the experiments. The animals were housed under standard conditions (22–24 °C, 12 h light/dark cycle with light starting at 6:00 a.m., up to four rats in one cage) with free access to conventional standard rodent chow and drinking water.

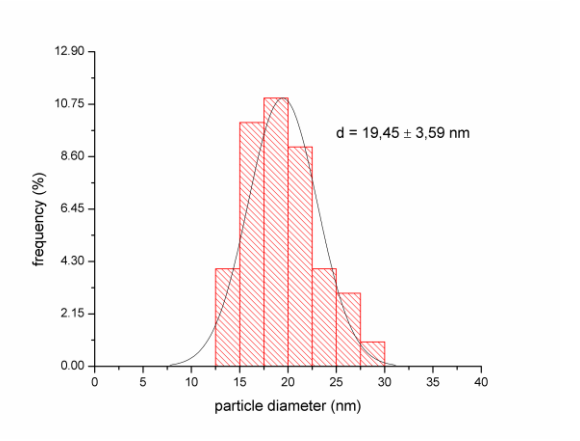
Metal nanoparticles were synthesized at the Department of Applied Chemistry, University of Szeged, Faculty of Science and Informatics. MnO_2 nanoparticles were made by a technique combining sonication and hydrothermal treatment. An appropriate amount of aqueous $KMnO_4$ solution was mixed with ethylene 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.

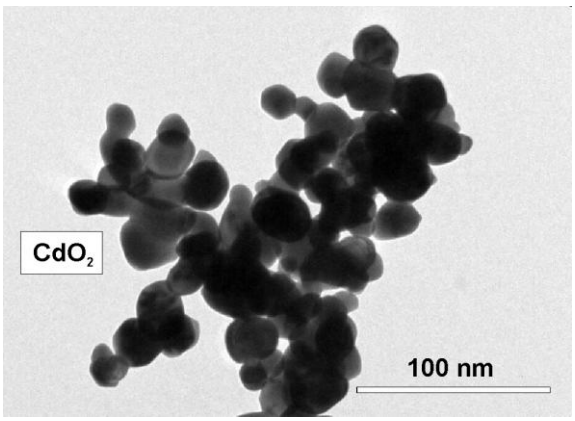
CdO_2 and PbO were produced in solid phase, by milling the base materials ($CdCl_2$ with Na_2CO_3; $Pb(CH_3COO)_2$ with NaOH) and calcining the intermediate product carbonate or hydroxide. The chemical purity of the nanoparticles was checked by X-ray diffraction, and their particle size, by X-ray diffraction and transmission electron microscopy (TEM). The mean diameter of MnO_2 particles was ca. 23 nm, of PbO was ca. 19 nm, of CdO_2 was ca. 20 nm (Fig. 1). For administration to the rats, the nanoparticles were suspended in distilled water. The suspension was sonicated to prevent aggregation, and was instilled into the rats' trachea 5 days a week, for 9 weeks (in case of Mn) or 6 weeks (in case of Pb and Cd). The instilled volume was 1.0 ml/kg b.w.; vehicle controls had distilled water only. For the brief anesthesia, diethyl ether was obtained from Central Pharmacy of the University if Szeged. Urethane, used for terminal anesthesia, was purchased from Reanal, Budapest.











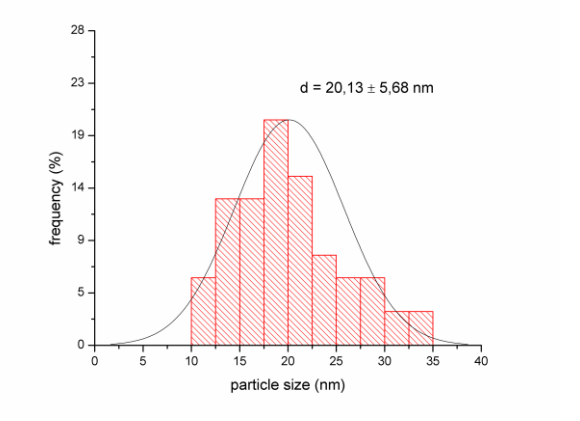


Figure 1: TEM image (left) and size histogram (right) of the MnO_2, PbO, CdO_2 nanoparticles. (Data kindly provided by Dr. Zoltán Kónya and his colleagues, Department of Applied Chemistry.)

2.2. Experimental protocol, intratracheal instillation

The experiments were done on groups of rats consisting of 8-10 animals per treatment dose and time (e.g. in the experiment with MnO_2 NPs, 8 rats received a particular dose for 3 weeks, another 8 rats for 6 weeks, and still another for 9 weeks). In the first experiment, done with Mn, the 9 weeks total treatment time was a compromise between adequately modelling long-term, low-dose human exposure – treatment times in earlier works were 12 weeks (Papp et al., 2003) or 10 weeks (Vezér et al., 2005) – and the tolerance of rats to the daily repeated treatment procedure (described below). In another, finally unpublished, experiment, too high repetition of the procedure resulted in significant mortality. Then some of the results with Mn indicated that a shorter treatment period may also be sufficient, so in the Pb and Cd experiment it was set to 6 weeks. The substances used, as well as doses and treatment durations, are summarized in Table I.

Table I: Treatment groups, corresponding doses and treatment durations.

Group	Code	Substance	Dose (mg metal / kg b.w.)	Duration of the treatment
Untreated control	*Con*	---	---	3, 6, 9 weeks
Vehicle control	*W*	Distilled water	---	3, 6, 9 weeks
Manganese low dose	*Mn-LD*	MnO_2 nanosuspension	2.63	3, 6, 9 weeks
Manganese high dose	*Mn-HD*		5.26	
Cadmium low dose	*Cd-LD*	CdO_2 nanosuspension	0.04	3, 6 weeks
Cadmium high dose	*Cd-HD*		0.4	
Lead low dose	*Pb-LD*	PbO nanosuspension	2	3, 6 weeks
Lead high dose	*Pb-HD*		4	

The doses were chosen on the basis of previous experience (Sárközi et al., 2008). For intratracheal instillation the animals were briefly anesthetized with diethyl ether in a glass jar with air-tight lid. When the anaesthesia was complete, the rat was suspended on an oblique board, standing at 60° to horizontal, with their upper incisors hung in a wire loop to hold the animal in place and keep its mouth open. 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 on non-traumatic forceps, and a custom-made laryngoscope was used to gain access to the glottis. The nanosuspension (or distilled water 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 drawn 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.

2.3. Behavioral investigation: open field test

At the end of the treatment period 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 between 8 and 11 hours in the morning. Just before the test, the animals were allowed to get acclimatized in the dimly lit test room for 30 minutes. The animals were placed individually into the centre of the box, and the instrument was recording their horizontal and vertical motor activity (in one 10 min session per rat) 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. Previous neurotoxicological works of the Department (e.g., Vezér et al., 2005) proved that this behavioral test was usable for investigating the impairment of higher nervous functions caused by heavy metals.

2.4. Electrophysiological investigation

2.4.1. Preparation of the animals

The animals were anaesthetized by intraperitoneal injection of 1000 mg/kg b.w. urethane (Mook, 2006). The head of the rats was fixed in a head holder, the skin was opened by a mid-sagittal cut and the muscles and connective tissues adhering to the skull were removed. Finally the left hemisphere was exposed by removing the temporal bone along the inner circumference by means of a mini drill. Wounds were sprayed with 10 % lidocaine and the exposed cortex was protected with a thin layer of petroleum jelly. After that, the animals were wrapped in a warm cloth to maintain the temperature and put aside for at least 30 min for recovery. Then, the rat was placed into the stereotaxic frame of the electrophysiological apparatus. For sustaining normal body temperature, a thermostated (+36.5°C) base plate was used to support the rat's underside during the recording procedure.

2.4.2. Recording procedure

To record spontaneous and evoked cortical activity, ball-tipped silver recording electrodes were positioned on the dura over the primary somatosensory (SS) area (projection of the whisker pad, barrel field), and over the primary visual (VIS) and auditory (AUD) area. These regions were determined on the basis of a somatotopic map (Zilles, 1984). A stainless steel clamp was attached to the cut skin edge as indifferent electrode. SS stimulation was done by a pair of needles inserted into the whiskery part of the nasal skin, delivering square electric pulses (for stimulation parameters, see below). VIS stimulation was performed by flashes delivered by a flash generator via an optical fibre conductor directed into the contralateral eye of the rat. For acoustic stimulation, sound clicks were applied into the ear of the rat. Compound action potential of the tail nerve was evoked by means of a pair of stimulating needle electrodes inserted at the base of tail (delivering similar electric stimuli as used to stimulate the whiskers), and the compound action potentials were recorded distally by another pair of needles at a distance of 50 mm.

Sensory stimuli were delivered by a digital time base and stimulator unit (Experimetria Ltd, Budapest, Hungary). All stimuli were set and applied as of just supramaximal strength (meaning that, e.g., the stimulus voltage was increased until the evoked response reached

maximal amplitude and ca. 5% was added) and well above background. Electrical stimulation of the whiskers and the base of tail was done by delivering rectangular electric stimuli (3-4 V, 0.05 ms). The intensity of the visual stimulation was ca. 60 lux, and that of the auditory stimuli, 40 dB. Trains of 50 stimuli were applied and the evoked potentials (EPs) recorded. The standard frequency of the stimulation was 1 Hz. Previous studies in our laboratory (Papp et al., 2001, 2004) demonstrated that varying the frequency of stimulation can sensitively detect the dynamic interaction of successive excitation processes in the sensory system which in turn reflects the actual state of the CNS. Accordingly, the frequency dependence in the parameters of the cortical evoked activity was determined by delivering stimuli to the somatosensory system (i.e. to the whisker pad) beyond the standard 1 Hz, also with 2 and 10 Hz frequency. In the stimulation of the tail nerve, 1, 20 and 50 Hz was used.

One session consisted of six minutes recording of spontaneous activity (electrocorticogram, ECoG) first, from the three sensory cortical areas simultaneously. Then EPs from the same cortical areas via the same surface electrodes were recorded, and finally the compound action potential of the tail nerve. The recorded biological signals were amplified (10^4x) fed into the digitizer interface of the recording setup, and stored on PC.

2.4.3. Evaluation

The complete recording and evaluation was executed by the software Neurosys 1.11 (Experimetria Ltd, Budapest, Hungary).

From the ECoG records, the relative spectral power of the frequency bands: delta, 0.5-4 Hz; theta, 4-7 Hz; alpha, 8-13 Hz; beta1, 13-20 Hz; beta2, 20-30 Hz; gamma, 30-50 Hz (Kandel and Schwartz, 1985) was determined by the software automatically.

From the relative band power data, the so-called “ECoG index” was calculated with the formula: ([delta]+[theta])/([beta1]+[beta2]). This proved to be a handy (albeit simplifying) single-figure descriptor of the ECoG spectrum in earlier works.

The recorded evoked responses were automatically averaged off-line, and their parameters were measured manually by means of screen cursors of the software. Exemplified on the SS EP, onset latency was measured between the stimulus artefact (designated **0** in Fig. 2.2) and onset of the first wave (**A** in Fig. 2). Duration of the EP was calculated as the difference of the **0-D** and **0-A** times. In case of the visual and auditory EPs, onset latency and duration was measured, in the same way. The tail nerve action potential had also a biphasic shape. There, onset latency was defined analogously with the **0-A** distance. Tail nerve conduction velocity was calculated from the onset latency and the distance of the electrodes.

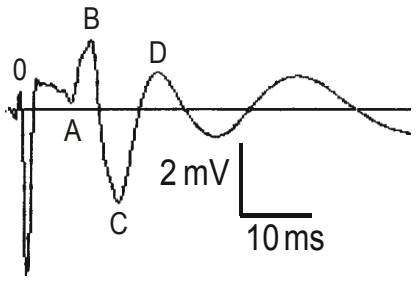


Figure 2: Typical example of the somatosensory evoked potential with the specific measuring points. See text for details.

Right after finishing the electrophysiological investigation, the animals were sacrificed by an overdose of urethane (and were dissected, see 2.5).

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

2. 5. General toxicological investigations

The animals were under continuous clinical observation during the experiment and abnormal reactions were noticed. Body weight, as a general indicator of the rats' health state, was measured once weekly. Following electrophysiological recordings the overdose of urethane, 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. During dissection, blood samples were

collected from the abdominal vein, 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.

