PhD Thesis

NEUROTOXICITY OF A MODELLED COMPLEX ENVIRONMENTAL HEAVY METAL EXPOSURE IN RATS

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The Applicant's Relevant Publications

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Abstracts:

Horváth Edina: Funkcionális idegrendszeri elváltozások ólom különböző formáival kezelt patkányokban. *MHT Ifjúsági Tagozatának VI. Fóruma; Debrecen, 2010.* Összefoglalók. 30. old. **Egészségtudomány, LIV(2), p. 105 (2010)**

Horváth E., Oszlánczi G., Máté Zs., Takács Sz., Szabó A., Vezér T.: Magatartási és elektrofiziológiai hatások oldott és nanopartikuláris állapotú kadmiummal szubakutan kezelt patkányokban. *MÉT LXXI Vándorgyűlése; Szeged, 2010.* Programfüzet 130. old. **Acta Physiologica Hungarica, 97(4), p. 445 (2010)**

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SUMMARY

Metals are major environmental pollutants due to the long periods of use (in case of lead, from ca. 3500 BC on) and the immense amounts produced. Many metals are xenobiotics because they used to have minimal presence (and, hence, bioavailability) before man-made emission into the environment had begun, and because they either are completely useless and toxic for the human organism (e.g. mercury, lead or cadmium) or are essential micronutrients but toxic when overdosed (manganese, chromium, copper, etc.).

Metal-containing dusts and fumes are generated along the complete life cycle of metal articles and are found in the workplace atmosphere. Airborne metals cause primarily inhalational exposure, the extent of which is influenced by the chemical form of the metals and the particle size. Environmental nanoparticles (NPs) have important health effects on their own. Their submicroscopic size and large specific surface area, together with the high numbers of NPs entering the organism in a typical exposure situation, result in great biological activity. Metal ions dissolved from the surface of NPs also must be considered among the mechanisms of action.

The second most important route of exposure to heavy metals is probably ingestion. Dust can contaminate food or drink. Further, incorporation and accumulation of toxic metals from the soil is characteristic for numerous plants, including ones used for staple food production, the case cadmium content in rice being an example.

Regarding the multitude of applications of metals, the broad spectrum of heavy metal toxicity and its consequences, as well as the modes of their possible entrance into the organism, it is of paramount importance study this problem further on, among others by animal experiments based on more and more adequate models. Based on practical importance and on previous experiences of the Department, lead, cadmium and manganese were chosen for the work subserving this thesis.

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. Airborne Pb causes significant internal exposure both in humans and in experimental animals, and the harms of Pb ingested with contaminated food is well-known. Its human nervous systems effects include encephalopathy, diminished learning ability and behavioral problems in children. 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. Cereals, especially rice, also tend to accumulate Cd from the soil, resulting in foodborne exposure. 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. Oral or parenteral overdosing of Mn can induce the same symptoms. Disorders with electrophysiological signs after Mn exposure include e.g. myoclonus in welders and epileptic activity in an accidentally exposed child.

In previous experiments it was found that recording and analysis of central and peripheral electrophysiological signals, and of certain behavioral phenomena, is sufficiently sensitive to detect the effects of lead, cadmium and manganese on the nervous system of rats, applied subacutely in dissolved form orally, or in the form of nanoparticles by intratracheal instillation. In order to obtain a more adequate model, these two forms of metal application were combined in the present work to imitate exposure coming both from environmental (food/waterborne) and occupational (inhalational) sources.

The aims of the work were specified in the following questions:

- Are the treatment schemes and doses used previously for application of manganese, lead and cadmium in dissolved form orally, and in nanoparticulate form intratracheally, usable also in combination exposure?
- What quantitative and qualitative differences can be observed in the general toxicological, electrophysiological and behavioral changes obtained by applying the mentioned metals only in dissolved oral form, only in nanoparticulate intratracheal form, , and in combining these forms of application?
- Is the dose-response relationship in oral-only, intratracheal-only, and combined application different?

Experimental work was done on young adult male Wistar rats. The three metals were administered to the rats (10 animals per group) in two physicochemical forms and two ways. Aqueous solution was given orally by gavage (per os, po.), while the suspension of NP form was instilled in the trachea (intratracheally, it.). The chemical identity and dose of the substances applied to the rats was as follows:

Metal	Compound	Doses (mg/kg b.w.)
Manganese		
dissolved form	$MnCl_2 \cdot 4H_2O$	15, 60
nanoparticulate form	MnO_2 (Ø 30.9±9.91nm)	2.63
Cadmium		
dissolved form	$CdCl_2 \cdot 2.5H_2O$	3.5
nanoparticulate form	CdO_2 (Ø 65.6±12.4 nm)	0.04
Lead		
dissolved form	$Pb(CH_3COO)_2 \cdot 3H_2O$	80, 320
nanoparticulate form	PbO (Ø 38.9±7.20 nm)	2

The doses were based on previous experience. For po. application, the compounds were dissolved in distilled water to 1 ml/kg b.w. administration volume and were given by gavage. The NPs were synthesized at the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics. For it. administration, the NPs were suspended in 1% hydroxyethyl cellulose (HEC) dissolved in PBS (pH 7.4), and instilled in the trachea of rats under brief diethyl ether anesthesia.

The po. doses were given once daily, 5 days per week, for 3 and 6 weeks (Mn and Pb) or for 3 weeks (Cd). One half of the rats underwent then the behavioral and electrophysiological investigation described below while the others had subsequent it. administration of NPs, again for 3 and 6 weeks (Mn and Pb) or for 1 week (Cd). There was another Cd- treated group receiving NPs first. For each treatment variation, untreated and vehicle-treated controls were also made. Cd treatment necessitated a different scheme because of too high general toxicity.

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 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. 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. 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.

Body weight gain was reduced by all three metals. With Mn and Cd, the effect of the NP form on body weight gain was disproportionately strong, regarding the dose applied, much stronger than the effect of the dissolved form and also stronger than that of the NP form given alone in previous experiments. The relative weight of the liver (on the basis of brain weight) was decreased by the NP form of Mn and by both forms of Cd. Lung weight was massively increased by it. application of each metal. In the OF test, ambulation and rearing was decreased by Mn, and the changes were stronger after 6+6 (po., then it.) than after 6 weeks treatment. The Pb-treated rats showed more overall time and longer periods of ambulation, but less rearing. Also Cd caused reduced OF motility, most efficiently in the po.+it. scheme.
In the ECoG, 6 weeks of po. Mm application caused a shift to higher frequencies. This change was not mad more intense by subsequent it. application. Orally applied Pb increased the slow and decreased the fast waves in the ECoG but in rats with po.+it. application this change was no more observed. Cd had no significant effect on the spontaneous cortical activity.
The cortical EPs generally showed latency lengthening on the effect of the metals. Pb also caused increased extra lengthening of the latency on higher frequency stimulation (10 vs. 1 Hz). It was conspicuous that the effect on the SS and VIS EP latency of Mn and Pb was about as strong after 3 weeks po. plus 3 weeks it. as after 6 weeks po. administration, although the summed dose was much lower in the former case.
The conduction velocity of the tail nerve was decreased by Mn, Pb and Cd, and the relative refractory period increased by Mn and Cd. The anomalous dose dependence seen on the EP latency was present also on the nerve conduction velocity in case of Mn.

Changes observed during the experiments described in this thesis suggested on several instances that there can be a more-than-additive interaction between the amounts of heavy metals given by po. and it. application. This can be due to the blood-brain barrier weakened by the op. given metal, being less able to exclude NPs, but the extreme mobility of NPs itself can result in higher metal levels in the CNS. The role of oxidative stress induced by the metals ond/or the NPs containing them must also be considered, and is one of the likely common mechanisms explaining the similar character of alteration induced by the three metals studied. Further such mechanisms are interference with Ca-dependent phenomena and with mitochondrial energy production.

Based on the results described and evaluated above, it can be stated that the attempt to model the complex exposure, coming both from environmental (food/waterborne) and occupational (inhalational) sources, was successful. And, investigation of general and nervous system effects of toxic environmental heavy metals in a combined exposure (dissolved form po. plus NP form it.) by means of a set of neuro-functional tests is apparently a model to which no direct parallel was found in the literature.

Having in mind the presence of xenobiotic heavy metals in the (occupational and residential) environment and in commodities of environmental origin (drinking water and food) the health effects in general, and in particular the effects on sensitive systems like the nervous system, are of primary concern. The occurrence of metals in nanoparticulate form, adds a new feature to the old problem.

The questions listed above can finally be answered as follows:

- The treatment schemes and doses used previously for oral and intratracheal application could be adapted without significant change in case of Mn and Pb treatment. In case of Cd, strong general toxicity required the development of a new scheme with shorter it. exposures. The doses, although caused comparable alterations in the electrophysiological (and partly in the behavioral) parameters, were at the systemic level not “equitoxic”.
- In quantitative aspect it became clear that the NP form of the metals, applied after weeks of oral exposure to the dissolved form, had disproportionately strong effect on the body weight gain (Mn, Cd) and on some of the open field behavioral parameters and on parameters of evoked electrophysiological responses (all three metals). Qualitative difference was seen mainly in the electrocorticograms, which possibly reflected the interference of NP-specific (oxidative stress) and metal-specific (altered synaptic transmission etc.) actions. Comparison with earlier intratracheal-only results also indicated that lower NP dose was enough to evoke the same effects when applied after oral treatment.
- The differences in dose-response relationship could be examined only in terms of the external dose. Measurements of internal dose (tissue metal levels) constitute the necessary next step of the studies. In case of or al application of the metals in aqueous solution, thee effects were of similar kind and magnitude as seen earlier. It was known from the preceding intratracheal NP exposures that in this form much lower per kg doses are sufficient in terms of internal dose and functional effects. Comparison of the results from the present study to those mentioned above indicated that a lower NP dose was enough to evoke the same effects when applied after oral treatment.

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ABBREVIATIONS

AUD	auditory
BBB	blood-brain barrier
C	untreated control
CNS	central nervous system
ECoG	electrocorticogram
EP	evoked potential
H	high dose
HEC	hydroxyethyl cellulose
it.	intratracheally
L	low dose
NP	nanoparticle
OF	open field
PBS	phosphate buffered saline
po.	per os
SOD	superoxide dismutase
SS	somatosensory
VC	vehicle control
VIS	visual

1. INTRODUCTION

1.1. General properties of metals. Heavy metals in history and human culture

The majority of the chemical elements belong to the metals. Metals have good heat and electric conductivity, metallic shine, and more or less plastic deformability. Most metals are solid at room temperature. Electric conductivity and shine are due to the loosely bound electrons on the outer shell of metal atoms which are easily delocalized. These electrons are also lost in chemical reactions so that metals themselves are typically oxidized while reducing the other reacting substance. Metal atoms with more than one oxidation state, e.g. iron and manganese, tend to be involved in redox interactions which, under protoplasmic conditions, may result in the generation of oxidative free radicals. To achieve octet state, numerous metal atoms tend to form coordinate bonds with partners having a lone electron pair. Of the complexes and chelates formed this way, many – e.g. metallothioneins – fulfil biological functions.

It is customary to group metals into light and heavy ones. This was originally based on density, and those with a density over 5 g/cm^3 were regarded as heavy metals, but this categorization has been oft times superseded by other aspects including toxicity, resulting in confusion (Duffus, 2002).

When prehistoric peoples started to use metals, it meant an unprecedented impact not only on technological development and life circumstances but also on the environment and their own health. Stone Age lasted until 4000 BC, when the use of copper (the first metal to be utilized by mankind) appeared and the cooper era began. Copper can be found in the nature, although rarely, in “native form” so using copper did not absolutely require the use of fire. All other metals are, however, found only in their ores, in form of compounds formed with oxygen, sulfur or other elements. Obtaining these metals required smelting them from the ores in fire, which was on one hand a substantial technical achievement but on the other hand a source of human exposure and environmental pollution. The exploitation of lead ore steadily increased in the antiquity, and at the Roman Empire’s heyday, lead production reached 80,000 tons/year. The symptoms – loss of appetite, fatigue, lead colic, irritability, nervous spasm – seen in lead-mine workers were documented by Hippocrates and are described the same way even today. The environmental effects were not recognized in those times but the emissions

from ancient mines and smelters can today be identified (Breitenlechner et al., 2010; Thevenon et al., 2011).

Around 3500 BC, lead was discovered, and around 3000 BC, tin. Tin and copper were melted together to bronze so that the period between 3000 BC and 800 BC is called the Bronze Age. Finally, in ca. 800 BC, iron was discovered and the list of metals with major practical applications was complete for centuries. Lead and its compounds were widely used in cosmetics, for preserving foodstuffs, or to prevent fermentation of the wine. Lead-containing alloys were made to “tin” cups, plates, pots and coins. This would be inconceivable today, for the known toxicity, but the human health effect was not obvious at that time and the “food-preserving” effect of metal kitchenware, due to the antimicrobial action of the dissolved metals, was appreciated. Romans e.g. liked to keep wine in lead pots because it became sweetened by the lead acetate (called for long lead sugar) generated in the interaction of the wine (that must have been rather sour, with so much acetic acid present) with the wall of the vessel. Lead was also used in building, in shipbuilding, and in the famous Roman water supply system. There are studies stating that the decline of the Roman Empire was promoted by their aristocracy’s chronic lead intoxication via wine and aqueduct water (Eaton and Robertson, 1994).

Mercury (for us, another notoriously toxic metal) was also popular in the antique and medieval times and was amply used in beauty products and medicines of those ages, but these often proved to be rather harmful. The first Chinese emperor, Qin Shi Huang-Di, died in multi-organ failure after drinking a liquid mercury preparation promising „eternal life”. Cinnabar (HgO, the main ore of this metal) is still in use in traditional Chinese medicine.

Today’s rapidly growing industry and agriculture is almost forced to use ever newer materials, including metal-based ones. The resulting chemical risk, manifested in environmental and human health damage, is present even today, in spite of the more and more strict regulations which have been evolving from the numerous negative lessons of past centuries. Xenobiotics – substances which are “alien” for the metabolism, which can be neither utilized nor neutralized by the organism in question – are found in soil, groundwater, drinking water, air and, consequently, in plants and animals. In this aspect, a lot of metals are xenobiotics because they used to have minimal presence (and, hence, bioavailability) before man-made emission into the environment had begun, and because they either are completely useless and toxic for the human organism (e.g. mercury, lead or cadmium) or are essential micronutrients but toxic when overdosed (manganese, chromium, copper, etc.). Regarding the multitude of applications of metals in our days, the broad spectrum of toxic effects of heavy

metals (see below) and their consequences; as well as the varieties of their possible entrance into the organism, it is of utmost importance to learn more about this problem, among others by animal experiments based on more and more adequate models.

1.2. Toxicity of heavy metals and nanoparticles: general aspects

1.2.1. Typical forms and ways of heavy metal exposure. Importance of nanoparticles

Exposure to heavy metals is mostly occupational. Metal-containing dusts and fumes are generated along the complete life cycle of metal articles, from ore mining through smelting and final product manufacturing to waste management and recycling, and are found in the workplace atmosphere, sometimes at hazardous concentrations. Airborne metals cause primarily inhalational exposure, the extent of which is influenced by numerous factors including the type (nasal/oral), volume and intensity of breathing on the exposed organism's side, and on the other side by the chemical form in which the metal is present in the particles (solubility, etc.) and the particle size. According to particle diameter, one can speak of sedimenting dust (>10 µm), suspended or fine dust (100 nm-10 µm; often called PM10) and ultrafine dust or nanoparticles (NPs, <100 nm). Traditionally, PM10 received especial attention because this "thoracic" fraction can reach the alveoli by inspiration and cause toxic alveolitis, and because they can cause systemic exposure due to particle-laden alveolar phagocytes being transported by lymph, then blood, circulation to other body parts. Larger grains are, on the contrary, trapped in the upper airways.

However, as soon as it became technically possible to investigate the origin and environmental presence of NPs and their interaction with living organisms, they turned out to have important health effects on their own. All high-temperature industrial processes – smelting, casting, welding, etc. of metals (Antonini et al., 2003) or plastics (e.g. Teflon: Seidel et al., 1991) – can generate NPs, and various combustion processes are not less important NP sources. NPs are emitted as primary (pre-formed) particles or are generated from gaseous precursors in an atmospheric chemical process called nucleation. Their composition is determined by the composition of the materials worked with or burnt. So, in the heavy metal exposure of the general population the metal content of petrol – lead (largely phased out, in Hungary since 1999: Kertész et al., 2001) or manganese (still in use as lead supplement, to increase octane rating, in a few countries: Davis, 1998) – played a major role

because most of the metal left the engine with the exhaust gases in form of microscopic and submicroscopic oxide particles.

Intentionally manufactured nanomaterials (that is, not the nano-sized byproducts from other industrial processes) contain at least one component that has at least one dimension in the 1-100 nm range. Nanomaterials may add to the load of primary NPs in the workplace atmosphere. Application of NPs and nanofibres in consumers' commodities (personal care products, household chemicals, electronics, tires, etc.: Oberdörster et al., 2005) means that routes of uptake such as ingestion and dermal absorption must be considered in addition to inhalation.

On inhalation, particles are deposited at different section of the airways, determined first of all by their size. NPs are either deposited in the nasopharynx or get down to the alveoli (ICRP, 1994). A fundamental difference against microscopic and larger particles is that NPs are not held back by the usual biological barriers like the alveolar and capillary wall, and reach other target organs, beside being phagocyted like the PM10 fraction, by different transfer routes and mechanisms. One such mechanism is transcytosis across epithelia of the respiratory tract into the interstitium (Oberdörster et al., 2005) by means of caveolae (50-100 nm sized invaginations of the cell membrane, serving endo- and transcytosis of a number of molecules and microstructures: Razani and Lisanti, 2001) formed around NPs. Having crossed the alveolar and capillary membrane, NPs are distributed throughout the body by the circulation (Oberdörster et al., 2005).

The NPs' small size and large specific surface area, together with the high numbers of NPs entering the organism in a typical exposure situation, result in great biological activity. In vivo and in vitro toxicological studies confirmed that even relatively inert materials become more toxic and inflammatogenic in NP form. It was found, e.g., 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). "Titanium white" as pigment is normally harmless enough to be applied in the coating of tablets. The mentioned problem is general, concerning both nano-sized environmental pollutants and nanotechnological products.

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 is supposed to prevent foreign particles from entering the brain; NPs of various compositions were, however, detected in the brain of rats after application through the airways (Kreyling et al., 2006). Such extrapulmonary effects of NPs depend on several factors including particle solubility, particle or aggregate size, the site of deposition, and the integrity of the alveolar epithelial lining

(Elder et al., 2006). 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. The applicant's results with Cd exposure supported the interpretation that the blood-brain barrier may be weakened by the toxic metal ions and so will be more permeable for NPs (Horváth et al., 2011)

Apart from inhalation, the second most important route of exposure to heavy metals is probably ingestion. Airborne particles can be ingested if expectorated airways secretions are swallowed instead of being spat. Dust can contaminate food or drink, e.g. under unhygienic eating conditions at the workplace or if dust particles are captured on the surface of raw-eaten vegetables and fruits that have not been washed adequately. The settled particles' metal content can also be directly incorporated into the edible parts of the plants, or soil pollution by the particles can result in toxic metals being absorbed from the soil to the plants.

Both mechanisms were evidenced by the samples taken near to the defunct metal reprocessing plant "Metallokémia" in Budapest. This plant was reprocessing copper, zinc, lead and other metals from 1908 on. Lead smelting was stopped in 1977 but other metals were processed until the end of activities in 1990 and the byproducts (slag containing heavy metals) were stored on the plant's premises further on (Pápay and Horváth, 1992). Windblown dust was settling on the soil and on plants grown in the local residents' gardens. Downwind, near the factory, more than 1000 mg/kg Pb was in the upper soil, 1000 m farther away it was 100 mg/kg, at 2000 m, 50 mg/kg. Cd level near the plant was 3-6 mg/kg. Pb content of the groundwater was three times, and Cd content eight times, the allowable limit (Szabó, 1991). Leafy vegetables contained Pb over the limit in 40-50%, root vegetables in 8-50%, and various fruits in 24-40%. Pb in the soil and in the produces was loosely correlated, but for Cd levels the correlation was robust. The local children's blood Pb level was higher if they lived nearer to the factory; and among those living within the 500 m radius, eating or not eating home-grown fruits or vegetables had a considerable influence on blood Pb (Groszmann et al., 1992).

Incorporation and accumulation of toxic metals from the soil is characteristic for numerous plants, including ones used for staple food production, the case cadmium content in rice being repeatedly cited (Shigematsu, 1984).

From the intestines, most heavy metals are absorbed to around 10%. Common metal transport mechanisms, responsible for the uptake of essential metals, are involved, which explains why individuals with calcium- or iron-deficiency absorb more of the toxic metals. Children,

requiring much calcium for bone growth, can absorb 50% of the ingested lead while adults absorb not more than 15% (Järup, 2003). Toxic metals are transported as “dead-heads” not only from the intestine to the blood but also from the blood through the blood-brain barrier to the CNS. Trivalent metal ions, like Mn^{3+} or Al^{3+}, use transferrin to pass the blood-brain barrier; aluminium being the culprit for “dialysis dementia”, a kind of brain damage seen in dialysed patients.

The dermal absorption route is less well described, but presence of soil particles in inguinal lymph nodes of persons who usually walk barefoot on the soil indicate that this is possible (Oberdörster et al., 2005). Also some viruses, being NPs by their size, can cause transdermal infection.

1.2.2. General mechanisms of heavy metal toxicity

Heavy metals exert a profound action on living matter, affecting the growth, metabolism, morphology of cells.

Denaturation of proteins is one of the general damaging effects of heavy metals. The metal ions interact with ionized moieties of amino acids in the polypeptide chain and disrupt the non-covalent polar and ionic interactions which stabilize the secondary (or higher order) structure of the protein. The covalent link provided by disulfide bridges is also attacked by numerous heavy metal ions. Lead and cadmium have an especial affinity to sulfur, even their most abundant ores are sulfides. This chemically-based destructive effect of heavy metals on the most essential constituent of all living organisms – that is, a type of toxicity – can also be exploited as a means of antimicrobial protection (e.g., in Credé’s solution and in organomercurial antiseptics), but for the purposes of this thesis the metals in question are regarded as human (neuro)toxicants.

Denaturing an enzyme protein means elimination its biocatalytic activity. Glutathion-S-transferase was, e.g., reported to be inactivated by lead (Daggett et al., 1997) or lead and cadmium (Planas-Bohne and Montserrat, 1992). This also exemplifies that disturbed redox balance and oxidative stress is another frequent consequence of heavy metal exposure. Some of the toxic heavy metals (iron, chromium, manganese) are capable of redox cycling while others (e.g. cadmium) affect cellular antioxidant defence (Valko et al., 2005). Apart from that, all NPs, with or without toxic metal content, tend to be absorbed to organic macromolecules (possibly resulting in altered functionality) and to induce oxidative stress (Li et al., 2003). 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., 2007; Stone et al., 2007).

It has long been known that many cellular functions (among others, muscle contraction, exocrine and endocrine secretion) are regulated by local changes in Ca^{2+} concentration. Increased intracellular Ca concentration is essential for intracellular enzyme activation and for neurotransmission in chemical synapses. Ca^{2+} ion is also a charge carrier, carrying inward current via selective voltage- and ligand-gated Ca channels. The latter are parts of the postsynaptic (e.g. glutamate or nicotine-type acetylcholine) receptors so that neither side of a chemical synapse can function properly in case another metal interferes with Ca (Malenka and Nicoll, 1999). Voltage-gated Ca channels are blocked by a variety of divalent and trivalent metal cations including manganese (Pumain et., 1987); as well as lead, mercury and zinc (Büsselberg, 1995). Disturbed neurotransmission also belongs to the toxic spectrum of certain neurotoxic heavy metals (Braga et al., 1999; Takeda et al., 2003).

In the trafficking of metals between parts of the cells, and between different cells or tissues, metallothioneins play an important role. These are relatively small (500-14000 Da) proteins unusually rich in cysteine units. By means of the sulfhydryl groups, metallothioneins bind a range of metal ions. Physiological metals (like zinc and copper) are bound, stored and transported to the appropriate sites, while toxic ones (mercury, cadmium, etc.) are neutralized by the binding. The presence of –SH groups provides metallothioneins with antioxidant properties. Both metal exposure and oxidative stress (which can also be linked, see above) can induce the synthesis of metallothioneins.

1.3. Properties and toxicity of manganese

In pure state, manganese (Mn) is a silvery-grey, hard and brittle metal. It finds practical application in a variety of alloys and compounds. Mn can have a series of oxidation states between 2 and 7. For all living organisms, Mn is an essential trace element, but toxic when overdosed. 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 a component in many enzymes, e.g. in one of the superoxide dismutases (Mn-SOD; Law et al., 1998) playing important role in counteracting oxidative stress. Glutamine synthetase, alkaline phosphatase, arginase, and pyruvate carboxylase also contain Mn (Quintanar, 2007). 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).

Mn can be found, to various amounts, in all soils; originating from weathering of rocks and volcanic activity. The latter emits also atmospheric Mn, but today the main Mn load comes from man-made pollution (mining and metal industry, the above-mentioned fuel additive, etc.). Mn is found in steel and other alloys, and also in the coating of welding rods (for protection of the glowing hot welded metal parts from oxidation). MnO_2 is used in the production of dry cells. Mn is also used in paints and for glass and ceramics. Methylcyclopentadienyl manganese tricarbonyl (MMT) was an anti-knock petrol additive the use of which is still allowed in certain countries. Semiconductor nanocrystals (Yang et al., 2005) and ZnS:Mn^{2+} nanoflowers (three-dimensional synthetic nanostructures; Chen et al., 2005) represent the use of Mn in nanotechnology. Mn is used in certain agricultural fungicides (Maneb, Mancozeb; Ferraz et al., 1988). A new application with direct human exposure is in magnetic resonance imaging for diagnostic use, in form of the contrast agent trisodium mangafodipir (Mn-DPDP, Teslascan).

Chronic inhalation of manganese compounds is the typical form of occupational Mn exposure. The resulting “manganese madness” among ore miners was first reported in the 19^{th} century (Couper, 1837). The Mn-related chronic neurological disorder is today called manganism, and it usually progresses in three stages (Saric et al., 1977; Calne et al., 1994). The symptoms are nonspecific 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. But, in spite of the similar symptoms, the site of damage in manganism and in Parkinson’s disease is different. The site of action for Mn are the striatal, and not the mesencephalic, dopaminergic neurons (Erikson and Aschner, 2003).

Inhalation is, however, not the only way of Mn exposure leading to CNS damage. Parkinson-like disorder 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. This indicated that other physicochemical forms of Mn and other routes of exposure are also relevant to the health of the CNS (and deserve being included in neurotoxicological studies). For geological reasons (e.g. in Greece: Kondakis et al., 1989) or due to man-made pollution (such as improper disposal of used dry cells in Japan: Kawamura et al., 1941) abnormally high Mn levels in the drinking water were observed,

causing CNS symptoms in the affected population. In regions of the USA with high-Mn drinking water, loss of visual and verbal memory, typical for Mn-induced brain damage, was described (Woolf et al., 2002). Foodborne overexposure by Mn in babies fed on cow milk- or soybean-based formulas was reported (Marlowe and Bliss, 1993) as was hypermanganesaemia following long term parenteral nutrition (Crook, 2001).

The neurotoxic spectrum of Mn is variable and goes beyond the classical manganism. Epileptic activity was observed in children following inhalational (Hernandez et al., 2003) or parenteral (Komaki et al., 1999) exposure; and myoclonus in welders (Ono et al., 2002). In young shipyard workers, EEG and visual evoked potential alterations were observed and blood Mn levels up to 14 μg/L were measured (Halatek et al., 2005), while in reference groups blood Mn is 5–7 μg/L (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).