2.6. Chemical and biochemical measurements

To prepare for biochemical analysis 1 g tissue sample was homogenized with 4 ml saline, and the samples were centrifuged under cooling for 10 min at 5000 rpm. The supernatant was drained into Eppendorf tubes and was centrifuged for 20 minutes at 14,000 rpm. The supernatant was collected and used in further work.

It was essential to quantify protein content as certain parameters were compared to protein amount. The samples were diluted (liver: 200x, other organs: 100 x). Determination was done according to Lowry et al. (1951) where proteins react with Folin reagent producing dark blue colour with absorption maximum at 750 nm. Protein content was determined by the built-in software of the spectrophotometer (Beckmann Du 640) on the base of a calibrating curve obtained using bovine serum albumin.

For GSH assay, method of Sedlak and Lindsey (1968) was used. SH groups bound to nonproteins were quantified by Ellmann reagent (DTNB) from protein-free samples. DTNB (5,5-dithio-bis-nitrobenzoic acid; 10 mM, dissolved in methyl alcohol) is a disulphide chemical compound that GSH can reduce. Reduced DTNB is bright yellow.

The supernatant was precipitated with 4x amount of 5% TCA. Samples were centrifuged for 10 minutes at 10,000 rpm. 400 µl TRIS buffer was added to 200 µl supernatant. Colour reaction was initiated by adding 20 µl DTNB. Spectrophotometry was done on 412 nm. Concentration was calculated with the following formula:

$$[GSH] = E \times V_{all} \times 10\text{-}3 / \varepsilon \times l \times V_{sample}$$

E: extinction, ε: molar extinction coefficient (131000 M-1cm-1), V_{all}: volume of the measuring solution (ml), V_{sample}: volume of the sample (ml), l: length of the beam (cm)

SOD assay was carried out by applying the Misra and Fridovich (1972) method, modified by Matkovics et al. (1982). The rationale for the technique is that superoxide dismutase enzyme in alkaline medium can inhibit the spontaneous adrenaline-adrenochrome transition depending on concentration. Control measures were also applied in order to define spontaneous adrenalin

change and to determine extinction change during one minute (ΔE/min). Wavelength of the measurement was 470 nm and the temperature was 37 °C. The formula used for calculation was the following:

$$\text{inhibition \%} = (E_{control} - E_{sample}) / E_{control} * 100 / 50$$

The unit of enzyme is the amount that can produce 50 % inhibition.

For metal level determination, ca. 1 g samples were dried at 80°C to constant weight, and were digested in 5 ml 65 % HNO_3 at 90°C for 90 min. The digested matter was washed quantitatively into 100 ml measuring flasks and Mn determination was done by inductively coupled plasma mass spectrometry (at the laboratory of the MOL Hungarian Oil and Gas Company).

2.6. Statistical analysis of the data

From the general toxicological, (bio)chemical, behavioral and electrophysiological data, group means (±SD) were calculated. All results were checked for normality by means of the Kolmogorov-Smirnov test, then tested for significance using one-way ANOVA with post hoc Scheffe's test by the SPSS 15.0 for Windows software package. The relationship between the bodily metal load in the rats and the measured neuro- and general toxicological parameters was tested by plotting these in correlation diagrams, and linear regression was calculated by the "linear fit" function of MS Excel. Significance was accepted at $p<0.05$.

3. RESULTS

3.1. Effects of manganese nanoparticle exposure

3.1.1. Body weight gain and organ weights

Intratracheal instillation of MnO_2 NPs significantly reduced the rats' body weight (Fig. 3). Untreated rats (*Con*) had normal weight gain. The growth in the vehicle control (*W*) group was also constant, even if at a lower rate (likely due to the repeated treatment procedure). The weight of rats in both treated groups (*Mn-HD*, *Mn-LD*) was, however, significantly lower already after one week treatment. From the 6th week on, there was no weight gain in the *Mn-LD* group, and the rats in *Mn-HD* showed even some weight loss.

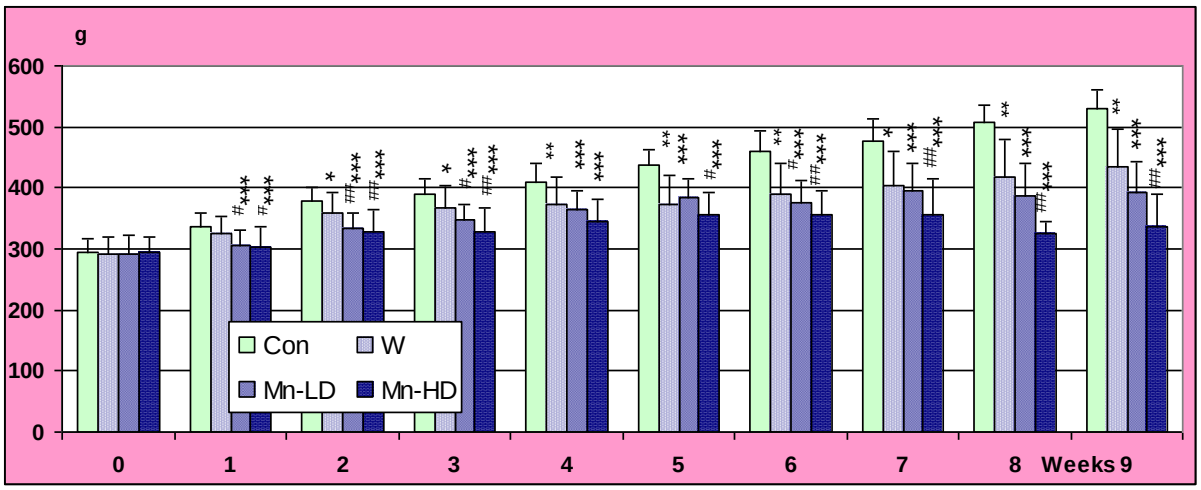


Figure 3: Body weight gain of the rats treated with Mn NPs and of the corresponding controls. Mean+SD, n=8. Insert: group codes, see Table I for explanation.
*, **, ***: p<0.05, 0.01, 0.001 vs. Con; #, ##: p<0.05, 0.01 vs. W.

In the organ weights, the relative weight of the lungs showed massive change (Table II). When calculated on the basis of body weight, (upper part of the table) the relative weight of the lungs strongly increased by the 9th week in both treated groups. In group *W*, however, the change was negligible which reassured us that distilled water instillation alone had no effect on the lung weight. Significant decrease in the relative weight of the liver, and increase in that of the kidney, was also seen.

The effects seen on the body weight-related data could be, even if significant, questionable due to the body weight effect of the treatment. The absolute brain weight data were less variable (after 9 weeks; *Con*: 2.157 ± 0.079 g, *W*: 2.026 ± 0.108 g, *Mn-LD*: 1.980 ± 0.092 g, *Mn-HD*: 1.935 ± 0.247 g) so that brain weight-related calculation was also done (lower part). This way, the increase of lung and decrease of liver weight remained significant (and the difference between relative liver weight change in the *W* vs. *Mn-LD* and *Mn-HD* groups was clearer, arguing against an artefact caused by the procedure). In the *Mn-HD* group, increased weight of the adrenals (probably due to stress) was seen.

Table II: Relative organ weights after 9 weeks intratracheal exposure by MnO_2 NPs.

Groups	***Con***	***W***	***Mn-LD***	***Mn-HD***
to 1/100 body weight				
Brain	0.404±0.020	0.470±0.060	0.490±0.048	0.538±0.086
Lung	0.321±0.011	0.370±0.024	0.841±0.249**$^{\#\#}$	1.112±0.336***$^{\#\#\#}$
Liver	3.037±0.228	2.804±0.206*	2.902±0.243**	2.729±0.283***
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
to brain weight				
Lung	0.797±0.055	0.797±0.093	1.614±0.468***$^{\#\#\#}$	2.047±0.426***$^{\#\#\#}$
Liver	7.528±0.665	6.080±1.104**	5.979±0.803**	5.134±0.546***$^{\#}$
Kidney	1.488±0.073	1.332±0.160	1.309±0.263	1.227±0.087**
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$^{\#\#}$	0.202±0.044
Adrenals	0.024±0.005	0.029±0.006	0.033±0.011	0.041±0.011**$^{\#}$

Mean±SD, n=8.

*p<0.05, **p<0.01, *** p<0.001 vs. *Con*; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. *W*.

3.1.2. Effects on open field behavior

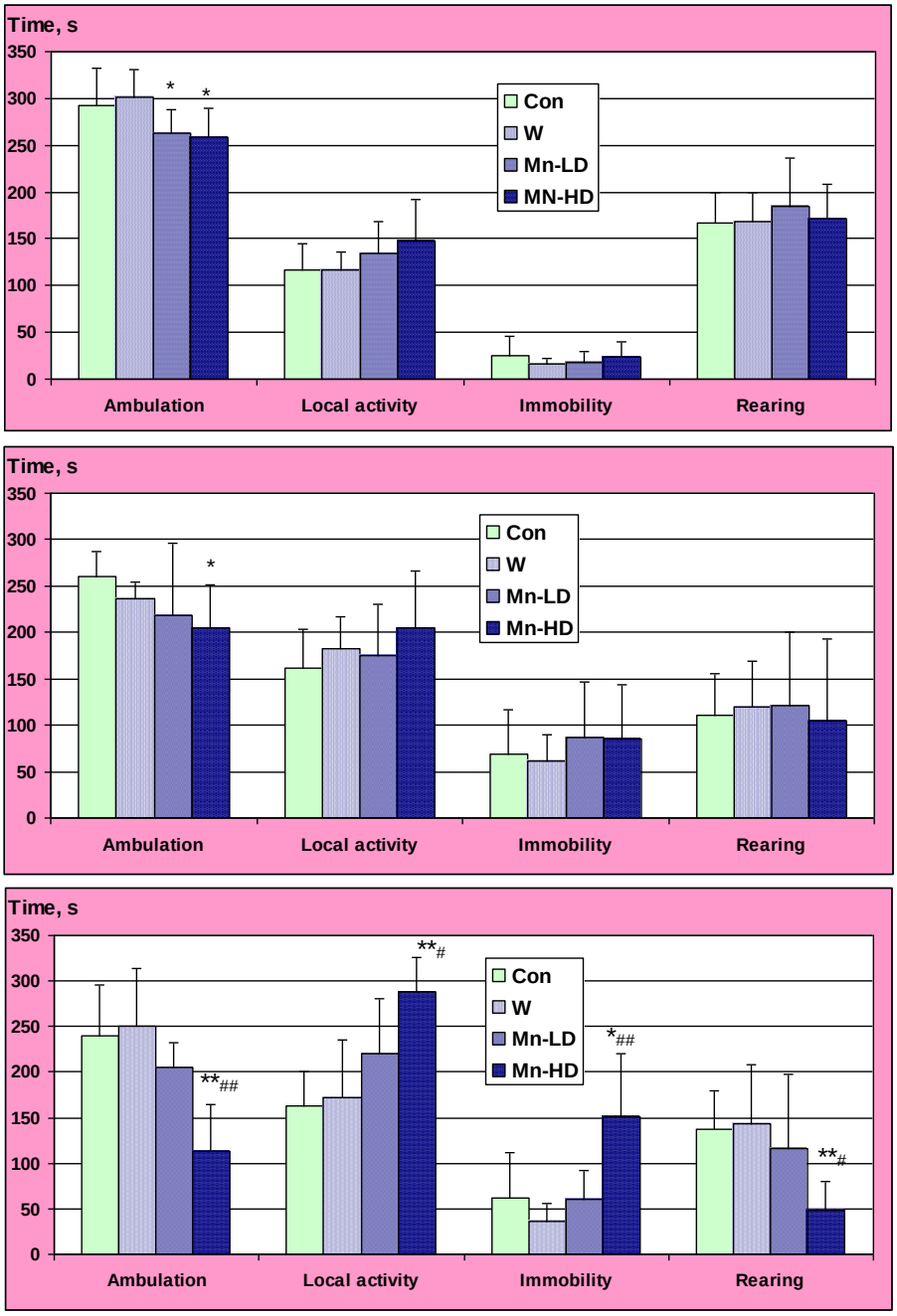


Figure 4: Open field activity of the rats treated with Mn NPs and of the corresponding controls after 3 (top graph) 6 (middle graph) and 9 (bottom graph) weeks exposure. The data (Mean+SD, n=8) represent the time spent in the given form of activity. Insert: group codes.
*, **: $p<0.05$, 0.01 vs. Con; $^{\#}$, $^{\#\#}$: $p<0.05$, 0.01 vs. W.