1.4. Properties and toxicity of lead

Lead (Pb) is a soft, malleable metal with low melting point. It is one of the oldest metals mined and used. Egyptians and Romans widely used this metal in everyday life (see above). In antique Rome, 4 kg lead was used per year and person, compared to 6 kg in the United States in 1965. From Gutenberg's times to the advent of photo typesetting, printing technology was based on types cast from Pb-based alloys. In spite of the known toxic effects, Pb remains to be used in several large-scale applications, an example being lead-acid batteries found in motor vehicles, in emergency power supplies, etc. Also, leaded car fuel disappeared from the market only in the last 15 years, so that in the mid-90's, approximately 90% of all Pb emission into the atmosphere was due to leaded petrol (Lovei, 1996). Lead-based pigments, like lead white ($PbCO_3$) and minium (Pb_3O_4) are no more used but demolition or renovation of old buildings or iron structures (bridges, ships) painted originally with such paints can expose the workers involved. On one criminal occasion, minium was used in Hungary to improve the color of ground red paprika (Kákosy et al., 1995, 1996). Today, half of the newly produced lead articles are made of recycled lead, which is good for the environment but the occupational hazard is the same. Hot molten lead emits metal fumes consisting of microscopic and submicroscopic aerosol particles. In absence of proper safety measures, working with lead results in serious intoxication, as shown, among others, by the case of the illegal battery recyclers in Heves (Epinfo, 1995).

Pb has no biological functions, it is purely toxic. It is also one of the most common environmental xenobiotics (present in soil, groundwater, air, and foodstuffs) for which the large-scale and careless use during previous centuries is to blame. Occupational health damage due to Pb used to be so grave that a separate “Lead Examination Station” (Ólomvizsgáló Állomás, an early predecessor of the present National Institute of Occupational Health) was set up in Hungary in 1934 (Galgóczy, 1999)

The general population is exposed mainly by contaminated drinking water or food: roots, leafy vegetables, meats, dairy products or fishes (ATSDR, 1999). High Pb level in home-grown vegetables was a major source of risk also in the above-mentioned case of the Metallokémia plant. Tobacco smoke is another notable source of Pb exposure. By the oral route, 5-50% of Pb is absorbed; but by inhalation, almost the complete amount. Before the ban of leaded petrol, the population had, mainly in urban areas and along main roads, significant airborne Pb exposure (Rudnai et al., 1998). Pb is accumulated in the central nervous system, first of all in the cortex and hippocampus (Gerhardsson et al., 1995), and produces encephalopathy at blood Pb levels of 1000-1200 μg/L in adults and 800-1000 μg/L 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, in exposed children, every 100 μg/L increase in blood Pb level is associated with a 1-5 point decrease in the IQ (Goyer, 1996). IQ differences in Hungarian schoolchildren, attributable to airborne Pb, were published by Füzesi (1997). Pb can also pass the placenta and cause serious damage to the nervous system before birth. In workers with chronic Pb exposure, less severe neurologic effects were documented with headache, lethargy, dizziness, diminished reaction time, worsened cognitive and visuomotor performance, and slowed nerve conduction (Lille et al., 1988; Araki et al., 2000).

1.5. Properties and toxicity of cadmium

Cadmium (Cd) is a soft, malleable bluish-white metal with low melting point. It has no biological function in higher-order organisms and is toxic even in small amounts. It is chemically related to zinc which explains several of its toxic effects.

Cd has been used for industrial purposes since the 19th century. The most significant application of Cd used to be in corrosion resistant electroplating layers on steel. Metallic Cd and its compounds were also used as pigments, UV-resistance stabilizers in plastics, coatings,

special alloys, Cd-based semiconductors, and on large scale in rechargeable Ni-Cd batteries. Such applications resulted in significant workplace exposure, mainly by inhalation. However, due to the toxic hazard involved, the use of Cd has been largely reduced in the last 20 years – but novel technologies and materials using Cd appeared in the meantime, like quantum dots (Rzigalinski and Strobl, 2009).

The presence of Cd in the elements of environment (air, soil, groundwater, etc.) is to a large part due to human activity, and the general population is exposed mainly by contaminated drinking water or food. Cd is emitted into the environment with solid and liquid industrial (and to a lesser extent, residential) waste. The fertilizer superphosphate can also contain significant amounts of Cd (tens of mg/kg) originating from the mineral raw material, leading to build-up of Cd in the treated fields (Syers et al., 1986).

Cd is one of the most toxic environmental and industrial pollutants because it can damage, all important organs (ATSDR, 2008a) and also the CNS (Méndez-Armenta, 2007). It is a Group 1 human carcinogen (IARC, 1993).

Inhalation of Cd containing aerosol is typically an industrial hazard. Depending on the size of the particles, airborne Cd is absorbed from the respiratory tract to 2-50% (Chaney et al., 2004). In a published acute case of intoxication due to Cd inhalation, the victim had first respiratory signs which transformed over 3-6 months to a Parkinson-like state (stiffness of the limbs, bradykinesia, muscle stiffness) that did not improve on antiparkinsonian medication (Okuda et al., 1997). 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).

At population level, Cd exposure is mostly foodborne, an important exception being tobacco use. Tobacco plants accumulate the metal in the leaves 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). By smoking 20 cigarettes day, a person's internal daily Cd dose is ca. 1 μg.

Several other cultivated plants, most notably cereals, also tend to take up Cd from the soil (Järup, 1998). Cd accumulates easily via the food chain: its level can reach 10 μg/kg in crops (grain, fruits, vegetables) but goes up to 100-1000 μg/kg in offal (kidney and liver). Shellfish can contain 200-2000 μg/kg Cd (Galal-Gorchev, 1991) but are themselves not poisoned, surprisingly.

Gastrointestinal absorption of Cd in humans is 5-20% (Chaney et al., 2004). Absorbed Cd is transported via the blood to the main target organs such as lung, kidney, liver, bones, brain,

testis, even to the placenta (Casalino et al., 2002; Méndez-Armenta et al., 2003; Morselt et al., 1991; Wier et al., 1990).

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).

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 h 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).

Beside the nervous system, Cd damages its typical sites of deposition, liver and kidneys, and the bones. The latter is due to a Zn/Cd competition at the active centre of bone alcalic phosphatase ($E + Zn^{2+}$, $Cd^{2+} \rightarrow CdE + Zn^{2+}$) which results in bone material loss and pathological fractures. Vertebral fracture and the subsequent pain (“itai-itai” is a painful cry in Japanese) were described from Japan where a rice field was flooded with metal-laden industrial wastewater (Shigematsu, 1984).

1.6. Aims

As described in the previous sections, human exposure to certain environmental heavy metals, where the workplace environment is a leading source of exposure, has been a major issue of hygiene at population level. Any effect of these metals on the nervous system deserves especial attention, because of the importance of the affected functions in the quality of life and productivity at the level of individuals, and because mental power (depending on healthy brains) is a most precious human resource.

In previous experiments at the Department it was found that recording and analysis of central and peripheral electrophysiological signals, and of certain behavioral phenomena, is sufficiently sensitive to detect the effects of lead, cadmium and manganese on the nervous system of rats, applied subacutely in dissolved form orally (Nagymajtényi et al., 1997; Papp et al., 2003; Vezér et al., 2005) or in the form of nanoparticles by intratracheal instillation (Oszlánczi et al., 2010a). In order to obtain a more adequate model, these two forms of metal application were combined in the present work to imitate exposure coming both from environmental (food/waterborne) and occupational (inhalational) sources.

The aims of the work were specified in the following questions:

- Are the treatment schemes and doses used previously for application of manganese, lead and cadmium in dissolved form orally, and in nanoparticulate form intratracheally, usable also in combination exposure?
- What quantitative and qualitative differences can be observed in the general toxicological, electrophysiological and behavioral changes obtained by applying the mentioned metals only in dissolved oral form, only in nanoparticulate intratracheal form, and in combining these forms of application?
- Is the dose-response relationship in oral-only, intratracheal-only, and combined application different?

2. MATERIALS AND METHODS

2.1. Experimental animals 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 on at 6:00 a.m., up to four rats in one cage) with free access to conventional pellet food and drinking water.

The three metals, Mn, Cd and Pb, were administered to the rats in two physicochemical forms and two ways. Aqueous solution was given orally by gavage (per os, po.), while the suspension of NP form was instilled in the trachea (intratracheally, it.). The chemical identity and dose of the substances applied to the rats is given in Table 1.

Table 1 The substances used for treatment

Metal	Compound	Doses (mg/kg b.w.)
Manganese		
dissolved form	$MnCl_2 \cdot 4H_2O$	15, 60
nanoparticulate form	MnO_2	2.63
Cadmium		
dissolved form	$CdCl_2 \cdot 2.5H_2O$	3.5
nanoparticulate form	CdO_2	0.04
Lead		
dissolved form	$Pb(CH_3COO)_2 \cdot 3H_2O$	80, 320
nanoparticulate form	PbO	2

For oral application, the compounds given in Table 1 were dissolved in distilled water to 1 ml/kg b.w. administration volume. Metal salts with high water solubility and non-toxic anion were chosen for po. administration to imitate the presence of metals in food and drink. The NPs used for it. application were oxides, because methods to produce them were found in the literature and because metal oxide NPs are found in real-world emissions e.g. in welding fumes. The final form, exerting toxic effects, is most probably a metal ion, absorbed from the intestine or dissolved from the surface of NPs in the phagosomes of alveolar macrophages. In

case of NP application, their own surface effects, resulting e.g. in oxidative stress, are also present.

The NPs were synthesized at the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics. MnO_2 NPs 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 hour.

CdO_2 NPs were synthesized by a dry process. Stochiometric amount of $CdCl_2$ and Na_2CO_3 were put, using NaCl as matrix, in the drum of a planetary ball mill (Fritsch Pulverisette 6) and rotated with 20 stainless steel mill balls at 400 rpm for 4 hours (reaction 1: $CdCl_2 + Na_2CO_3 \rightarrow CdCO_3 + 2\ NaCl$). The mixture milled this way was then calcined at 480 °C for 4 hours in air (reaction 2: $CdCO_3 + ½\ O_2 \rightarrow CdO_2 + CO_2$). After calcination, the synthesis mixture was filtered (0.45 μm PTFE membrane filter) and washed with 80°C preheated water to remove any unreacted starting material and the soluble NaCl matrix. The precipitate was dried at 100 °C for 1 hour.

PbO NPs were produced in a similar way, milling a mixture of $Pb(CH_3COO)_2$ and NaOH (reaction 1: $Pb(CH_3COO)_2 + 2\ NaOH \rightarrow 2\ Na(CH_3COO) + Pb(OH)_2$). After calcination (reaction 2: $(Pb(OH)_2 \rightarrow PbO + H_2O$), the mixture was washed and filtered as above.

The chemical purity of the nanoparticles was checked by X-ray diffraction, and their particle size, by X-ray diffraction and transmission electron microscopy. The size histograms and images of the NPs are shown in Fig. 1.

For intratracheral administration, the NPs were suspended in 1% hydroxyethyl cellulose (HEC) dissolved in PBS (pH 7.4). This vehicle was physiologically neutral and slowed the aggregation of the NPs. The suspension was intensively sonicated as it was made, and was sonicated again before administration.

Diethyl ether (used for brief anesthesia) and HEC was obtained from the Central Pharmacy of the University of Szeged. Materials for synthesis of the NPs and for po. metal treatment, and urethane for terminal anesthesia (see below), were purchased from Reanal, Budapest.

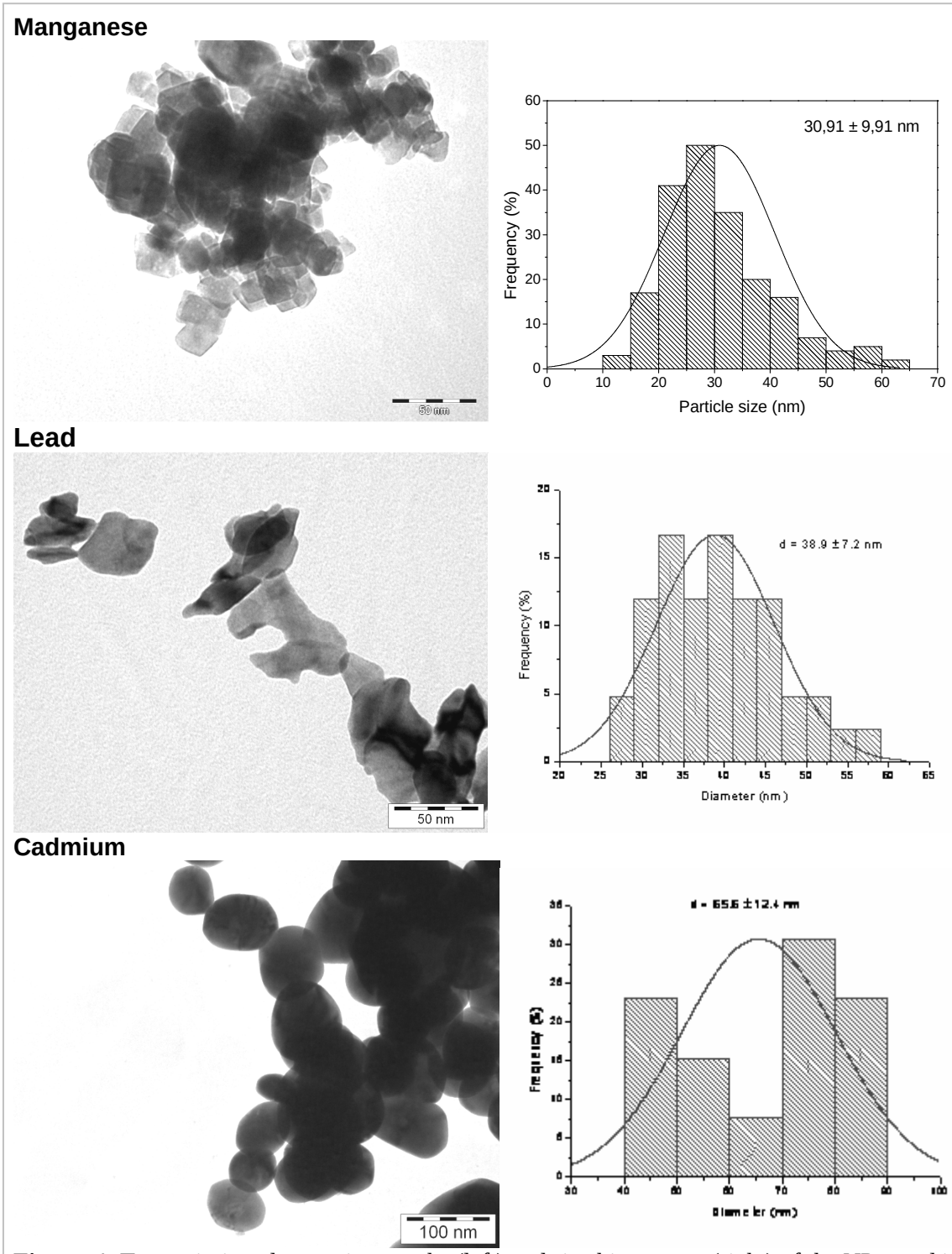

Figure 1 Transmission electromicrographs (left) and size histograms (right) of the NPs used in the experiments. Mean diameters of the NPs are also indicated.

2.2. Modes and time schemes of treatment

Gavage (for po. application) was performed using an appropriately bent and fire-polished thin glass tube attached to a 1 ml syringe. Gavage could be performed on awake rats.

For it. instillation, the animals had a brief diethyl ether anesthesia. The rat was put in a glass jar with air-tight lid, saturated with ether vapor. The completely anesthetized rat was suspended, on a board tilted to 60° from horizontal, by hanging its upper incisors in a wire loop. Keeping this way the rat in place and 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 of non-traumatic forceps, and a custom-made laryngoscope was used to gain access to the glottis. The NP suspension (or the vehicle, 1% HEC in 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 vapors.

The time scheme of treatment was, based on previous experience, determined so that detectable effects could be obtained in a reasonable time span. The basic idea, outlined in Aims, was to treat the rat first orally to imitate food/waterborne background exposure, then to apply intratracheal treatment, as if it were by workplace metal fumes. This was to be realized in a 3 and 6 weeks treatment scheme presented in Table 1. The treatment started with 3 or 6 weeks po. administration of the doses given in Table 1, to 2 x 10 rats. Then half of the group was finished (underwent behavioral and electrophysiological investigation, and was dissected) while the other half was further treated by it. administration for an equal length of time (3 or 6 weeks). This scheme worked well with Mn and Pb but produced too high general toxicity and excessive loss of animals in case of po.+it. Cd treatment. So, a different scheme with shorter it. application period was used instead, with only one po. dose, and it. application was done before, and also after, po. treatment (in two different groups, see Table 2C). All the same, the controls and the **Cd3** group of thee first (abandoned) experiments with Cd exposure were usable and are included in the evaluation.

Table 2 Treatment schemes

A. Manganese treatment

Group code	Substance and dose (mg/kg b.w.)	Treatment time
MnC3	untreated	3 weeks
MnVC3	distilled water, per os	3 weeks
MnL3	$MnCl_2$ 15 mg/kg b.w. po.	3 weeks
MnH3	$MnCl_2$ 60 mg/kg b.w. po.	3 weeks
MnC33	untreated*	6 weeks
MnVC33	distilled water, po. + HEC it.	3 weeks 3 weeks
MnL33	$MnCL_2$ 15 mg/kg b.w. po. + MnO_2 NPs 2.63 mg/kg b.w. it.	3 weeks 3 weeks
MnH33	$MnCL_2$, 60 mg/kg b.w. po. + MnO_2 NPs 2.63 mg/kg b.w. it.	3 weeks 3 weeks
MnC6	untreated*	6 weeks
MNVC6	distilled water, po.	6 weeks
MnL6	$MnCl_2$ 15 mg/kg b.w. po.	6 weeks
MnH6	$MnCl_2$ 60 mg/kg b.w. po.	6 weeks
MnC66	untreated	12 weeks
MnVC66	distilled water, po. + HEC it.	6 weeks 6 weeks
MnL66	$MnCl_2$, 15 mg/b.w. kg po. + MnO_2 NPs 2.63 mg/kg b.w. it.	6 weeks 6 weeks
MnH66	$MnCl_2$, 60 mg/b.w. kg po. + MnO_2 NPs 2.63 mg/kg b.w. it.	6 weeks 6 weeks

* C33 and C6 were two different groups.

B. Lead treatment

Group code	Substance and dose (mg / kg b.w.)	Treatment time
PbC3	untreated	3 weeks
PbVC3	distilled water, po.	3 weeks
PbL3	$Pb(CH_3COO)_2$, 80 mg/kg b.w. po.	3 weeks
PbH3	$Pb(CH_3COO)_2$, 320 mg/kg b.w. po.	3 weeks
PbC33	untreated*	6 weeks
PbVC33	distilled water, po. + HEC it.	3 weeks 3 weeks
PbL33	$Pb(CH_3COO)_2$, 80 mg/kg b.w. po. + PbO NPs 2 mg/kg b.w. it.	3 weeks 3 weeks
PbH33	$Pb(CH_3COO)_2$, 320 mg/kg b.w. po. + PbO NPs 2 mg/kg b.w. it.	3 weeks 3 weeks
PbC6	untreated*	6 weeks
PBVC6	distilled water, po.	6 weeks
PbL6	$Pb(CH_3COO)_2$, 80 mg/kg b.w. po.	6 weeks
PbH6	$Pb(CH_3COO)_2$, 320 mg/kg b.w. po.	6 weeks
PbC66	untreated	12 weeks
PbVC66	distilled water, per os + HEC intratracheally	6 weeks 6 weeks
PbL66	$Pb(CH_3COO)_2$, 80 mg/kg b.w. po. + PbO NPs 2 mg/kg b.w. it.	6 weeks 6 weeks
PbH66	$Pb(CH_3COO)_2$, 320 mg/kg b.w. po. + PbO NPs, 2 mg/kg b.w. it.	6 weeks 6 weeks

* C33 and C6 were two different groups of rats.

C. Cadmium treatment

Group code	Substance and dose (mg / kg b.w.)	Treatment time
CdV3	distilled water, po.	3 weeks
Cd3	$CdCl_2$ solution, 3.5 mg/kg b.w. po.	3 weeks
CdV13	HEC, it. + distilled water, po.	1 week 3 weeks
Cd13	CdO_2 NPs, 0.04 mg/kg b.w. it. + $CdCl_2$ solution, 3.5 mg/kg b.w. po.	1 week 3 weeks
CdV31	distilled water, po. + HEC, it.	3 week 1 weeks
Cd31	$CdCl_2$ solution, 3.5 mg/kg b.w. po. + CdO_2 NPs, 0.04 mg/kg b.w. it.	3 week 1 weeks

The doses given in Table 2 have been used in previous works of the Department (Pb oral: Pecze et al., 2005; Mn oral: Vezér et al., 2005; Cd oral: Papp et al., 2003; NP doses: Oszlánczi et al., 2010a), and were found to produce clear neuro-functional effects.

The animals were randomized between groups to avoid significant differences in the starting body weight. Body weight was measured before every administration to determine the exact daily doses and to follow weight gain. The total dose applied to one rats during the entire treatment period was also calculated using the daily weight data. During the treatment period, there was one administration every workday (that is, 5 times a week). While handling the animals, any sign of general toxicity in their appearance and behavior were observed and noted.

2.3. Behavioral investigation by the open field method

At the end of the treatment period (that is, after 3, 6 or 12 weeks metal exposure), open field test was done to assess the rats' spontaneous locomotor activity. The test was performed one or two days after the last metal administration, po. or it. An open field box (OF) of 48x48x40 cm size was used, 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. One by one, the animals

were placed into the centre of the box, for one 10-min session. The instrument recorded the animal's horizontal and vertical motor activity 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 ambulation (i.e. walking, horizontal activity), less shift, as local activity (motions without changing the place), and no shift at all, as immobility. Rearing was recorded if beams at floor level and at the higher level were interrupted simultaneously. It was known form previous experience (e.g., Vezér et al., 2005) that the OF test was suitable for investigating the impairment of higher nervous functions caused by heavy metals.

2.4. Electrophysiological investigation

The electrophysiological recording was done on the same day after the OF test or on the following day. 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 (galea aponeurotica) attached to the skull were removed. Finally the left temporal bone was cut along its inner circumference by a dental drill bit attached to a mini drill, and the left hemisphere was thus exposed. Lidocaine spray (10%) was applied to the wounds and the exposed cortex was protected with a thin layer of petroleum jelly. The animals were then wrapped in a warm cloth to maintain body temperature and were put aside for at least 30 min for recovery. For recording, the rat was placed into the stereotaxic frame of the electrophysiological setup. To stabilize body temperature, a thermostated (+36.5°C) base plate was used to support the rat's underside during the 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.

The recorded biological signals were amplified (10^4x), fed into the digitizer interface of the recording setup, and stored on PC. The complete recording and evaluation was executed by the software Neurosys 1.11 (Experimetria Ltd, Budapest, Hungary).

The session started with six minutes recording of spontaneous activity (electrocorticogram, ECoG) first, from the three sensory cortical areas simultaneously. 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.

Then EPs from the same cortical areas were recorded via the same surface electrodes. 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.

Finally, compound action potentials of the tail nerve were recorded. These were 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 were recorded distally by another pair of needles at a distance of 50 mm.

Evoked activity (cortical responses and tail nerve action potential) 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) 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. From double-pulse stimulation of the tail nerve, relative refractory period was determined.

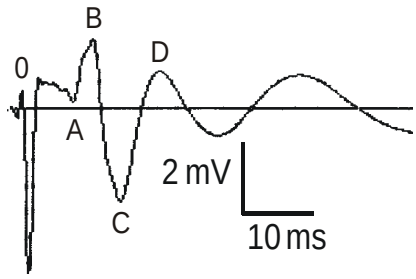

Figure 2. Typical example of the somatosensory evoked potential with the specific measuring points. See text for details.

2.5. General toxicological investigations

Beyond body weight and clinical observation mentioned above, organ weights were also used as indicators of general toxicity. Following electrophysiological recording, the animals were sacrificed by an overdose of urethane, and were dissected. Organs – brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals – were removed and weighed. Relative organ weights, on the basis of 1/100 of body weight or brain weight, were calculated.

2.6. Statistical analysis of the data

From the general toxicological, 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. Significance was accepted at $p<0.05$.

During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed. The methods used in the experiments were licensed by the authority competent in animal welfare issues under No. XXI./02039/001/2006.

3. RESULTS

3.1. Effects of manganese

3.1.1. Body and organ weights

Oral Mn treatment had no effect on the time course of the body weight in 3 weeks, and had a slight effect in 6 weeks treatment (Fig. 3). But, as soon as intratracheal administration of Mn NPs started (4th and 7th week, respectively), the weight gain in the treated groups got substantially slower than either in the untreated (**MnC**) or in the vehicle-treated (**MnVC**) control.

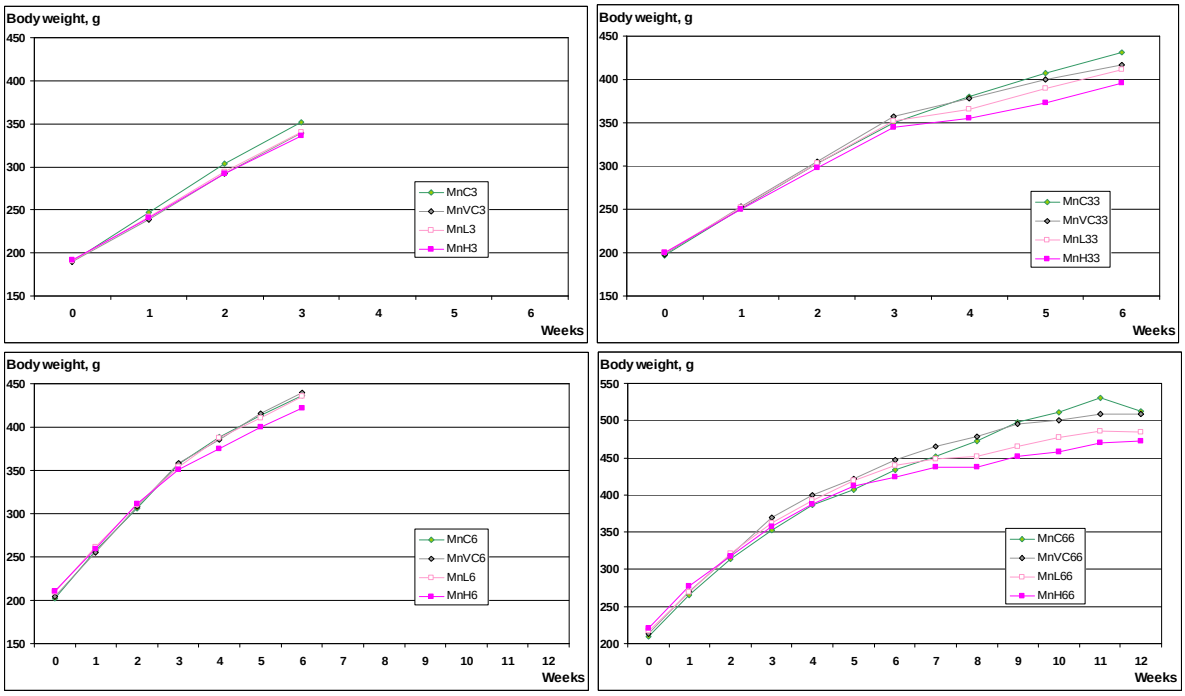

Figure 3 Time course of the control and treated rats' body weight over the 3, 6 and 12 weeks of Mn exposure. Mean values, n=8. Error bars are omitted for clarity.

Body weight gain over the entire treatment period (the 3, 6 or 12 weeks), shown in Table 3, was significantly reduced vs. control mostly in groups with combined treatment. These data indicate also that the procedure of it. administration in itself had minimal effect. The groups **MnL6** and **MnH6**, and **MnL33** and **MnH33**, received Mn for equally long treatment periods but in different chemical form and amount. By plotting the calculated daily weight gain against the total applied Mn amount (calculated summed dose in Table 3) for each animal in

the relevant groups (Fig. 4) one can see that the weight gain in rats with combined, po. + it., treatment was mostly less than in the comparable only-po. group, though the total amount of Mn administered was lower.