The general trend in the open field motor behavior was dose- and time-dependently decreasing motility. The time spent with ambulation decreased significantly already after 3

weeks treatment (Fig. 4, top graph). The corresponding increase in the time share of local activity and immobility increased moderately after 3 and 6 weeks treatment but by the 9th week (bottom graph in Fig. 4) this change, and the decrease in rearing, also became significant.

The complete set of numerical data of OF activity (Table III) shows that not only the time of ambulation but also the distance covered, and the speed of walking, were reduced in the Mn-treated rats. The time/count calculated values indicate that there were not only more events of local activity and immobility among the treated rats but these were also longer; the rats were apparently reluctant to change to ambulation once they were only locally active or not active at all.

Table III: Open field performence data of the Mn-NP treated rats and their controls after 9 weeks exposure.

Groups	Con	W	Mn-LD	Mn-HD
Ambulation distance (cm)	195.35±45.14	185.05±51.89	155.14±26.17	72.44±40.21**##∘
Ambulation time (s)	240.60±55.29	250.20±63.03	204.50±28.18	113.83±50.70**##∘
Ambulation count	29.80±6.98	29.00±7.35	29.00±4.20	20.00±5.44
Ambulation speed (cm/s)	8.14±0.68	7.40±0.90	7.63±1.10	6.17±0.64***#
Ambulation time/count (s)	8.16±2.87	9.42±4.78	6.87±0.89	5.68±1.59
Local activity time (s)	162.20±38.75	171.60±63.01	220.00±61.06	288.00±38.11**#
Local activity count	68.80±15.83	71.60±12.56	81.17±13.23	95.17±5.27*#
Local activity time/count (s)	2.42±0.05	2.35±0.40	2.61±0.46	3.04±0.54*#
Immobility time (s)	61.60±50.16	35.80±19.68	60.17±32.11	150.50±69.38*##∘
Immobility count	31.20±16.96	22.60±11.39	32.17±16.77	65.67±16.74*##∘
Immobility time/count (s)	1.93±0.44	1.54±0.12	1.79±0.25	2.21±0.63#
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**#
Rearing time/count (s)	2.61±0.72	2.57±0.54	2.66±0.84	1.83±0.59

Mean+SD, n=8.
*, **: $p<0.05$, 0.01 vs. Con; #, ##: $p<0.05$, 0.01 vs. W.

3.1.3. Electrophysiological effects

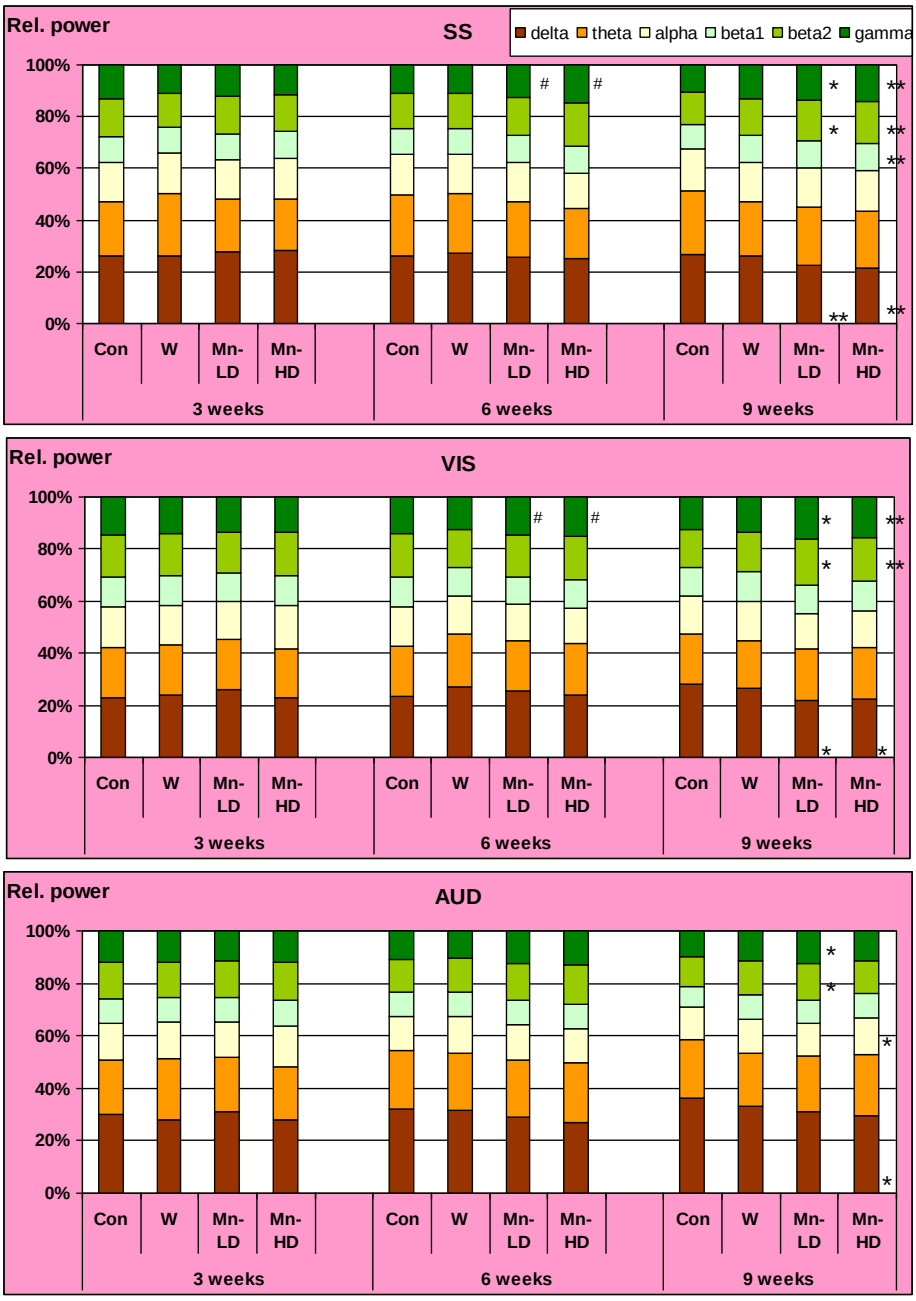


Figure 5: Band spectrum of the electrocorticogram recorded from the three cortical areas (SS, somatosensory; VIS, visual; AUD, auditory) after 3, 6 and 9 weeks Mn NP exposure. Abscissa: treatment groups and time. Bands: see insert in the top graph.
*, **: p<0.05, 0.01 vs. Con; #:p<0.05 vs. W; °: p<0.05 vs. LD.

In the spontaneous cortical activity after 3 weeks, there was a slight shift towards higher frequencies in the VIS and AUD, but not SS, area (Fig 5). After 6 weeks treatment this was

more pronounced and became significant in the gamma band of the ECoG in the SS and VIS area. Finally, after 9 weeks, both the decrease of delta, and increase of beta and gamma activity was significant, not only in the *Mn-HD* but partly also in the *Mn-LD* group. There was no qualitative difference between the spectrum changes seen in the recorded cortical areas.

From the data of the cortical EPs, onset latency was the most sensitive to the NP exposure. As seen in Fig. 6, both doses caused latency lengthening of the SS EP after 9 weeks exposure but the effect of the high dose was stronger. Also the frequency-dependent latency increase was more expressed in the Mn NP treated groups. On the VIS and AUD EP latency, only the

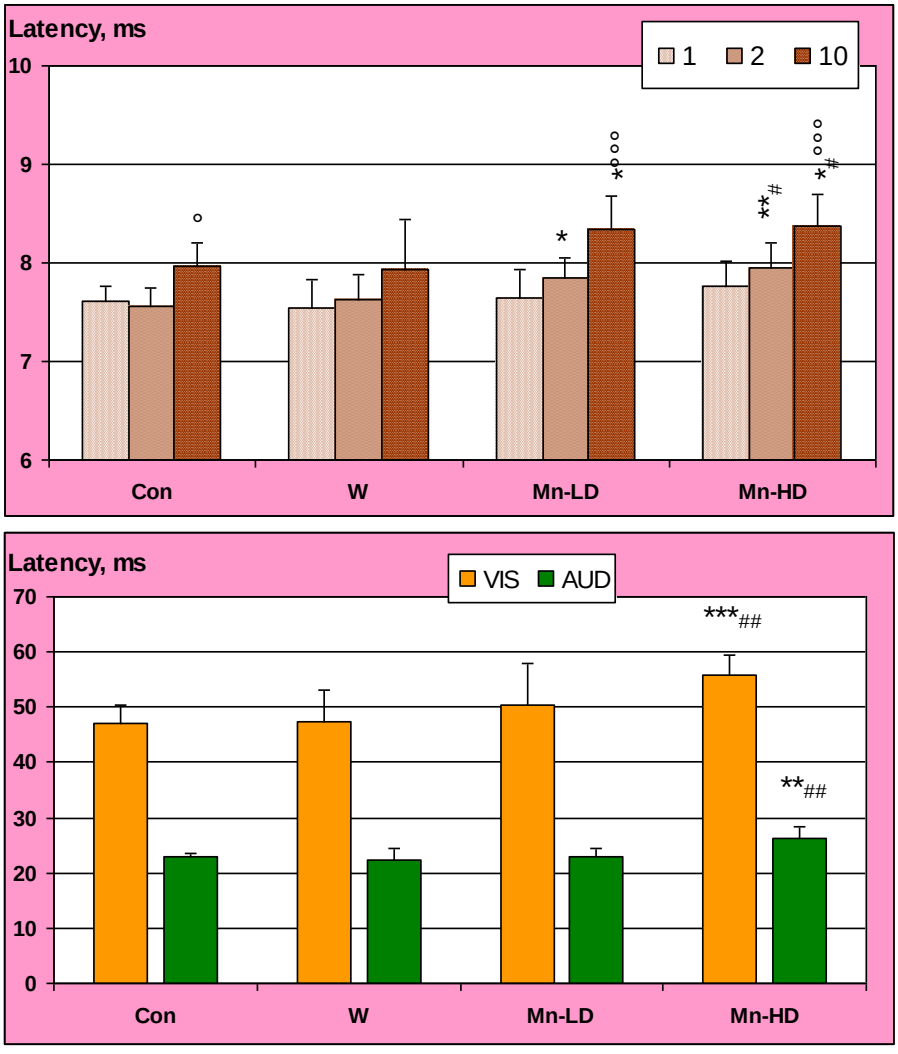


Figure 6: Onset latency of the somatosensory evoked potential at various stimulation frequencies (top graph, see insert for frequency in Hz), and of the visual and auditory evoked potential (bottom graph) after 9 weeks Mn NP exposure. Mean+SD, n=8.
*, **, ***: p<0.05, 0.01, 0.001 vs. Con; $^{\#}$, $^{\#\#}$: p<0.05, 0.01 vs. W (between identical frequencies among groups at the SS EP); °, °°, °°°: p<0.05, 0.01, 0.001 (vs. 1 Hz stimulation within a group at the SS EP)

higher dose caused significant lengthening.

The conduction velocity of the tail nerve was significantly decreased after 9 weeks exposure (Fig 7), but the dependence of this parameter on the frequency of stimulation was not altered.

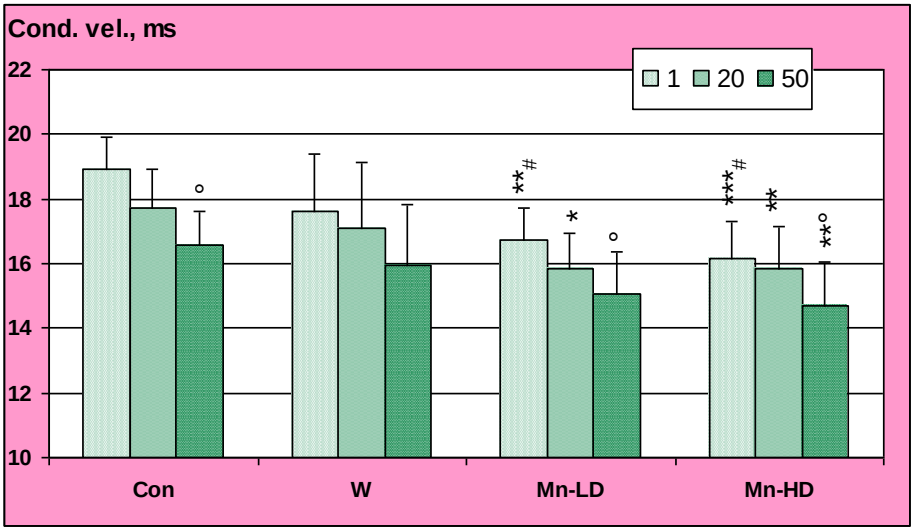


Figure 7: Conduction velocity of the tail nerve after 9 weeks treatment by Mn NPs, at various stimulation frequencies (see insert). Mean+SD, n=8.
*, **, ***: p<0.05, 0.01, 0.001 vs. Con; #: p<0.05 vs. W (between identical frequencies among groups); °: p<0.05 (vs. 1 Hz stimulation within the same group)

3.1.4. Results of chemical and biochemical measurements

Significant Mn deposition was detected in the treated rats' brains after 9 weeks exposure, which was apparently saturating as the difference between *Mn-HD* and *Mn-LD* groups was little. Blood levels were more proportional to the dose. After 6 weeks only, the differences were similar but still not significant.

In the total SOD and Mn-SOD activity of the brain samples, the Mn NP exposure caused an increase with some dose-dependence (Table IV). Only the increase of total SOD was significant. The level of reduced glutathione, another indicator of the local redox conditions, was moderately decreased in the treated rats' brains.

Table IV: Mn levels and oxidative stress parameters in the brain and blood samples of rats treated for 9 weeks with MnO_2 NPs or the empty vehicle.

Groups	***W***	***Mn-LD***	***Mn-HD***
Brain Mn, ppb (n=8)	1931±476	7151±2863**	7230±2910**
Blood Mn, ppb (n=8)	514±217	782±169	1022±206*
Brain SOD, U/mg prot. (n=5)	46.65±3.26	49.83±4.83	70.57±9.52***
Brain Mn-SOD, U/mg prot. (n=5)	10.97±4.89	14.94±9.51	25.54±21.01
Brain GSH, μM (n=5)	0.0937±0.0058	0.0894±0.0038	0.0825±0.0043*

Mean±SD, n=8 or 5/group as indicated.

*, **, ***: $p<0.05$, 0.01, 0.001 vs. W.

The correlation of Mn load and certain toxicological parameters is shown on the plots in Fig. 8. Only the correlation of the ECoG index and the nerve conduction velocity with the brain Mn level was significant (and also the relationship of ECoG index and brain GSH level, not shown).

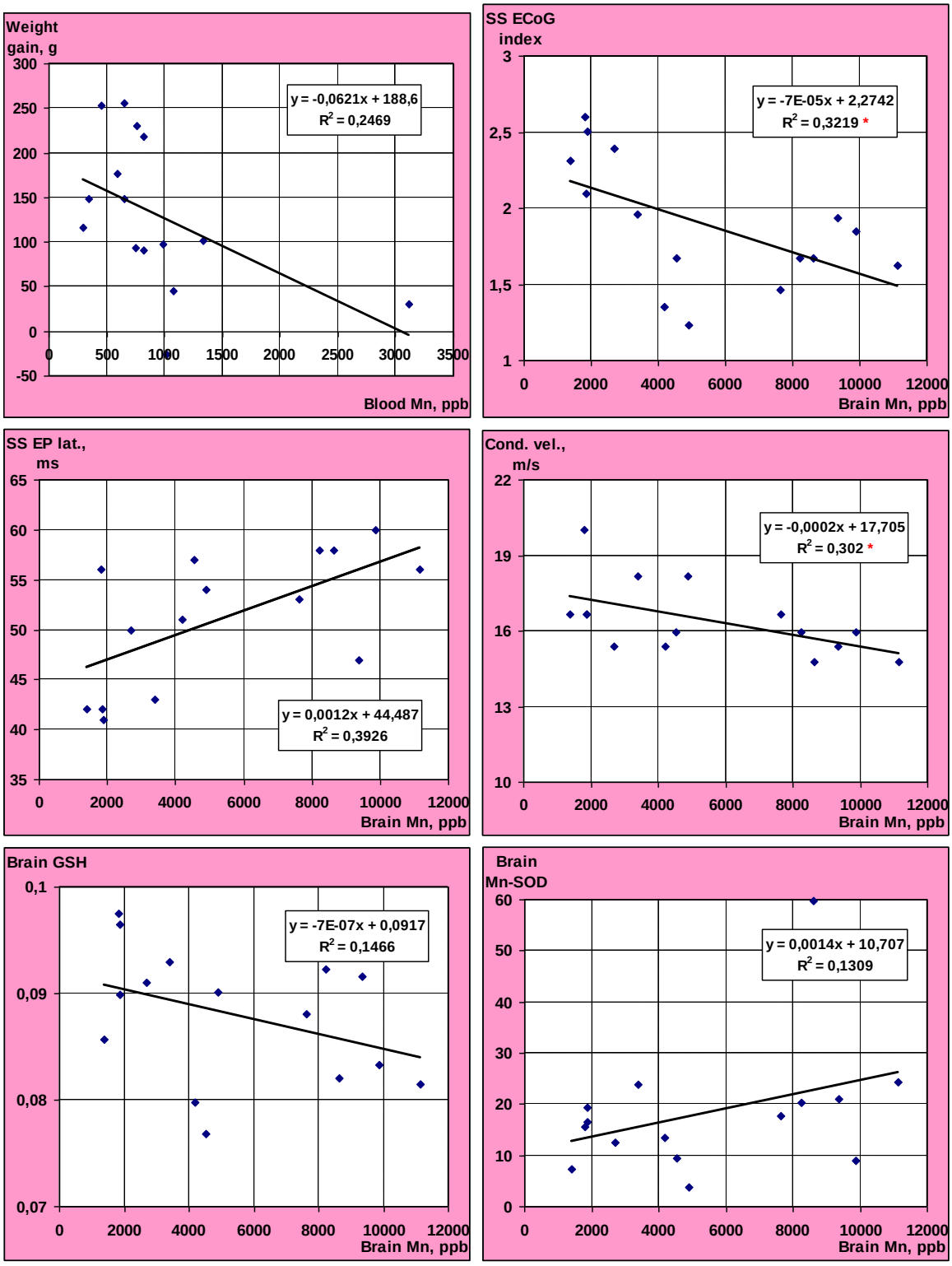


Figure 8: Correlation of the brain or blood Mn level to certain measured neuro-functional and toxicological parameters.
*: $p<0.05$ for the linear fit

3.2. Effects of lead nanoparticle exposure

3.2.1. Body weight gain and organ weights

Intratracheal treatment by Pb NPs caused significant retardation in the rats' body weight gain from the 2nd treatment week on (Fig. 9). The difference between the *Con* and *W* groups was, however, moderate which indicated again that the procedure of repeated ether anesthesia and instillation, in itself, had some effect but it was not really negative.

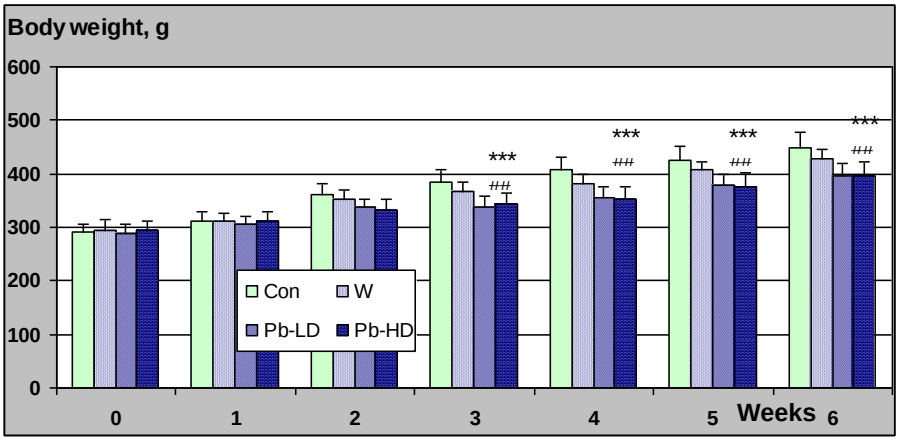


Figure 9: Body weight gain of the rats treated with Pb NPs and of the corresponding controls. Mean+SD, n=10. Insert: group codes.
***: $p<0.001$ vs. Con; ##: $p<0.01$ vs. W.

The relative organ weights showed various significant changes when calculated on the basis of 1/100 body weight (Table V, upper part). This was due, like in case of Mn (see 3.1.1), at least partly to the massive effect of PbO NP administration on the body weight. On the basis of brain weight (lower part of Table V) only the lungs showed significant change of relative weight after 6 weeks treatment. In the *Pb-HD* group, the lungs had also a strongly emphysematous appearance. There was some decrease in the relative weight of the liver, and increase in that of the kidneys, but these remained below significance as did all organ weight changes after only 3 weeks exposure (not shown). The change of the relative brain weight seen in the upper part of the table was partially real (originating from the absolute weights, which were: *Con*, 2.164±0.088 g; *W*, 2.086±0.068 g; *Pb-LD*, 2.092±0.159 g; *Pb-HD*, 2.058±0.062 g, $p<0.05$ vs. *Con*) but much lower than the changes of the body weight in the

same groups, so that the brain-relative organ weight data indicated the effect of NP administration on the organs more correctly.

Table V: Relative organ weights after 6 weeks intratracheal exposure by PbO NPs.

Groups	***Con***	***W***	***Pb-LD***	***Pb-HD***
to 1/100 body weight				
Brain	0.474±0.029	0.480±0.022	0.529±0.0562*$^{\#}$	0.520±0.0476*$^{\#}$
Lung	0.334±0.036	0.343±0.028	0.515±0.0760***$^{\#\#\#}$	0.583±0.0586***$^{\#\#\#}$
Liver	3.111±0.208	3.201±0.333	3.342±0.3666	3.324±0.1640*
Kidney	0.605±0.037	0.618±0.038	0.721±0.0961**$^{\#\#}$	0.686±0.0382***$^{\#\#\#}$
Heart	0.255±0.017	0.265±0.019	0.282±0.0323*	0.267±0.0158
Spleen	0.188±0.029	0.176±0.026	0.199±0.0388	0.187±0.0211
Thymus	0.090±0.011	0.095±0.023	0.103±0.0209	0.104±0.0284
Adrenals	0.010±0.003	0.012±0.004	0.013±0.0045	0.013±0.0035
to brain weight				
Lungs	0.707±0.080	0.714±0.061	0.976±0.113***$^{\#\#\#}$	1.128±0.125***$^{\#\#\#}$
Liver	6.599±0.740	6.680±0.798	6.353±0.719	6.446±0.667
Kidney	1.281±0.112	1.288±0.081	1.366±0.118	1.328±0.120
Heart	0.541±0.054	0.551±0.043	0.536±0.055	0.517±0.049
Spleen	0.396±0.059	0.367±0.062	0.379±0.085	0.361±0.041
Thymus	0.191±0.023	0.199±0.054	0.196±0.043	0.199±0.040
Adrenals	0.020±0.006	0.026±0.008	0.025±0.008	0.024±0.007

Mean±SD, n=10.

*,**,***: $p<0.05$, 0.01, 0.001 vs. Con; $^{\#}$, $^{\#\#}$, $^{\#\#\#}$: $p<0.05$, 0.01, 0.001 vs. W.

3.2.2. Effects on open field behavior

After 6 weeks exposure, rats in both treated groups (*Pb-LD* and *Pb-HD*) spent significantly more time with ambulation than the in controls (*Con* and *W*: Fig. 10). The decrease in rearing, and even more the increase in local activity, was less characteristic and was significant only vs. *Con*. After 3 weeks exposure only, the same trend was seen but without significance.