Table 3 Body weight gain over the 3, 6 and 12 weeks of Mn exposure, and summed Mn dose calculated for the same periods.

	Groups			
	MnC3	**MnVC3**	**MnL3**	**MnH3**
Body weight gain (g)	161.67±14.45	149.80±11.21	149.17±13.85	143.71±23.61
Calculated summed dose (mg Mn/rat)	--	--	17.94±0.51	71.58±2.93
	MnC33	**MnVC33**	**MnL33**	**MnH33**
Body weight gain (g)	234.17±19.68	218.44±20.95	210.89±17.18*	195.20±27.60**$^{\#}$
Calculated summed dose (mg Mn/rat)			28.46±0.55	83.42±2.95
	MnC6	**MnVC6**	**MnL6**	**MnH6**
Body weight gain (g)	234.18±29.09	235.17±15.26	226.14±17.30	211.00±19.28$^{\#}$
Calculated summed dose (mg Mn/rat)			44.55±1.00	178.43±8.53
	MnC66	**MnVC66**	**MnL66**	**MnH66**
Body weight gain (g)	303.67±36.31	281.25±43.16	267.57±22.77*	251.88±23.62*
Calculated summed dose (mg Mn/rat)			67.14±5.21	201.73±7.51

Mean±SD, n=8.
*, **: p<0.05, 0.01 vs. **VC**, $^{\#}$: p<0.05 **MnH** vs. **MnL**.
The summed dose was calculated by adding individual daily doses which were calculated on the basis of daily body weights.

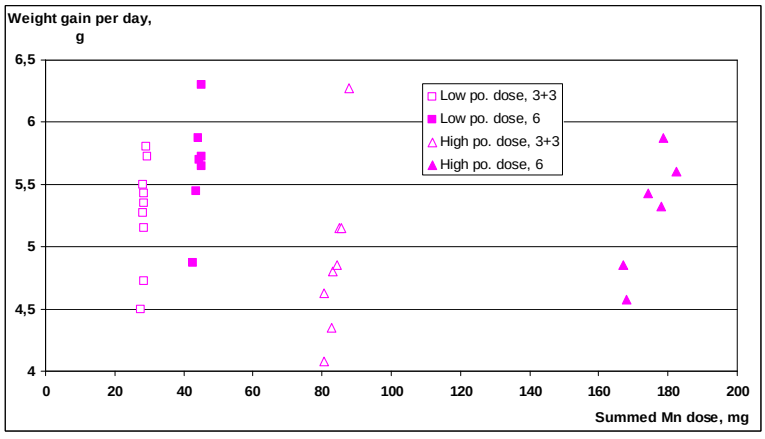

Figure 4 Calculated daily weight gain (total weight gain divided by the length of treatment period) of the individual rats plotted against their calculated summed Mn dose.

Among the relative organ weights, that of the lung, liver and adrenals showed significant changes (Table 4; the table also lists the absolute data of brain weight to show the basis of calculation). Lung weight was strongly affected by the it. Mn treatment (**MnL33**, **MnH33**) and less strongly also by the it. vehicle administration (**MnVC33**). The lungs excised from rats with it. Mn exposure had typically an emphysematic appearance with visible dark spots of Mn deposition.

Table 4 Relative organ weights of the lungs, liver and adrenals, and the absolute brain weights used as calculation basis.

	Brain absolute weight (g)	**Relative organ weights**		
Groups		***Lungs***	***Liver***	***Adrenals***
MnC3	1.989±0.061	0,170±0.009	1,856±0.104	0,0070±0.0010
MnVC3	1,999±0.072	0,200±0.021*	1,606±0.103*	0,0085±0.0027
MnL3	2,038±0.095	0,167±0.017$^{\#}$	1,837±0.207$^{\#}$	0,0070±0.0008
MnH3	1,974±0.111	0,224±0.055*^{X}	1,840±0.299	0,0081±0.0011^{X}
MnC33	2,076±0.061	0,169±0.020	1,534±0.101	0,0061±0.0013
MnVC33	2,092±0.067	0,214±0.009**	1,563±0.113	0,0062±0.0007
MnL33	2,053±0.043	0,391±0.056***$^{\#\#\#}$	1,626±0.133	0,0078±0.0014*$^{\#}$
MnH33	2,106±0.077	0,372±0.018***$^{\#\#\#}$	1,450±0.075$^{\#X}$	0,0073±0.0014*
MnC6	2,127±0.047	0,151±0.013	1,512±0.102	0,0057±0.0006
MnVC6	2,092±0.028	0,154±0.013	1,443±0.088	0,0064±0.0014
MnL6	2,124±0.076	0,149±0.011	1,546±0.081	0,0070±0.0009*
MnH6	2,103±0.060	0,165±0.022	1,430±0.106	0,0063±0.0008
MnC66	2.117±0.106	0.134±0.013	1.283±0.124	0.0057±0.0007
MnVC66	2.157±0.081	0.264±0.016	1.240±0.126	0.0055±0.0011
MnL66	2.160±0.138	0.431±0.027***$^{\#\#\#}$	1.459±0.129*	0.0062±0.0014*
MnH66	2.095±0.132	0.451±0.045***$^{\#\#\#}$	1.235±0.106	0.0062±0.0016*

For group codes, see Table 1; for the calculation, see Methods. The data are mean±SD (n=8).
*, **, ***: p<0.05, 0.01, 0.001 vs. **C**; $^{\#}$, $^{\#\#\#}$ p<0.05, 0.001 vs. **VC**; ^{X}: p<0.05 **MnH** vs. **MnL**

3.1.2. Open field motility

Mn treated rats showed decreased motility only after 6 and 6+6 weeks exposure; the effect after 3 and 3+3 weeks was absent or minimal (not shown). As seen in Table 5, 6 weeks po. treatment caused some decrease in motility but this was rarely significant. After 6 weeks po. + 6 weeks it. exposure, high dose caused significant decrease in ambulation time and count, and increase in the time of local activity and immobility. The length of individual events of local activity and immobility also increased in **MnH66**.

Table 5 Data of open field motility in rats treated with Mn by the 6 and 6+6 weeks scheme and the corresponding controls.

A. 6 weeks oral exposure.

Groups	MnC6	MnVC6	MnL6	MnH6
Ambulation distance (cm)	2230.45±266.45	2543.38±309.44	2293.59±372.49	2255.68±415.08
Ambulation time (s)	255.33±7.87	298.33±13.29	268.88±25.54#	253.88±32.24#
Ambulation count	37.17±5.78	32.50±3.02	33.25±4.74	33.75±5.20
Ambulation speed (cm/s)	8.76±1.19	8.50±0.70	9.02±0.99	9.03±1.13
Ambulation time/count (s)	7.02±1.16	9.25±0.95	7.88±1.64	8.32±1.87
Local activity time (s)	113.17±18.55	116.67±14.62	115.50±34.32	112.75±30.77
Local activity count	55.00±5.14	52.33±6.02	57.88±12.38	50.50±10.70
Local activity time/count (s)	2.05±0.24	2.24±0.32	2.15±0.23	2.21±0.25
Immobility time (s)	14.83±6.01	18.17±7.70	20.25±15.87	13.50±7.91
Immobility count	9.83±3.19	10.67±4.68	13.00±8.00	9.13±4.09
Immobility time/count (s)	1.52±0.38	1.91±0.94	1.47±0.30	1.44±0.29
Rearing time (s)	217.67±23.47	167.83±23.02	196.38±32.64	200.88±35.56
Rearing count	82.67±16.03	65.00±10.94	70.75±13.31	73.75±9.18
Rearing time/count (s)	2.72±0.59	2.60±0.26	2.81±0.44	2.72±0.30

B. 6 weeks oral + 6 weeks intratracheal exposure

Groups	MnC66	MnVC66	MnL66	MnH66
Ambulation distance (cm)	1741.82±650.61	2184.59±469.23	1610.48±355.60#	1631.40±234.60#
Ambulation time (s)	248.67±71.11	264.13±33.27	245.20±32.55	222.50±22.88#
Ambulation count	33.00±6.63	32.63±4.81	30.90±4.61	31.00±3.30
Ambulation speed (cm/s)	6.81±1.38	7.81±0.85	7.32±0.66	6.89±0.85
Ambulation time/count (s)	7. 86±3.02	8.29±1.89	7.42±1.43	8.23±1.11*
Local activity time (s)	154.17±80.23	137.38±26.60	168.40±29.95##	177.90±24.04*##
Local activity count	66.33±21.77	60.25±8.26	70.90±11.59	69.60±9.97
Local activity time/count (s)	2.22±0.44	2.28±0.28	2.39±0.28	2.56±0.55
Immobility time (s)	27.67±22.05	19.13±9.52	36.38±18.24*##	34.50±13.63*#
Immobility count	16.17±10.01	12.25±5.52	23.60±12.08#	16.80±4.42
Immobility time/count (s)	1.58±0.31	1.55±0.32	1.31±0.81	1.71±0.49
Rearing time (s)	170.50±62.65	180.38±35.78	168.30±42.67	141.10±47.18#
Rearing count	67.50±17.38	65.88±11.56	61.90±13.25	56.70±14.35*#
Rearing time/count (s)	2.48±0.57	2.78±0.60	2.72±0.42	2.45±0.43

Mean+SD, n=8.
*, **: p<0.05, 0.01 vs. **C**; #, ##: p<0.05, 0.01 vs. **VC**.

3.1.3. Cortical electrical activity

The general trend in the ECoG band spectra was a decrease in the delta, and increase in the theta and in the fast (beta, gamma) bands. Similarly to the OF effects, these changes were relatively more pronounced only after 6 and 6+6 weeks Mn treatment (Fig. 5). Data of the untreated controls are not displayed, in order to avoid crowded graphs and because the difference between **MnC** and **MnVC** was slight.

The somatosensory EPs showed increased latency on the action of Mn. This increase was seen at all stimulation frequencies but the steepness of frequency dependence of the latency was not much altered by Mn. Fig. 6 shows latency data in relative values, normalized to the latency of the SS EP obtained with 1 Hz stimulation in the corresponding **MnVC** group. This kind of display was chosen in order to eliminate the differences between control values in the various treatment schemes and so to bring the effect of Mn dose and of treatment scheme in the foreground. The SS latency lengthening was of similar magnitude in **MnL33** and **MnL6** (and was only slightly more different in **MnH33** vs. **MnH6**) in spite of the dissimilar summed

dose (see Table 3) which was approximately in accordance with the relationship of summed Mn dose and body weight. VIS latency in the groups **MnH33** and **MnH6** was in similar relationship (Fig. 7). AUD latencies changed less consequently.

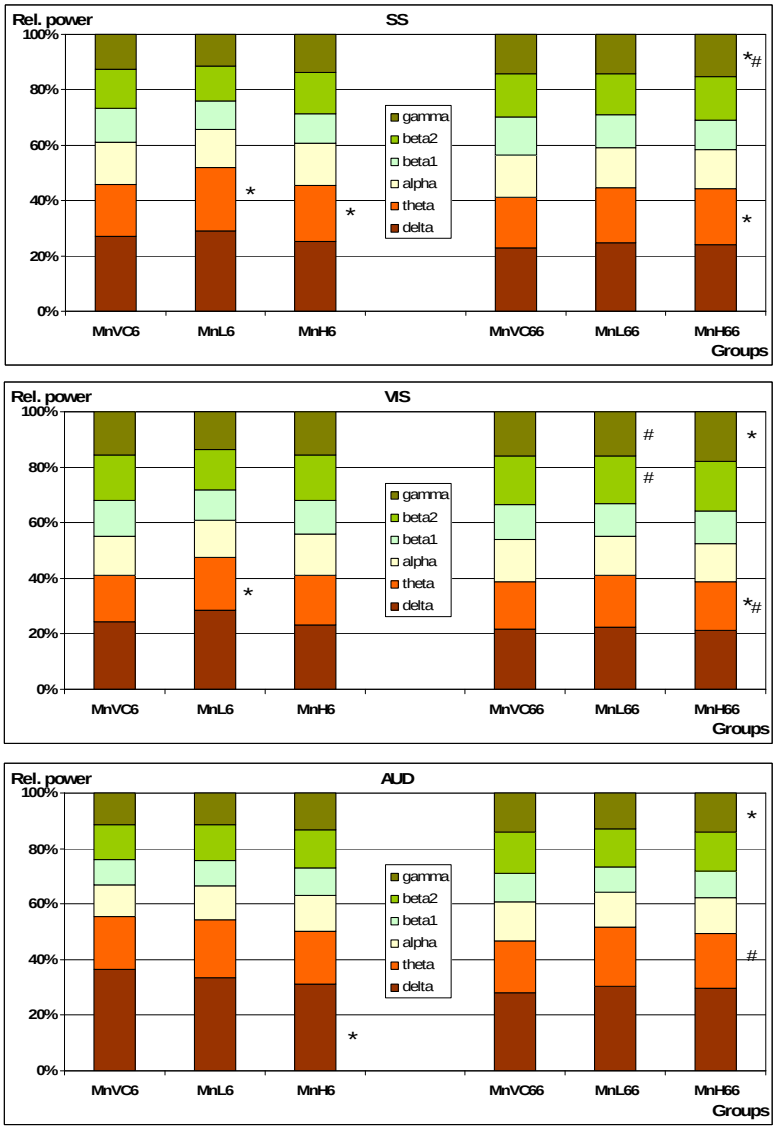

Figure 5 Band spectrum of the ECoG in rats treated with Mn by the 6 and 6+6 weeks scheme. *: p<0.05 vs. **MnVC**; #: p<0.05 high vs. low dose.