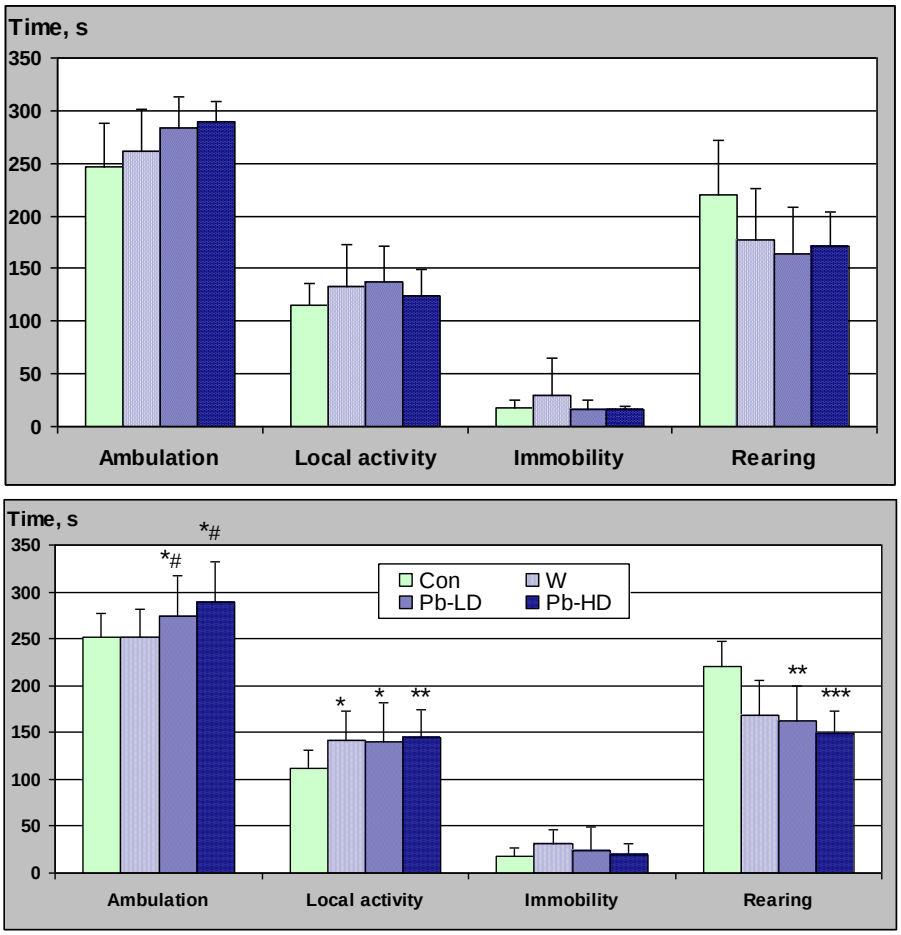


Figure 10: Open field activity of the rats treated with Pb NPs and of the corresponding controls after 3 (top graph) and 6 (bottom graph) weeks exposure. The data (Mean+SD, n=10) represent the time spent in the given form of activity. Insert: group codes.
*, **, ***: $p<0.05$, 0.01, 0.001 vs. *Con*; $^{\#}$: $p<0.05$, vs. *W*.

The data of Table VI show that the rats in *Pb-HD* group covered a longer path over the longer ambulation time but their speed was not higher. They did not start walking more times than the rats in *Con* or *W* but the average time of one ambulation event was longer. Also, the

changes in local activity and rearing were present both in the time and count data (and hence, in the mean event length).

Table VI: Open field test data of the Pb-NP treated rats and their controls after 6 weeks exposure.

Groups	Con	W	Pb-LD	Pb-HD
Ambulation distance (cm)	2014.94±289.01	2148.18±171.88	2115.70±534.04	2263.99±516.65°
Ambulation time (s)	252.10±25.53	251.50±30.31	274.70±42.05	288.50±43.83*#°
Ambulation count	32.60±4.62	30.10±3.78	32.20±4.21	28.80±6.30
Ambulation speed (cm/s)	7.98±0.64	8.6±1.22	7.60±0.99#	7.79±0.87
Ambulation time/count (s)	7.92±1.68	8.45±1.37	8.681±1.86	10.62±3.46*
Local activity time (s)	111.00±19.83	141.10±31.80	140.20±40.94*	143.80±29.85*
Local activity count	58.10±8.02	64.00±6.85	64.20±14.24	62.40±13.68*#
Local activity time/count (s)	1.90±0.16	2.19±0.36	2.16±0.24*	2.32±0.20***
Immobility time (s)	17.70±8.50	30.70±15.75	24.30±24.32	19.30±11.57°
Immobility count	12.30±5.76	18.50±7.26	15.20±10.30	12.40±6.33°
Immobility time/count (s)	1.44±0.26	1.59±0.41	1.43±0.30	1.57±0.50
Rearing time (s)	220.20±26.67	167.70±38.25	161.80±38.10**	149.40±23.51***
Rearing count	80.00± 6.82	67.30±14.62	67.70±12.50*	62.90±8.80***
Rearing time/count (s)	2.77±0.38	2.49±0.18	2.39±0.48	2.39±0.35*

Mean+SD, n=10.

*, **, ***: $p<0.05$, 0.01, 0.001 vs. Con; #: $p<0.05$, 0.01 vs. W; °: $p<0.05$ vs. LD.

3.2.3. Electrophysiological effects

In the ECoG activity, a weak trend of decreased intensity in the low and increase in the high frequency bands was seen after 6 weeks Pb NP exposure (Fig. 11), but this was significant mostly in the *Pb-HD* group only, and not in every recorded area. After 3 weeks only, the data showed no trend.

The latency of the SS EP was hardly different in the *Con* and *W* groups, and noteworthy frequency-dependent increase was seen only with 10 Hz stimulation (Fig. 12, upper graph). In the *Pb-LD* group, there was only minor lengthening of latency but the frequency-dependent increase at 10 Hz stimulation became significant (vs. 1 Hz). In the *Pb-HD* group, significant latency increase was seen and the frequency-dependent increase was also more pronounced.

The VIS and AUD EP also had increased latency in the *Pb-HD* group (Fig. 12, lower graph) but in case of VIS EP the variance between *Con* and *W* made it less clear.

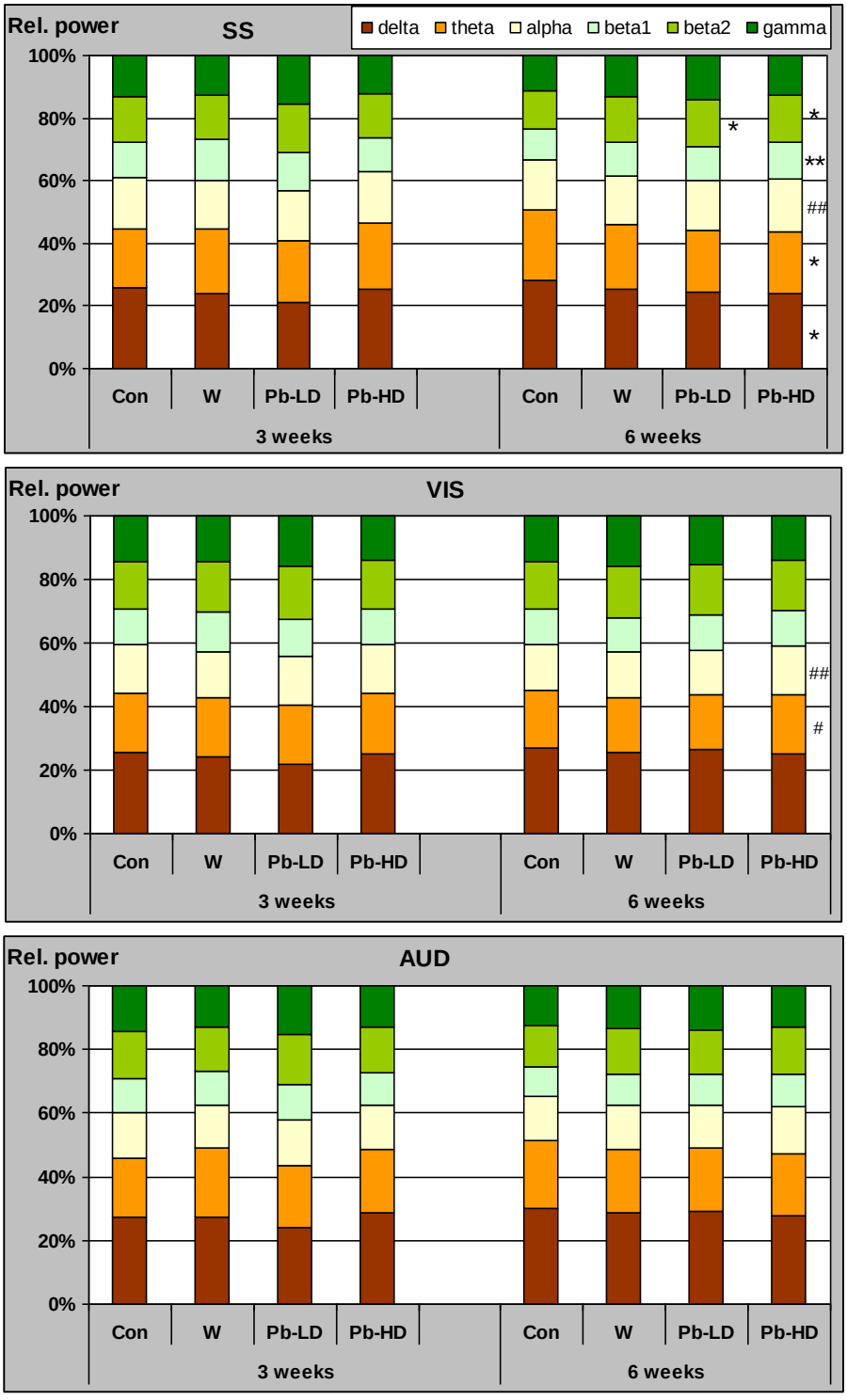


Figure 11: Band spectrum of the electrocorticogram recorded from the three cortical areas (SS, somatosensory; VIS, visual; AUD, auditory) after 3 and 6 weeks Pb NP exposure. The same display as in Fig. 5.
*, **: p<0.05, 0.01 vs. Con; $^{\#}$,$^{\#\#}$: p<0.05, 0.01 vs. W.

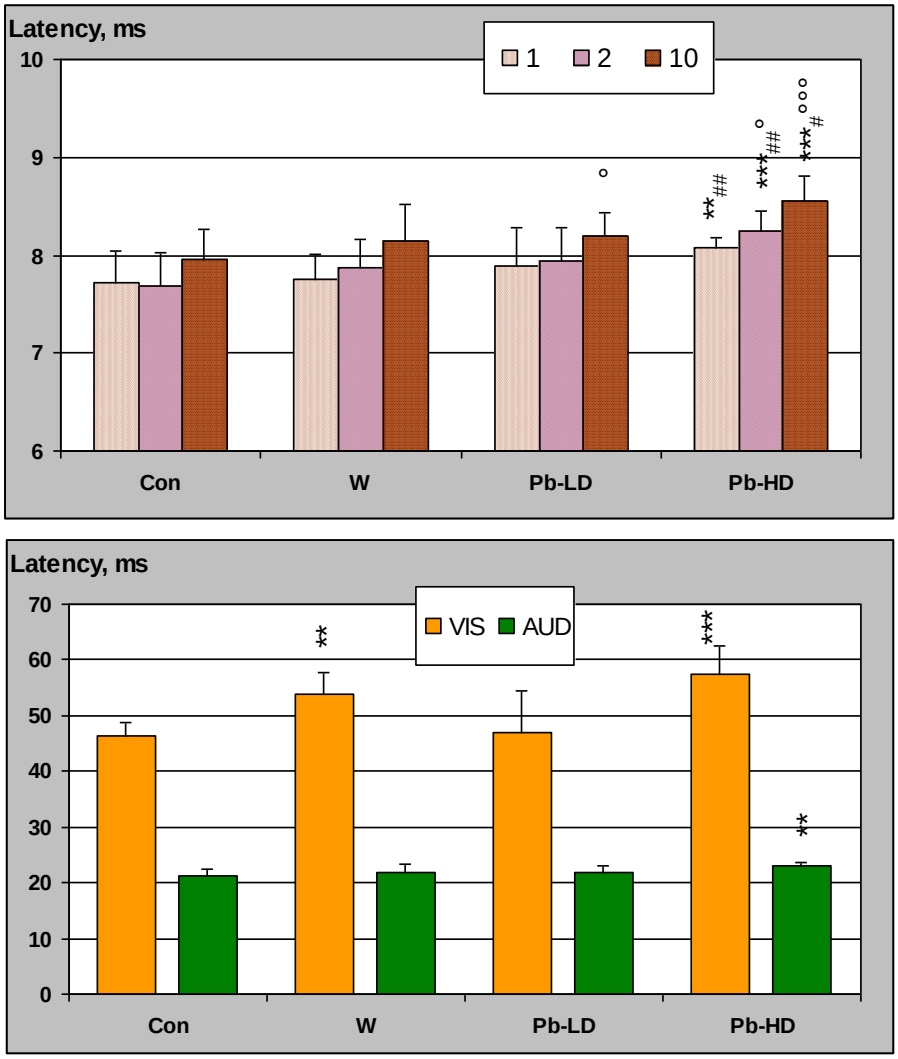


Figure 12: Onset latency of the somatosensory evoked potential at various stimulation frequencies (top graph, see insert for frequency in Hz), and of the visual and auditory evoked potential (bottom graph) after 6 weeks Pb NP exposure. Mean+SD, n=10.
**, ***: p<0.01, 0.001 vs. Con; #, ##: p<0.05, 0.01 vs. W (between identical frequencies among groups at the SS EP); °, °°°: p<0.05, 0.001 (vs. 1 Hz stimulation within a group at the SS EP).

In line with the lengthened cortical latencies, the conduction velocity of the tail nerve was reduced in the treated groups (Fig. 13). There was some difference also between *Con* and *W* but the decrease in *Pb-LD* and *Pb-HD* was highly significant against both. In the frequency dependence of the conduction velocity there was, however, no significant alteration.

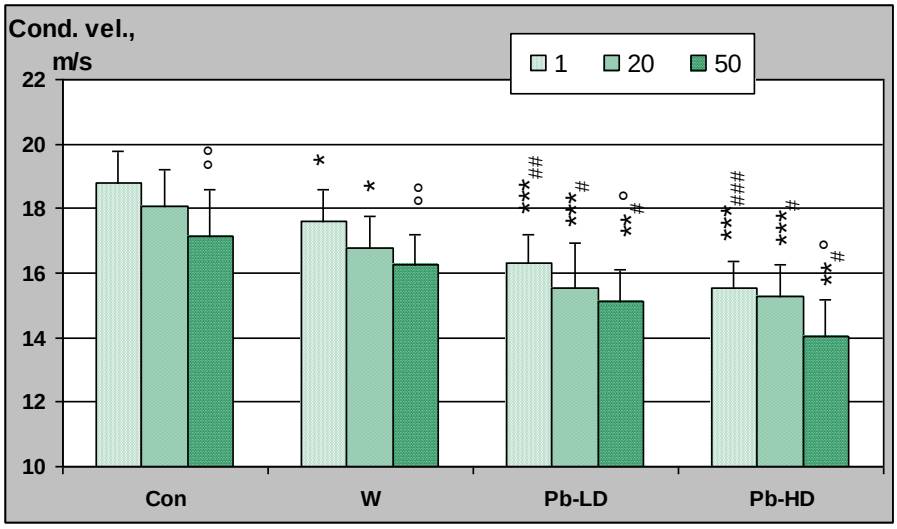


Figure 13: Conduction velocity of the tail nerve after 6 weeks treatment by Pb NPs, at various stimulation frequencies (see insert). Mean+SD, n=10.
*, **, ***: p<0.05, 0.01, 0.001 vs. Con; #, ##, ###: p<0.05, 0.01, 0.001 vs. W (between identical frequencies among groups); °, °°: p<0.05, 0.01 (vs. 1 Hz stimulation within the same group).

3.2.4. Results of chemical and biochemical measurements

Six weeks of intratracheal application of the PbO NPs resulted in massive increase of the measurable Pb content of the tested organs, and the metal levels were roughly proportional to the dose (Table VII). The levels in *W* reflect environmental background (in group *W* rats of the Mn NP experiment, the Pb levels were similar).

Pb NP treatment diminished the SOD activity in each organ included in the biochemical analysis but this change must have happened in the Mn-independent SOD fraction because the Mn-SOD activity had no similar trend. GSH level significantly increased in liver and lung, but not brain samples.

Table VII: Pb levels and oxidative stress parameters in the brain and blood samples of rats treated for 6 weeks with PbO NPs or the empty vehicle.

	Treatment groups		
Pb levels (ppb)	***W***	***Pb-LD***	***Pb-HD***
Blood	226±125	1694±290***	4012±1031***$^{\#\#}$
Brain	175±49	1056±209***	1870±443***$^{\#\#}$
Liver	80±12	2166±696***	4690±695***$^{\#\#\#}$
Lung	5418±4482	2778249±1232587**	4369691±2060069**
SOD (U/mg prot.)	***W***	***Pb-LD***	***Pb-HD***
Brain	39.57±3.59	39.03±6.55	34.58±4.79
Liver	61.99±5.71	51.80±7.91	48.61±9.65*
Lung	22.27±2.34	22.43±4.43	18.21±1.97*
Mn-SOD (U/mg prot.)	***W***	***Pb-LD***	***Pb-HD***
Brain	10.58±1.83	13.13±0.96	11.52±5.01
Liver	33.07±4.30	29.46±9.58	32.50±10.65
Lung	7.74±3.43	6.81±3.37	9.49±3.28
GSH (μM)	***W***	***Pb-LD***	***Pb-HD***
Brain	0.1034±0.0039	0.0924±0.0038***	0.0986±0.0020*
Liver	0.1909±0.0573	0.3245±0.1426*	0.4363±0.0631***
Lung	0.0928±0.0038	0.0909±0.0017	0.0996±0.0071*

Mean±SD, n=6.

*, **,***: p<0.05, 0.01, 0.001 vs. *W*; $^{\#\#}$, $^{\#\#\#}$: p<0.01, 0.001 *Pb-HD* vs. *Pb-LD*.

In Fig. 14, showing the correlation of Pb load and certain toxicological parameters, it can be seen that body weight gain and two of the electrophysiological parameters (ECoG index and nerve conduction velocity) were in quite strong and significant correlation with the Pb level in the blood or the brain. Biochemical parameters showed much weaker correlation.

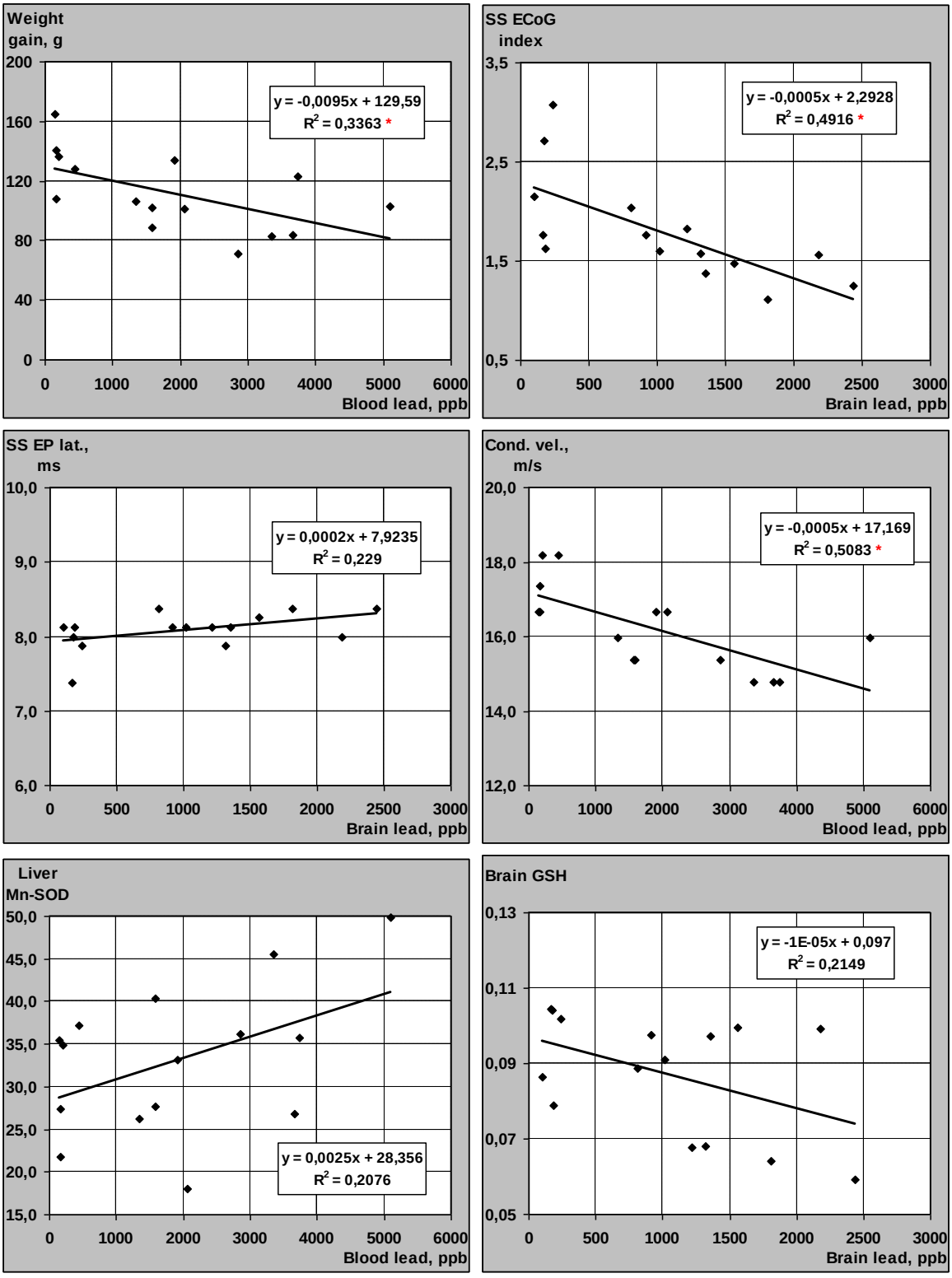


Figure 14: Correlation of the brain or blood Pb level to certain measured neuro-functional and toxicological parameters.
*: $p<0.05$ for the linear fit.

3.3. Effects of cadmium nanoparticle exposure

3.3.1. Body weight gain and organ weights

Body weight gain in the high dose treated rats (*Cd-HD*) was practically halted in the first two weeks of treatment and the difference vs. *Con* remained high during the whole treatment period. The more slowly developing lag of weight gain in the *Cd-LD* group became more pronounced towards the end of the 6 weeks.

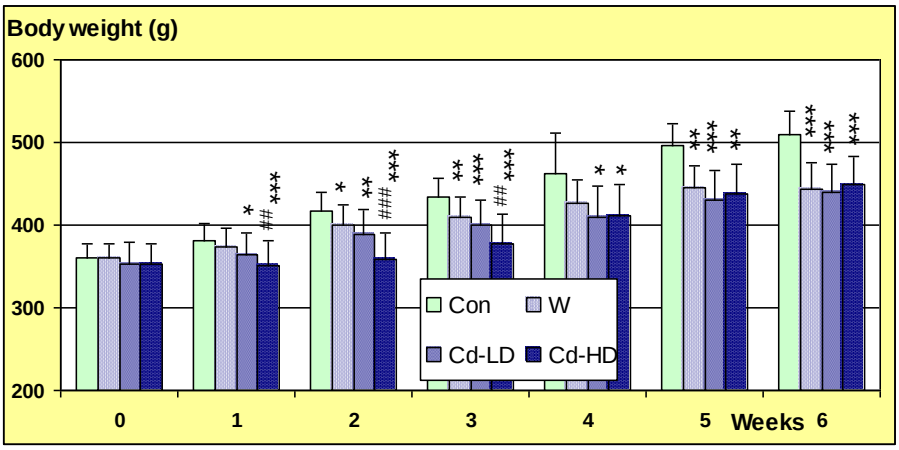


Figure 15: Body weight gain of the rats treated with Cd NPs and of the corresponding controls. Mean+SD, n=8. Insert: group codes.
*, **, ***: p<0.05, 0.01, 0.001 vs. Con; $^{\#}$, $^{\#\#}$: p<0.05, 0.01 vs. W.

From the significant relative organ weight effects (Table VIII) seen on comparison to 1/100 body weight, the increase of lung and thymus weight, and decrease of spleen weight, seemed to be real as these were seen also if brain weight was the basis of calculation. Brain weight itself was little influenced by the Cd NP treatment (*Con*, 1.278±0.054 g; *W*, 1.156±0.91 g; *Cd-LD*, 1.169±0.091 g; *Cd-HD*, 1.189±0.134 g).

Table VIII: Relative organ weights after 6 weeks intratracheal exposure by CdO_2 NPs.