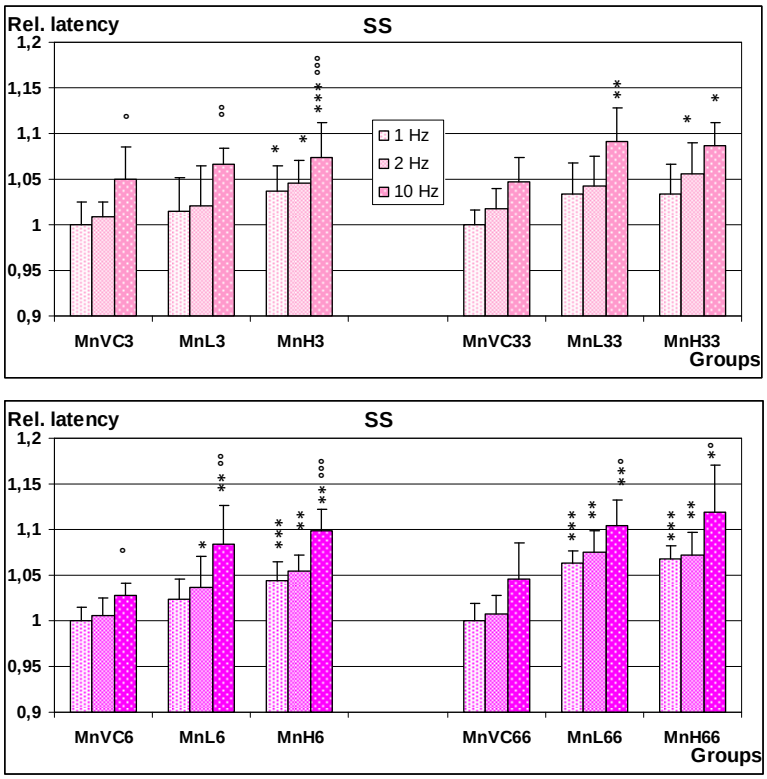

Figure 6 Latency of the somatosensory EP in rats treated with Mn according to the various schemes (top: 3 and 3+3, bottom: 6 an 6+6 weeks). Relative values, normalized to the latency of the SS EP obtained with 1 Hz stimulation in the corresponding **MnVC** group. Mean+SD, n=8.
*,**,***: p<0.05, 0.01, 0.001 vs. **MnVC**, at identical stimulation frequency (see insert)
°,°°,°°°: p<0., .01, 0.001 vs. latency with 1 Hz stimulation within a treatment group

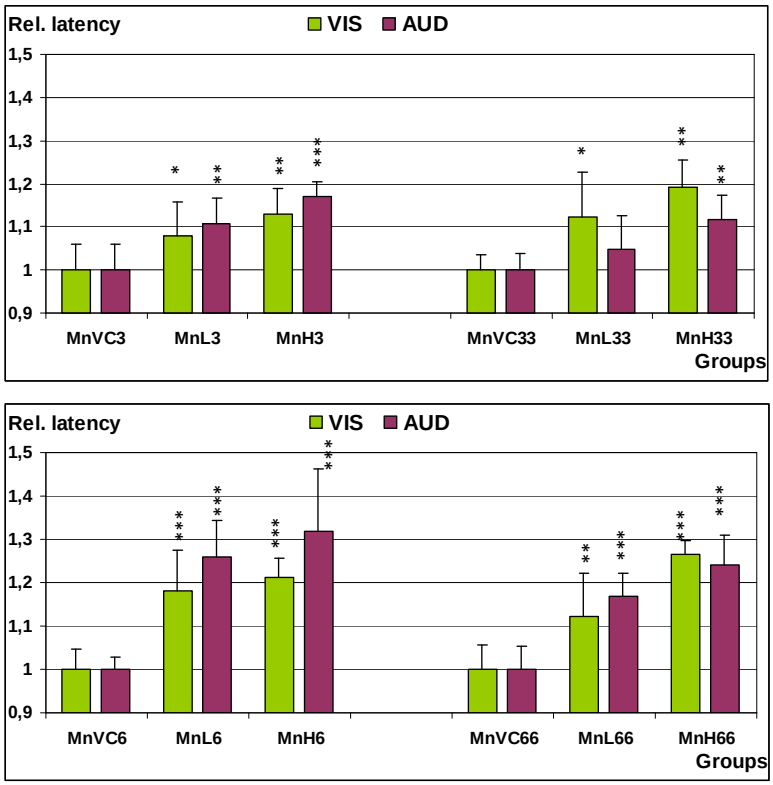

Figure 7 Latency of the visual and auditory EPs in rats treated with Mn according to the various schemes. Relative values, normalized to the latency in the corresponding **MnVC** group. Mean+SD, n=8.
*,**,***: p<0.05, 0.01, 0.001 vs. **MnVC**

3.1.4. Tail nerve action potential

Conduction velocity in the tail nerve was significantly decreased by Mn treatment (Fig. 8, top), even at the lowest dose applied (that is, in **MnL3**). Beyond the general dose dependence, the velocity reduction in **MnL33** and **MnH33** was again stronger or ca. equal to that in **MnL6** and **MnH6**, respectively – similarly to the case of the SS EP. Relative refractory period, which characterizes the frequency following ability of the nerve (Fig. 8, bottom), increased with a roughly similar dependence on the dose as the conduction velocity did.

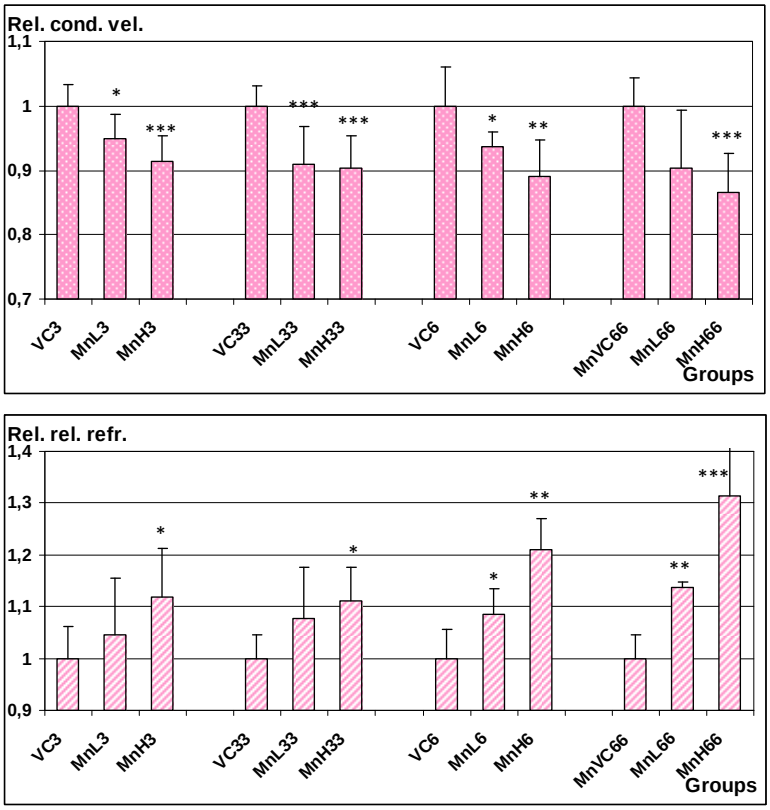

Figure 8 Conduction velocity (top) and relative refractory period (bottom) in rats treated with Mn according to the various schemes. Mean+SD, n=8. Relative values were normalized to the value measured in the corresponding **VC** group.
*,**,***: p<0.05, 0.01, 0.001 vs. corresponding **VC.**

3.2. Effects of lead

3.2.1. Body and organ weights

The effect of Pb administration on the body weight gain was visible already after 3 weeks po. treatment, but only with the high dose (Fig. 9). Starting the it. administration of Pb NPs (3+3, 6+6 weeks treatment) caused a marked drop in the weight gain, both after the high and low po. dose. This is reflected also in the weight gain data in Table 6, where the weight gain reduction was significant also in **PbH3** and **PbH6**. The relationship between weight gain and summed dose (Fig. 10) was dissimilar to that seen with Mn in Fig 4, and indicated a dependence of the weight gain more on the summed dose – which was ca. the twofold in 6 weeks po. treatment compared to that in 3 weeks po. + 3 weeks it. (see Table 6) – and less on the physicochemical form of Pb.

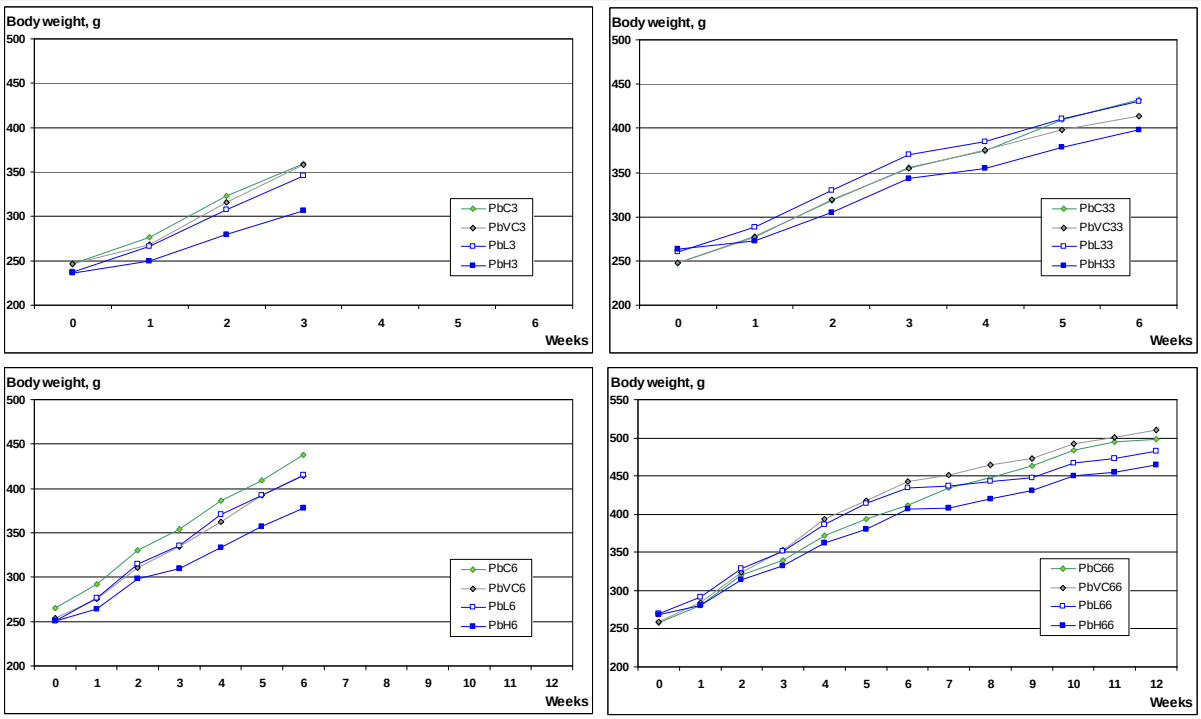

Figure 9 Time course of the control and treated rats' body weight over the 3, 6 and 12 weeks of Pb exposure. Mean values, n=8. Error bars are omitted for clarity.

Table 6 Body weight gain over the 3, 6 and 12 weeks of Pb exposure, and summed Pb dose calculated for the same periods.

	Groups			
	PbC3	**PbVC3**	**PbL3**	**PbH3**
Body weight gain (g)	112.17±14.29	111.67±11.00	108.33±12.03	78.00±8.77**$^{X\#}$
Calculated summed dose (mg Pb/rat)	--	--	193.98±9.67	710.29±50.55
	PbC33	**PbVC33**	**PbL33**	**PbH33**
Body weight gain (g)	185.17±25.87	166.33±12.01	170.13±17.29	144.57±25.57
Calculated summed dose (mg Pb/rat)			219.43±8.62	796.41±57.81
	PbC6	**PbVC6**	**PbL6**	**PbH6**
Body weight gain (g)	172.33±31.99	160.60±16.41	164.17±13.47	127.50±23.42*$^{\#}$
Calculated summed dose (mg Pb/rat)			450.58±17.32	1675.23±67.67
	PbC66	**PbVC66**	**PbL66**	**PbH66**
Body weight gain (g)	240.67±35.56	251.33±22.98	212.22±25.83$^{\#}$	195.38±25.12**^{X}
Calculated summed dose (mg Pb/rat)			499.58±25.70	1819.80±83.51

Mean±SD, n=8.
*,**: p<0.05, 0.01 vs. **PbC**; $^{\#}$: p<0.5 vs. **PbVC**; ^{X}: p<0.05 high vs. low dose.

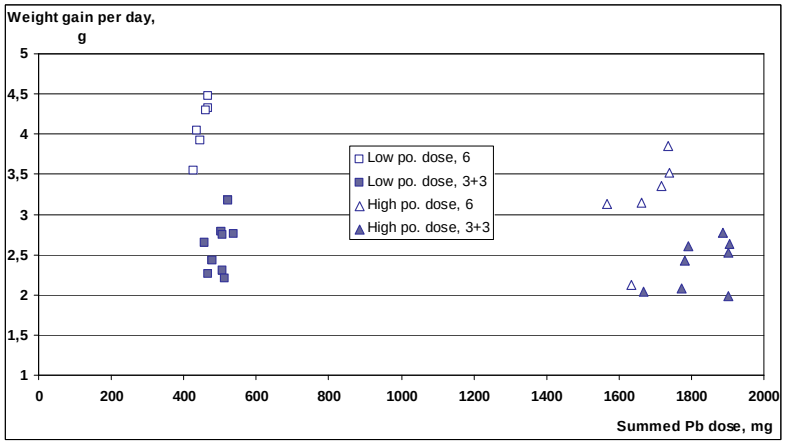

Figure 10 Calculated daily weight gain of the individual rats plotted against their calculated summed Pb dose. Displayed as in Fig. 4

The relative weight of the lungs increased massively in all groups with it. Pb administration, and less strongly also in the corresponding vehicle controls (Table 7). In the groups receiving the highest summed dose (**PbH3**, **PbH33**, **PbH6**, **PbL66**, **PbH66**) kidney weight increased significantly. Femur weight had a decreasing trend but without significance, a similar trend in the liver weight was not clearly present.

Table 7 Relative organ weights of the lungs, liver and adrenals, and the absolute brain weights used as calculation basis.