Groups	***Con***	***W***	***Pb-LD***	***Pb-HD***
to 1/100 body weight				
Brain	0.431±0.021	0.484±0.030	0.479±0.031**	0.477±0.026**
Lung	0.339±0.031	0.333±0.036	0.397±0.034**	0.650±0.137***$^{\#\#\#}$
Liver	3.146±0.217	3.480±0.603	3.124±0.133	2.963±0.390
Kidney	0.607±0.044	0.651±0.050	0.625±0.069	0.657±0.068
Heart	0.251±0.017	0.261±0.024	0.267±0.018	0.264±0.024
Spleen	0.202±0.030	0.172±0.015	0.159±0.026**	0.184±0.020
Thymus	0.091±0.010	0.094±0.019	0.099±0.010	0.132±0.029***$^{\#\#}$
Adrenals	0.012±0.003	0.013±0.003	0.014±0.004	0.014±0.004
to brain weight				
Lungs	0.787±0.067	0.692±0.084	0.831±0.088	1.359±0.254***$^{\#\#\#}$
Liver	7.323±0.718	7.198±1.071	6.549±0.502*	6.198±0.595
Kidney	1.407±0.070	1.350±0.089	1.311±0.172	1.379±0.146
Heart	0.583±0.031	0.542±0.043	0.558±0.039	0.556±0.066
Spleen	0.468±0.062	0.358±0.040	0.334±0.056***	0.387±0.041**
Thymus	0.213±0.026	0.194±0.033	0.207±0.017	0.277±0.056**$^{\#\#}$
Adrenals	0.028±0.008	0.027±0.007	0.029±0.008	0.029±0.008

Mean±SD, n=10.

*, **, ***: $p<0.05$, 0.01, 0.001 vs. Con; $^{\#\#}$, $^{\#\#\#}$: $p<0.01$, 0.001 vs. W.

3.3.2. Effects on open field behavior

The Cd NP exposure applied in the experiment caused no noteworthy change in the rats' open field behavior.

3.3.3. Electrophysiological effects

After 3 weeks Cd NP exposure, there was no clear change in the band spectrum of the treated rats' ECoG. After 6 weeks, a shift to higher frequencies was seen, which was significant in the delta, beta2 and gamma bands in the *Cd-HD* group (Fig. 16).

The latency of the SS EP was significantly lengthened after 6 weeks treatment only with the high dose of Cd NP (Fig. 17), and in this group the frequency dependence was also stronger

than in the controls. The VIS and AUD EPs also had lengthened latency, in VIS in both treated groups vs. *Con*.

In the tail nerve the Cd NP exposure had no significant effect.

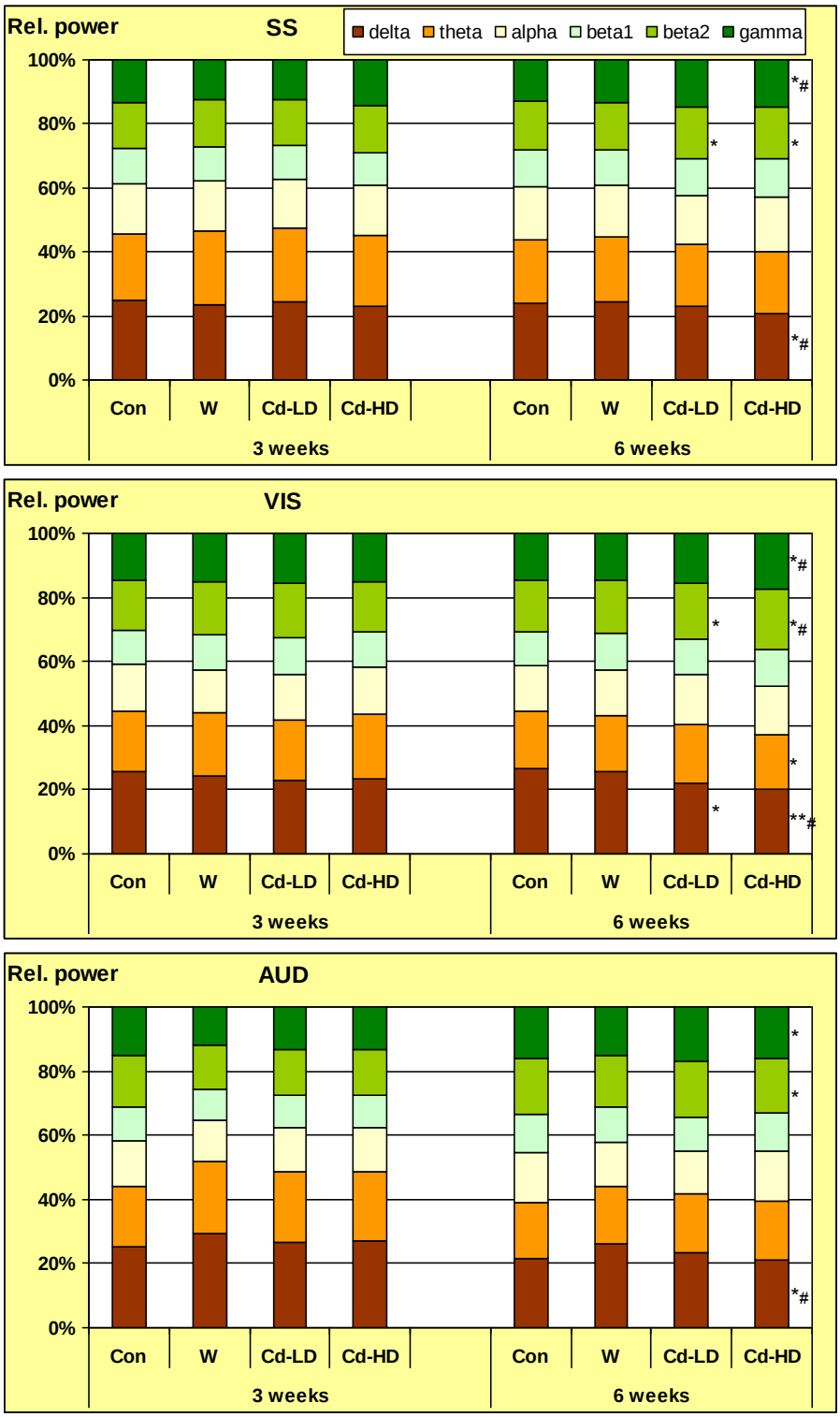


Figure 16: Band spectrum of the electrocorticogram recorded from the three cortical areas after 3 and 6 weeks Cd NP exposure. The same display as in Fig. 5.
*, **: $p<0.05$, 0.01 vs. Con; #: $p<0.05$ vs. W.

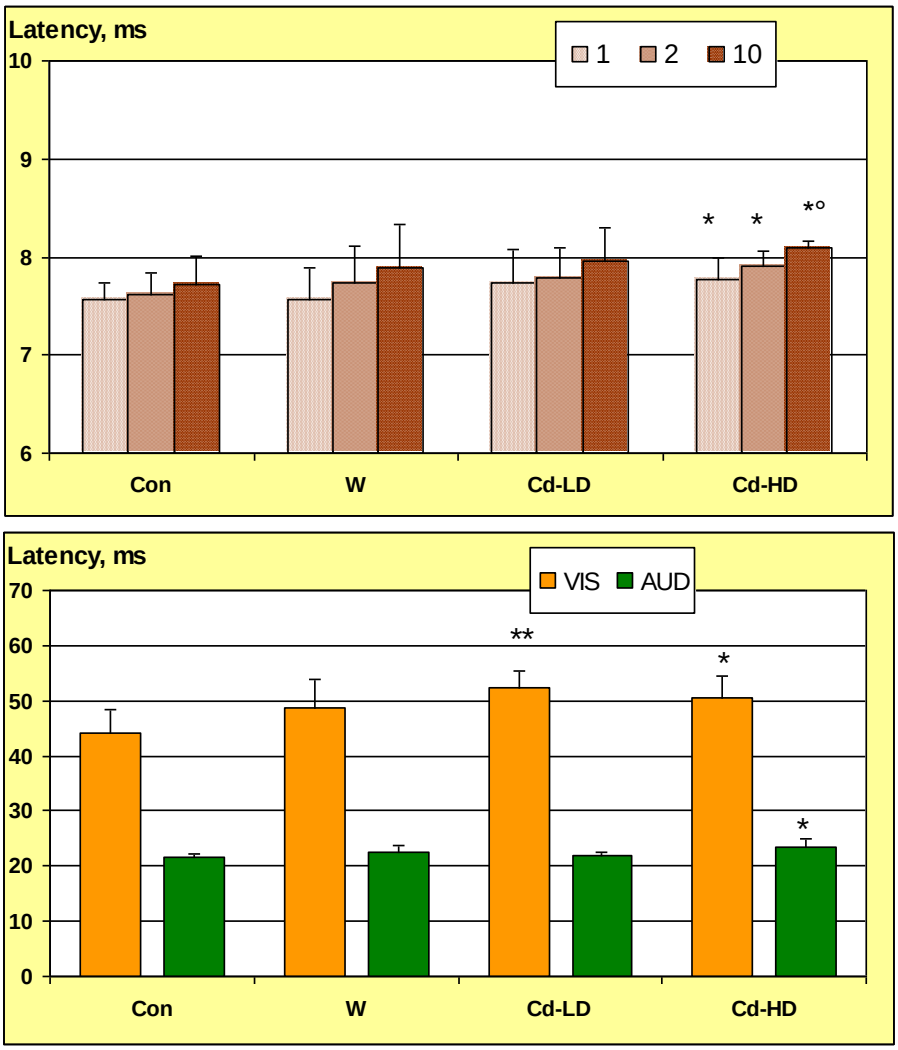


Figure 17: Onset latency of the somatosensory evoked potential at various stimulation frequencies (top graph, see insert for frequency in Hz), and of the visual and auditory evoked potential (bottom graph); after 6 weeks Cd NP exposure. Mean+SD, n=10.
*, **: $p<0.05$, 0.01 vs. Con (between identical frequencies among groups at the SS EP);
°: $p<0.05$ (vs. 1 Hz stimulation within a group at the SS EP).

3.3.4. Results of chemical and biochemical measurements

In the brain (and blood) samples, no Cd could be detected. The massive increase of lung and liver doses indicated all the same that the treatment was technically correct and effective.

Mn-SOD levels decreased in the samples from the Cd-treated rats but mostly only moderately. GSH increased in the brain and liver, but decreased in the lung samples (Table IX).

Table IX: Metal levels and oxidative stress parameters in the brain and blood samples of rats treated for 6 weeks with CdO_2 NPs or the empty vehicle.

	Treatment groups		
Cd level (ppb)	***W***	***Cd-LD***	***Cd-HD***
Brain	0	0	0
Liver	26±26	683±271**	9986±4171**$^{\#\#}$
Lung	1707±1391	43020±23904*	268399±199844*$^{\#}$
SOD (U/mg prot.)	***W***	***Cd-LD***	***Cd-HD***
Brain	43.76±6.47	43.62±9.89	44.53±10.91
Liver	62.20±8.23	63.53±11.84	61.48±11.61
Lung	24.72±4.60	23.41±2.53	22.44±6.14
Mn-SOD (U/mg prot.)	***W***	***Cd-LD***	***Cd-HD***
Brain	25.69±4.89	19.71±3.57*	18.12±10.22
Liver	46.09±7.10	48.76±10.99	40.95±10.51
Lung	16.98±7.30	15.25±5.131	11.35±5.975
GSH (µM)	***W***	***Cd-LD***	***Cd-HD***
Brain	0.0895±0.0037	0.0914±0.0075	0.1071±0.0035***$^{\#\#\#}$
Liver	0.0729±0.0040	0.0806±0.0065	0.1062±0.0020
Lung	0.2080±0.1780	0.2398±0.1335	0.1681±0.1620***$^{\#\#\#}$

Mean±SD, n=6.

*, **, ***: p<0.05, 0.01, 0.001 vs. W; $^{\#}$, $^{\#\#}$, $^{\#\#\#}$: p<0.05, 0.01, 0.001 vs. Cd-LD.

4. DISCUSSION

Investigation of the nervous system effects of NPs in rats using subacute intratracheal instillation and a set of neuro-functional tests (supplemented with chemical ones), as done in this thesis, apparently constitutes a model of human exposure and its consequences to which no direct parallel was found in the literature.

In terms of internal exposure, the model proved adequate; that is, the intratracheal application of metal NPs for several weeks caused significant increase of the Mn, Pb and Cd level in the tissue samples of the treated rats (Tables IV, VII and IX).

Comparison to levels found in exposed humans is possible only on the basis of blood metal levels. Data on airborne levels and inhaled amounts are insufficient for that, first of all because the rate of retention of the inhaled particles is usually unknown.

The blood Mn levels in our rats were, both in control and treated, much higher than levels in human samples described in the literature. But the relationship between the levels of individuals with heavy symptoms and unexposed ones was about 2 to 1 in both cases: ca. 14 μg/L in heavily exposed workers (Bader et al., 1999; Halatek et al., 2005) vs. 5–7 μg/L in reference groups (Bader et al., 1999), and 1022±206 ppb (HD-Mn) vs. 514±217 ppb (W) in our rats. The calculated airborne equivalent of the higher Mn dose, 5.26 mg/kg b.w., is ca. 10 mg/m^3 airborne Mn in continuous exposure (for the calculation, see below). This is higher than levels reported from real industrial settings (e.g. Bader et al., 1999) but the blood level in our untreated rats compared to those found in unexposed humans shows that the dose was not the only reason of the ca. 100-fold difference.