	Brain absolute weight (g)	Relative orghan weights			
Groups		**Lungs**	**Liver**	**Kidney**	**Femur**
PbC3	2.03±0.13	0.64±0.10	6.19±0.54	1.31±0.11	0.42±0.04
PbVC3	2.09±0.06	0.65±0.04	6.02±0.94	1.18±0.04	0.41±0.06
PbL3	2.03±0.08	0.67±0.07	6.20±0.58	1.24±0.07	0.36±0.02 *
PbH3	1.98±0.09 #	0.68±0.06	5.92±0.47	1.36±0.19 #	0.38±0.04
PbC33	2.18±0.08	0.96±0.24	6.11±0.81	1.34±0.14	0.49±0.07
PbVC33	2.05±0.08	1.21±0.29	6.42±1.01	1.43±0.12	0.51±0.07
PbL33	2.15±0.04	2.52±0.30***$^{\#\#\#}$	7.14±0.51*	1.50±0.10	0.50±0.02
PbH33	2.05±0.13 *	2.38±0.32 ***$^{\#\#\#}$	6.11±0.20^{x}	1.54±0.18*	0.49±0.09
PbC6	2.05±0.08	0.85±0.26	7.84±2.89	1.77±0.59	0.60±0.23
PbVC6	1.83±0.37	0.67±0.07	6.19±0.87	1.29±0.18	0.48±0.03
PbL6	2.04±0.03	0.85±0.27	6.69±0.62	1.51±0.15	0.51±0.04
PbH6	1.97±0.11	0.81±0.12	6.81±0.54	1.76±0.46 #	0.51±0.06
PbC66	2.03±0.06	0.71±0.11	6.79±0.63	1.46±0.10	0.64±0.05
PbVC66	2.15±0.08	1.42±0.11**	6.63±0.90	1.32±0.16	0.61±0.05
PbL66	2.08±0.08	2.87±0.35 ***$^{\#\#\#}$	7.51±1.22	1.59±0.16 $^{\#\#}$	0.61±0.06
PbH66	2.06±0.07 #	3.02±0.56 ***$^{\#\#\#}$	6.98±0.83	1.63±0.12*$^{\#\#}$	0.57±0.07

Mean+SD, n=8.
*, **, ***: p<0.05, 0.01, 0.001 vs. **C;** $^{\#}$, $^{\#\#}$, $^{\#\#\#}$: p<0.05, 0.01, 0.001 vs. **VC**
^{x} : p<0.05 **PbL3** vs **PbH3**

3.2.2. Open field motility

Pb-treated rats spent more time with ambulation and covered longer paths. The speed of movement was not changed (or was even reduced in **PbH66**) but single events of walking lasted longer (Table 8). There was also some increase in the time spent in local activity and such events were significantly longer also in **PbH66**. Rearing was significantly reduced in the treated rats, and these also spent more time in immobility.

Table 8 Data of open field motility in rats treated with Pb by the 6 and 6+6 weeks scheme and the corresponding controls.

A. 6 weeks oral exposure.

Groups	PbC6	PbVC6	PbL6	PbH6
Ambulation distance (cm)	2259.85±308.11	2532.42 ±228.02	2341.86±126.77	2495.10±282.65
Ambulation time (s)	262.67±19.04	284.60±12.46	284.40±24.54	298.75±28.94*****
Ambulation count	33.83±4.17	37.20±6.22	34.60±4.83	29.25±5.56$^{\#}$
Ambulation speed (cm/s)	8.58±0.78	8.89±0.56	8.26±0.51	8.36±0.50
Ambulation time/count (s)	7.89±1.41	7.80±1.16	8.42±1.89	10.60±2.81*****$^{\#}$
Local activity time (s)	112.83±11.91	107.20±45.25	100.17±18.32	124.25±11.06
Local activity count	52.33±6.09	48.80±11.43	49.83±3.87	60.25±1.89*****^{x}
Local activity time/count (s)	2.16±0.19	2.13±0.50	2.00±0.24	2.06±0.21
Immobility time (s)	19.83±8.95	14.20±9.98	18.00±12.20	20.25±7.85
Immobility count	14.17±5.64	9.60±5.77	11.33±5.47	12.50±5.07
Immobility time/count (s)	1.37±0.19	1.42±0.31	1.49±0.29	1.63±0.16
Rearing time (s)	205.67±27.03	195.00±46.07	202.33±25.77	157.75±31.64*****
Rearing count	60.83±6.34	76.20±14.58	77.33±11.52	76.75±9.22*****
Rearing time/count (s)	3.42±0.64	2.55±0.29	2.64±0.25	2.05±0.23 ******^{x}

B. 6 weeks oral + 6 weeks intratracheal exposure.

Groups	PbC66	PbVC66	PbL66	PbH66
Ambulation distance (cm)	2171.78±386.72	2058.16±451.43	2027.28±55.14	2241±138.47
Ambulation time (s)	261.50±28.79	252.83±44.02	279.17±21.94	313.17±22.12**$^{\#\#}$
Ambulation count	31.83±3.66	31.17±2.71	32.67±4.13	24.00±3.58 **$^{\#\#xx}$
Ambulation speed (cm/s)	8.27±0.87	7.66±0.83	6.16±3.11	7.31±0.19 ***$^{\#\#\#}$
Ambulation time/count (s)	8.29±1.27	8.18±1.75	8.71±1.63	13.36±2.64 ^{xxx}
Local activity time (s)	127.50±41.26	139.83±54.76	150.00±16.53	161.83±13.99
Local activity count	64.67±11.78	64.17±13.79	62.50±5.36	68.33±4.03
Local activity time/count (s)	1.94±0.36	2.12±0.41	2.40±0.17	2.38±0.27*$^{\#}$
Immobility time (s)	19.83±9.87	24.50±12.83	18.83±7.36	24.83±11.27
Immobility count	13.83±5.91	14.83±6.21	12.83±4.71	14.00±4.05
Immobility time/count (s)	1.39±0.15	1.61±0.27	1.47±0.29	1.72±0.43***$^{\#\#\#}$
Rearing time (s)	192.17±38.00	183.83±30.36	153.00±29.50	101.17±30.41 ^{x}
Rearing count	79.67±16.45	69.83±9.77	64.33±9.14	48.17±10.15***$^{\#\#x}$
Rearing time/count (s)	2.44±0.40	2.63±0.22	2.39±0.39	2.07±0.27$^{\#\#}$

Mean+SD, n=8.
*, **, ***: p<0.05, 0.01, 0.001 vs. **C;** $^{\#}$, $^{\#\#}$, $^{\#\#\#}$: p<0.05, 0.01, 0.001 vs. **VC**
^{X}: p<0.05 high vs. low dose

3.2.3. Cortical electrical activity

After 3 and 3+3 weeks, there were no noteworthy changes in the band spectrum of the ECoG (not shown). In the groups **PbL6** and **PbH6**, increased slow and decreased fast activity was seen, compared to their control **PbVC6**. However, in the rats receiving 6 weeks it. Pb exposure after 6 weeks po. treatment, this frequency shift disappeared (Fig. 11).

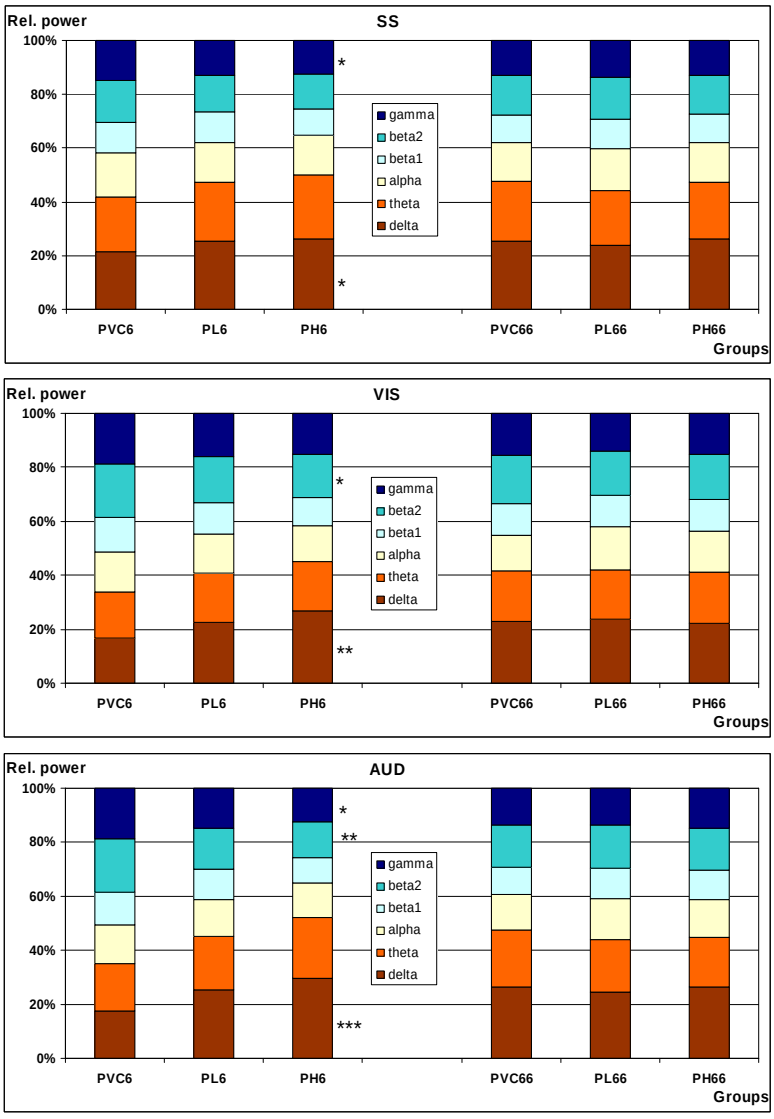

Figure 11 Band spectrum of the ECoG in rats treated with Pb by the 6 and 6+6 weeks scheme. *,**: p<0.05, 0.0 vs. **PbVC**.

Latency of the SS EP was increased in the Pb-treated rats (Fig. 12). The change was significant vs. vehicle control in rats with 3+3, 6 and 6+6 weeks treatment. The change in **PbH33** and **PbH6** was nearly equal, and in **PbL33** was somewhat bigger than in **PbL6**.

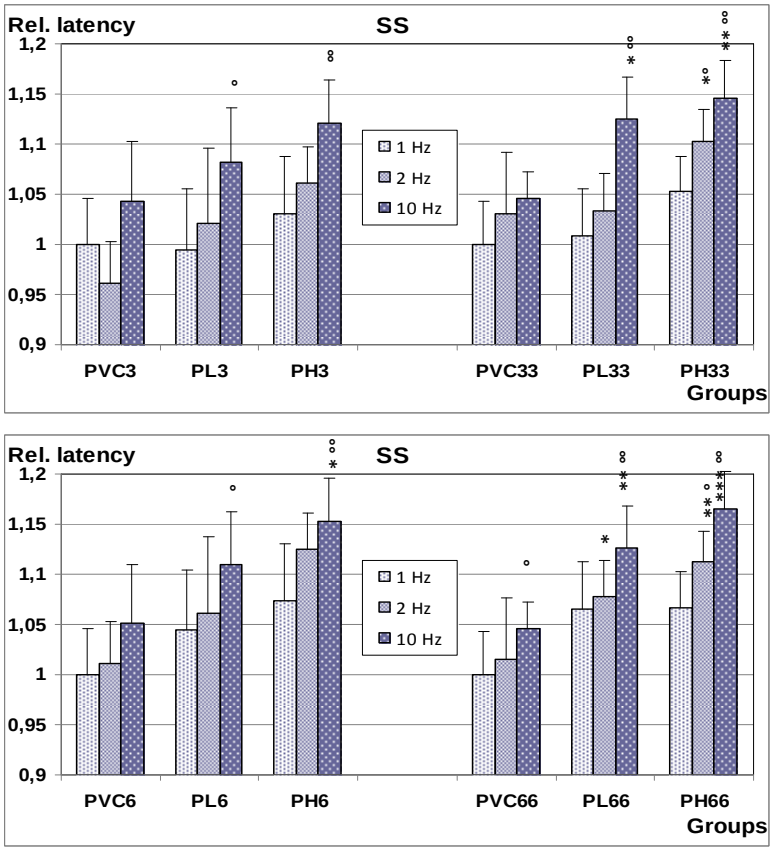

Figure 12 Latency of the somatosensory EP in rats treated with Pb according to the various schemes. Relative values, normalized to the latency of the SS EP obtained with 1 Hz stimulation in the corresponding **PbVC** group. Mean+SD, n=8.
*,**,***: p<0.05, 0.01, 0.001 vs. **PbVC**, at identical stimulation frequency (see insert).
°,°°: p<0.05, 0.01 vs. latency with 1 Hz stimulation within a treatment group.

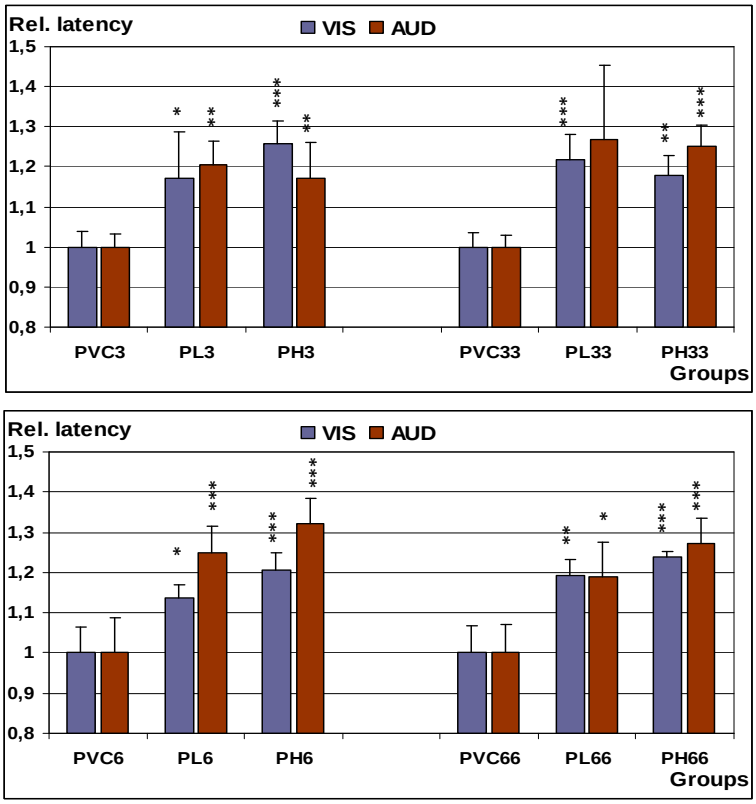

Figure 13 Latency of the visual and auditory EPs in rats treated with Pb according to the various schemes. Relative values, normalized to the latency in the corresponding **PbVC** group. Mean+SD, n=8. *,**,***: p<0.05, 0.01, 0.001 vs. **PbVC**

In both combination groups, the frequency dependent lengthening of the latency became also more intense. The relationship of latencies in **PbH33** and **PbH6** was dissimilar to that of body weight gain in these groups (Fig. 12). Also in the VIS EP, the latency in **PbH33** and **PbH6** was nearly equal. The latency of AUD EP was also lengthened but in a rather irregular way (Fig. 13).