The Pb doses were originally derived from the papers by Coffigny et al. (1994) and Pinon-Lataillade et al. (1993) who obtained 500-700 ppb blood Pb levels in rats by continuous exposure to air containing 5 mg/m^3 lead oxide dust. This external dose corresponds to ca. 2.5 mg/kg b.w. per day if a daily ventilation volume of 0.5 m^3/kg b.w. is supposed (based on Strohl et al., 1997), which led to choosing 2 and 4 mg/kg dose. In actual industrial exposures, however, similar blood levels were caused by airborne concentrations much lower than 5 mg/m^3, such as 1.06 mg/m^3 Pb in fumes (probably including NPs) in a lead battery workshop and 635 ppb Pb in the exposed workers' blood (controls: 87 ppb; Jiang et al., 2008); or 0.6

mg/m^3 Pb in another lead battery plant, and up to 500 ppb Pb in the exposed workers' blood (Lormphongs et al., 2004).

The absence of detectable Cd level in the rats' brains exposed by CdO_2 NPs might be due, on one hand, to the low dose. However, based on the daily ventilation volume mentioned above, our intratracheal dose is somewhat above those found in industrial settings (ca. 30 $\mu g/m^3$, indoors in car body repair shops; Vitayavirasuk et al., 2005 – or 1-19 $\mu g/m^3$, outdoors in bridge maintenance: Conroy et al., 1995), and is in the same range as the 70 and 550 $\mu g/m^3$ used by Takenaka et al. (2004) in a rat inhalation experiment. The internal dose arisen in the liver (and not only in the lungs) of the Cd NP treated rats showed that the NPs and/or their Cd content were absorbed – but were not transported to the brain. Rapid clearance of Cd from the blood to the liver and kidney in rats after inhalation has been described (Dill et al., 1994), and it is also known that under healthy conditions the entry of Cd into the brain is minimal, and the gradual breakdown of the blood-brain barrier under Cd effect needs long time (Clark et al., 1985). That in the Cd-treated rats several of the tests used for detecting nervous system effects showed no change supports all the same that the Cd level in the treated rats' brains was very low indeed.

Compared to the results of earlier works of the Department applying oral administration of heavy metals, it seems that administration of NPs into the trachea was – at least in case of Mn and Pb – especially efficient in inducing increased metal levels in tissue samples. In the present work PbO NPs (4.0 mg/kg into the trachea for 6 weeks) resulted in ca. 1870 ppb brain Pb level whereas orally by gavage a dose of 500 mg/kg Pb-acetate was given for 10 weeks to achieve ca 2500 ppb (Vezér, unpublished). In case of Mn, 5.26 mg/kg. b.w., instilled in NP form generated ca. 7000 ppb Mn level in the brain, compared to ca. 290 ppb after 10 weeks oral exposure by the tenfold dose (59 mg/kg b.w.; Vezér et al., 2005). There are two basic mechanisms by which the instilled NPs can generate increased metal levels in various organs. NPs – unlike bigger, microscopic particles – translocate readily to extrapulmonary sites and reach other organs by transcytosis across the respiratory epithel into the interstitium, and access the blood circulation directly or via the lymph drainage (Oberdörster et al., 2005). From the blood, NPs enter e.g. the brain through the capillary endothelial cells in the blood-brain barrier, and through the choroid plexuses (Crossgrove et al., 2005). NPs of various

compositions were, indeed, detected in the brain after application to the airways of rats (Kreyling et al., 2006).

Alternatively, the metal content of the NPs can be dissolved in ionic form from the surface of the particle (Handy et al., 2008). MnO_2, CdO_2 and PbO are water insoluble in bulk but will dissolve after phagocytosis by the alveolar macrophages, in the acidic environment (ca. pH 4.5) of the phagolysosomes (Lundborg et al., 1985). The released ions then can e.g. pass the blood-brain barrier.

The qualitative similarity of the majority of neuro-functional alterations seen in rats treated with the three different metal NPs suggest that common mechanisms of action exist. These can be first of all oxidative stress, interference with Ca-dependent phenomena, and effects on transmitter systems.

The ability of Mn-containing welding fumes to induce oxidative stress was proven in vivo (inflammation markers in the bronchoalveolar lavage fluid) and in vitro (depletion of glutathione) by McNeilly et al. (2004), who also stated that the soluble metal content was responsible for this effect. CdTe NPs (quantum dots) are cytotoxic (Zhang et al., 2007) and Cd increases oxidative stress by enzyme inhibition (Casalino et al., 2002). Metabolites indicating oxidative damage and induction of antioxidant enzymes upon Pb exposure were detected both in animals (Adonaylo and Oteiza, 1999) and in humans (Ahamed and Siddiqui, 2007). Even the reduced body weight gain observed in the treated animals might be due to oxidative stress, more exactly to the metabolic disturbance caused by the presence of free radicals (Merry, 2002).

In case of all three metals, ROS generation was detected in the brain (Mn: Zhang et al., 2010; Cd: Kumar et al., 1996; Pb: Patra et al., 2001) resulting in membrane lipid peroxidation (Mn: Avila et al., 2008; Cd and Pb: Zanchi et al., 2010; Pb: Ahamed and Siddiqui, 2007). Damage to these important functional units of the cell membrane results, in turn, in changes of fluidity and probably in altered neuronal membrane functions. That will then disturb a number of processes, including all receptor-bound phenomena, such as synaptic transmission, which have some likelihood to be reflected e.g. in various forms of cortical activity. For Pb, altered activity of the neuron membrane bound enzymes acetylcholinesterase and monoamine oxidase, in parallel with altered membrane fluidity, was reported by Flora and Seth (2000), however, no specific relationship of the two was mentioned. Kumar et al. (1996) described

membrane damage and increase of intracellular Ca^{2+} on Cd treatment of rats but without further functional implications. Also in a paper on the effect on rats of traffic-generated dust, containing Pb and Cd, (Zanchi et al., 2010) impaired short-term memory and oxidative stress as its possible cause was just mentioned. In our experiments, as shown by Figures 8 and 14, the measured oxidative stress parameters were much less strongly associated to the metal levels than the neuro-functional parameters so that, except for the significant correlation of ECoG index and brain GSH level in the Mn-treated rats, any connection between the two could not be directly demonstrated.

Among the electrophysiological phenomena recorded and analyzed in this thesis, cortical evoked potentials can reflect alterations in synaptic transmission (a likely consequence of membrane damage, see above) the most directly. The increased latency observed in the rats treated with each of the metal NPs might be, at least partly, due to decreased synaptic efficiency.

Pb^{2+} ions block the voltage-gated Ca-channels in presynaptic endings, impeding this way intracellular Ca^{2+} rise and depolarization on arrival of an axonal discharge, and preventing synchronous emptying of vesicles. Inside, however, Pb^{2+} ions can activate a number of Ca-dependent processes including those involved in exocytosis, so the spontaneous release of the transmitter will be more likely; and the same has been reported also for Cd^{2+} ions (Suszkiw et al., 1984). For Mn^{2+}, a similar mechanism (blocking of membrane Ca-channels and increase of intracellular Ca release at the same time) was reported by Kita et al. (1981).

The turnover of the main excitatory transmitter of the CNS, glutamate, is another site of action of metal xenobiotics. Having fulfilled its role, glutamate is taken up and converted to glutamine by the astrocytes. On action of Mn^{2+}, both the uptake (Erikson and Aschner, 2003) and the transformation to glutamine (Normandin and Hazell, 2001) is reduced. The transporter (astrocytic GLT-1) is inhibited also by Pb^{2+} (Struzynska et al., 2005), and Cd^{2+} (Liu et al., 2008). Excess glutamate may desensitize the postsynaptic receptors leading to weaker and/or slower postsynaptic excitation (and, hence, to smaller/slower cortical evoked potentials as it was observed indeed) and may also exert excitotoxicity (Coyle and Puttfarcken, 1993).

Excess glutamatergic activity could have also contributed to the observed increase of cortical activation (shift of the ECoG to higher frequencies), via the glutamatergic collaterals from the specific afferents to the ascending reticular formation. The activating modulatory effect of the

latter is cholinergic and the studied metals are known to affect the release of ACh (stimulus-evoked release is decreased but spontaneous release is increased, as described above).

The decrease of conduction velocity in the tail nerve, observed in Mn- and Pb-treated rats, was in line with the lengthened latency of the cortical EPs and was probably caused by the metal ions' effect on the channels involved in action potential propagation and/or by energetic shortage due to mitochondrial inhibition. First of all the increased sensitivity to frequent stimulation, seen primarily on the somatosensory cortical EPs (increased difference between identical parameter of the response measured at 1 Hz vs. at much faster stimulation), suggested the latter. Mn is a well-documented mitochondrial toxin, acting on complex II (Malecki, 2001) and complex III (Zhang et al., 2003). The mitochondrial damage by Pb was described by Lidsky and Schneider (2010). The energetic shortage could have an effect also on the basal cortical activity (ECoG) – but that would have been probably slowing (as in cases of mitochondrial encephalopathy: Smith and Harding, 1993) and we have seen no such change (it may have been present but covered by the above-described opposite effect of increased reticular activation).

The changes in open field motor behavior, also observed in the Mn- and Pb-treated rats, showed effects on several cortical systems, most notably the dopaminergic one. Motivation, determining spontaneous open field locomotor activity, is regulated by mesolimbic/mesocortical dopaminergic structures (Alexander et al., 1990). Dopaminergic neurons are especially vulnerable to oxidative stress due to the auto-oxidizing tendency of dopamine and to the presence of monoamine oxidase producing hydrogen peroxide (Alexi et al., 2000). The tendency of all three metals (including Cd with which we failed to detect an effect on motor behavior) to induce oxidative stress is well known and was mentioned above as a common point in their effects on the nervous system. Vertical motility in particular is a sensitive indicator of striatal dopaminergic activity (Sedelis et al., 2001), and diminished rearing was observed in the rats exposed both to Mn NPs (Fig. 4) and Pb NPs (Fig.10). In case of Mn, also horizontal movements were reduced which fits well in thee picture of general hypomotility, a phenomenon possibly analogous to what has been described in welders suffering from the Mn-dependent Parkinson-like syndrome (Bowler et al., 2006). The increased locomotion of the Pb-treated rats is analogous to what was reported by Ma et al. (1999) in rats following Pb exposure during intra-and extrauterine development, and was

explained by decreased cortical D2 receptor level. A possible human analog is “attention deficit hyperactivity disease” found to be more frequent among children with elevated blood Pb (Needleman and Gatsonis, 1990).

The results of this work can be summarized, and the questions listed in 1.6. be answered, as follows:

- Intratracheal instillation of the suspension of metal (manganese, lead and cadmium) containing NPs proved technically possible.
- Significant internal exposure developed after 6 or 9 weeks of instillation, indicated first of all by the metal level of the treated rats’ brains; although the Cd content of the NPs was detected only in the lungs and liver, but not the brain of the animals.
- Functional alterations in the rats’ nervous system were in fact observed. The cortical evoked potentials were the most sensitive, altered significantly after Mn, Pb and Cd exposure. Spontaneous cortical activity, peripheral nerve conduction velocity as well as open field behavior was significantly altered by Mn and Pb, but not by Cd.
- Oxidative stress could be detected in the treated rats, although the changes in superoxide dismutase activity and reduced glutathione level were moderate.
- In the Mn- and Pb-treated rats – where significantly elevated brain and blood metal levels were measured – correlation with neuro-functional alterations could be shown, and it was in several cases significant. The measured oxidative stress parameters were, however, much less strongly associated to the metal levels, so that no direct relationship of oxidative state and functional alterations could be shown, except for the significant correlation of ECoG index and brain GSH level in the Mn-treated rats. Cd application failed to cause measurable metal load in the brain and blood, so the correlation could not be studied, although some neurophysiological and biochemical effects were clearly present.

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7. APPENDIX

Oszlánczi, G., Vezér, T., Sárközi, L., Horváth, E., Szabó, A., Horváth, E., Kónya, Z., Papp, A.: Metal deposition and functional neurotoxicity in rats after 3 to 6 weeks nasal exposure by two physicochemical forms of manganese.
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