3.2.4. Tail nerve action potential

Conduction velocity in the tail nerve was generally decreased in the Pb-treated rats (Fig. 14). Again it seemed that, as in the case of weight gain, the change reflected more the dose and less the chemical form of Pb. The length of relative refractory period showed some changes but no clear tread (not shown).

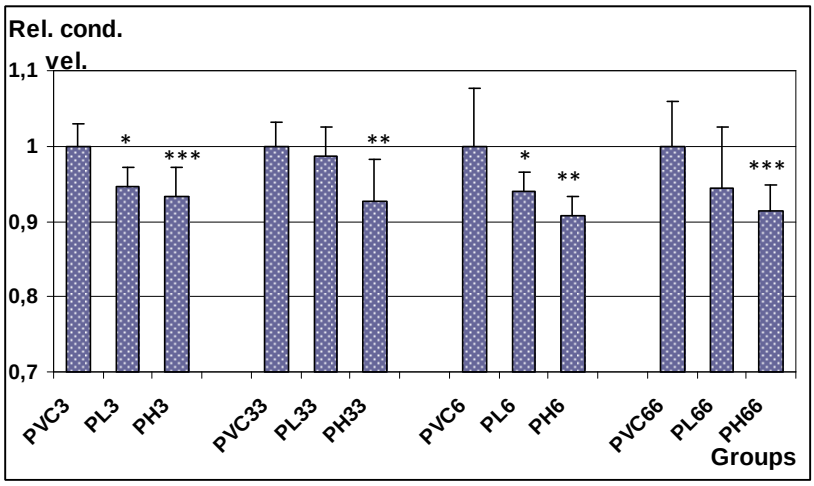

Figure 14 Conduction velocity in rats treated with Pb according to the various schemes. Mean+SD, n=8. Relative values were normalized to the value measured in the corresponding **VC** group.
*,**,***: p<0.05, 0.01, 0.001 vs. corresponding **VC**

3.3. Effects of cadmium

3.3.1. Body and organ weights

The time course of the body weight was markedly different in the groups **Cd13** and **Cd31**, and suggested that the NP form of Cd had, in spite of the much lower dose, more pronounced effect on this toxicological parameter (Fig. 15; **Cd3** and **CdV3** are not included because these are practically identical to the first 3 weeks of **Cd31** and **CdV31**). The effect is also seen in the weight gain and Cd dose data given separately for the po. and it. administration weeks in Table 9.

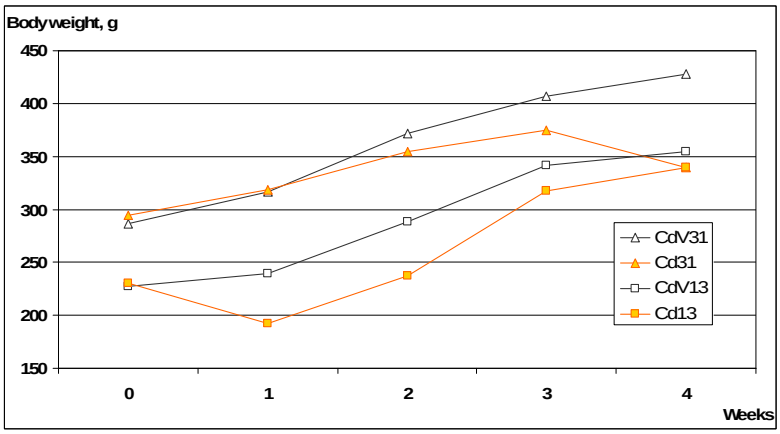

Figure 15 Time course of the control and treated rats' body weight treated by the 1+3 and 3+1 schemes (see Table 8). Mean values, n=8. Error bars are omitted for clarity.

Table 9 Body weight gain and summed Cd doses in the oral and intratracheal treatment periods.

Groups	***Body weight gain (g)***	***Calculated summed dose (mg Cd/rat)***	***Body weight gain (g)***	***Calculated summed dose (mg Cd/rat)***
	Week 1 - 3		Week 4	
CdV31	120.50±15.66	--	21.33±4.89	--
Cd31	80.38±35.58*	8.603±0.269	-27.14±24.07***	0.042±0.003
	Week 1		Week 2 - 4	
CdV13	11.67±6.65		97.67±26.09	
Cd13	-37.86±13.52***	0.032±0.004	138.51±19.57*	6.425±0.589

Mean+SD, n=8. *,***: p<0.05, 0.001 vs. corresponding control.

The relative weight of the lungs was massively influenced by the it. application (Table 9). Weight decrease of the liver and kidneys was also significant only in the combined treatment groups, and was strongest in **Cd31**, suggesting a disproportionately high toxicity of nanoparticulate Cd.

Table 10 Relative organ weights of the lungs, liver and adrenals, and the absolute brain weights used as calculation basis

	Brain absolute weight (g)	**Relative organ weights**		
Groups		***Lungs***	***Liver***	***Kidneys***
CdV3	2.093±0.059	0.650±0.036	6.025±0.940	1.181±0.042
Cd3	2.022±0.074	0.655±0.047	5.697±0.345	1.199±0.084
CdV31	2.064±0.061	0.908±0.051	7.238±0.569	1.387±0.074
Cd31	1.997±0.086	1.615±0.220***	5.910±1.233*	1.153±0.133**
CdV13	1.918±0.186	0.940±0.095	6.521±0.222	1.353±0.089
Cd13	1.976±0.118	1.338±0.162**	6.254±0.647	1.191±0.077*

Mean+SD, n=8.
*,**,***: $p<0.05$, 0.01, 0.001 vs. corresponding control.

3.3.2. Open field motility

Decreased motility was observed in all Cd-treated rats vs. the corresponding controls. In **Cd3**, only the run length was reduced; but in **Cd31**, nearly all parameters were altered including slower ambulation speed and longer events of local activity and immobility. In **Cd13** the changes were similar but less strong and mostly not significant. The data are shown in Table 11.

Table 11 Data of open field motility in rats treated with Cd by the various treatment schemes.

A. 3 weeks oral exposure.

Groups	CdC3	CdV3	Cd3
Ambulation distance (cm)	2848.64±175.82	2631.98±585.19	2420.33±201.35 *xxx
Ambulation time (s)	324.00±45.40	289.33±61.72	292.67±18.38 xxx
Ambulation count	26.00±6.36	32.00±10.00	27.33±4.23
Ambulation speed (cm/s)	8.46±0.63	9.11±1.10	8.28±0.64 xx
Ambulation time/count (s)	13.53±5.31	10.60±6.83	11.00±2.37
Local activity time (s)	129.33±30.47	115.33±43.03	144.67±25.52 xxx
Local activity count	58.50±11.48	50.17±12.66	61.83±6.68 #xx
Local activity time/count (s)	2.22±0.33	2.26±0.47	2.33±0.23 xx
Immobility time (s)	26.33±11.84	16.00±13.97	25.83±6.62 xx
Immobility count	16.00±5.69	10.33±6.47	14.50±3.27 xxx
Immobility time/count (s)	1.67±0.39	1.41±0.29	1.82±0.50 x
Rearing time (s)	121.33±35.59	180.33±69.88	137.83±36.27 xx
Rearing count	57.00±15.26	65.17±19.21	57.67±17.96 xx
Rearing time/count (s)	2.13±0.39	2.72±0.62	2.42±0.35

B. Combined exposures

Groups	CdV31	Cd31	CdV13	Cd13
Ambulation distance (cm)	2493.07±289.12	392.38±358.48 ###	2705.97±108.61	2226.76±363.10 #ooo
Ambulation time (s)	290.67±26.27	210.17±44.83 ##	297.17±21.84	265.86±19.39 o
Ambulation count	29.33±2.07	26.67±6.35	30.67±1.63	31.43±4.43
Ambulation speed (cm/s)	8.59±0.91	6.58±0.43 ###	9.15±0.77	8.34±0.81 ooo
Ambulation time/count (s)	9.95±1.14	8.24±2.71	9.73±1.10	8.59±1.21
Local activity time (s)	146.50±33.64	241.00±34.30 ###	129.83±33.62	156.14±37.65 ooo
Local activity count	61.17±9.99	81.50±8.87 ##	53.00±8.20	65.86±9.60 #oo
Local activity time/count (s)	2.39±0.40	2.96±0.29 #	2.44±0.50	2.37±0.38 oo
Immobility time (s)	17.50±5.24	87.00±52.22 ###	15.33±6.92	18.71±8.71 ooo
Immobility count	12.00±2.28	34.17±13.44 ###	11.67±3.83	13.29±4.89 ooo
Immobility time/count (s)	1.44±0.21	2.41±0.67 ###	1.29±0.26	1.40±0.21 ooo
Rearing time (s)	146.33±23.64	62.83±42.96 ##	158.67±41.35	160.29±41.01 ooo
Rearing count	69.17±11.62	26.67±12.04 ###	70.67±12.97	61.71±17.16 ooo
Rearing time/count (s)	2.13±0.27	2.20±0.71	2.23±0.22	2.65±0.58

*: p<0,05 **CdC3** vs. **Cd3**

#, ##, ###: p<0.05, 0.01, 0.001 **CdV31** vs. **Cd31** or **CdV13** vs. **Cd13**

x, xx, xxx: p<0.05, 0.01, 0.001 **Cd31** vs. **Cd3**

o, oo, ooo: p<0.05, 0.01, 0.001 **Cd31** vs. **Cd13**

3.3.3. Cortical electrical activity

In the band spectrum of the ECoG, there was practically no change in any of the Cd-treated groups.

In the somatosensory EP, increase of the latency was seen in all treated groups. In **Cd31**, the increase was significant at all stimulation frequencies (1, 2 and 10 Hz) vs. **CdV31**; whereas in the other treatment schemes (**Cd3**, **Cd13**) only at the highest stimulation frequency (Fig.16, top). The body weight gain difference was also the highest in **Cd31** vs. **CdV31** around the time of electrophysiological recording. The frequency-dependent extra lengthening of latency was, however, more pronounced also in **Cd3** and in **Cd31** than in the corresponding controls.

The VIS and AUD EPs were also significantly lengthened by Cd application in the groups **Cd3** and **Cd31**, but not in **Cd13** (Fig. 16, bottom).

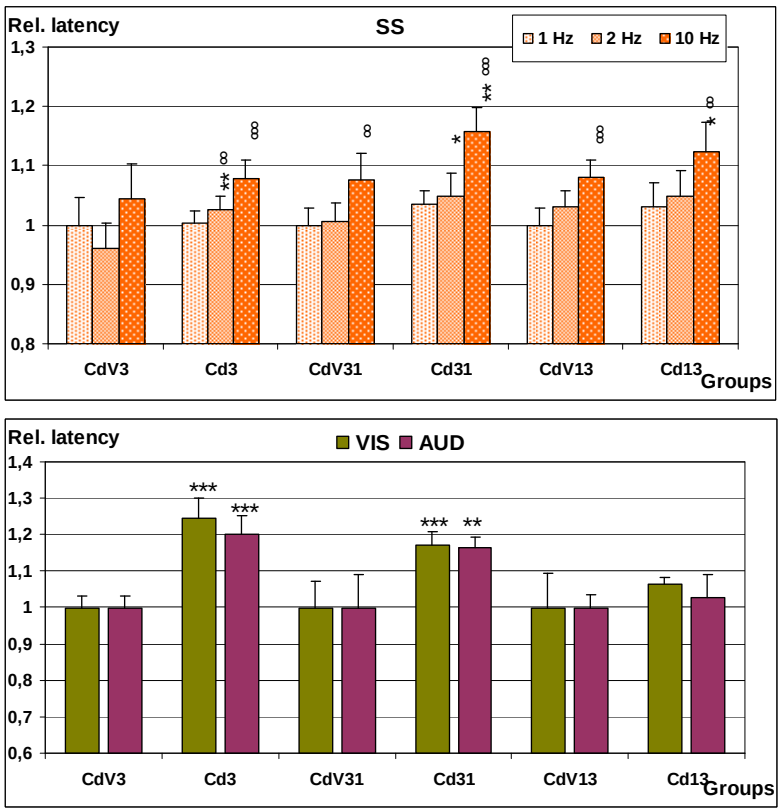

Figure 16 Latency of the somatosensory EP (top) and visual and auditory EP (bottom) in rats treated with Cd according to the various schemes. Relative values, normalized to the latency of the EP in the corresponding control group (for SS: with 1 Hz stimulation). Mean+SD, n=8.
*,**: p<0.05, 0.01 vs. control (**V**) at identical stimulation frequency (see insert).
°,°°,°°°: p<0.05, 0.01, 0.001 vs. latency with 1 Hz stimulation within a treatment group.

3.3.4. Tail nerve action potential

Decreased conduction velocity of the tail nerve was found in all treated groups (Fig. 17) but here there was no difference between various treatments, unlike what was seen in the cortical evoked responses. The lengthening of the relative refractory period, which indicated increased fatigability of the nerve in the treated rats, was significant in **Cd3** and **Cd13**.

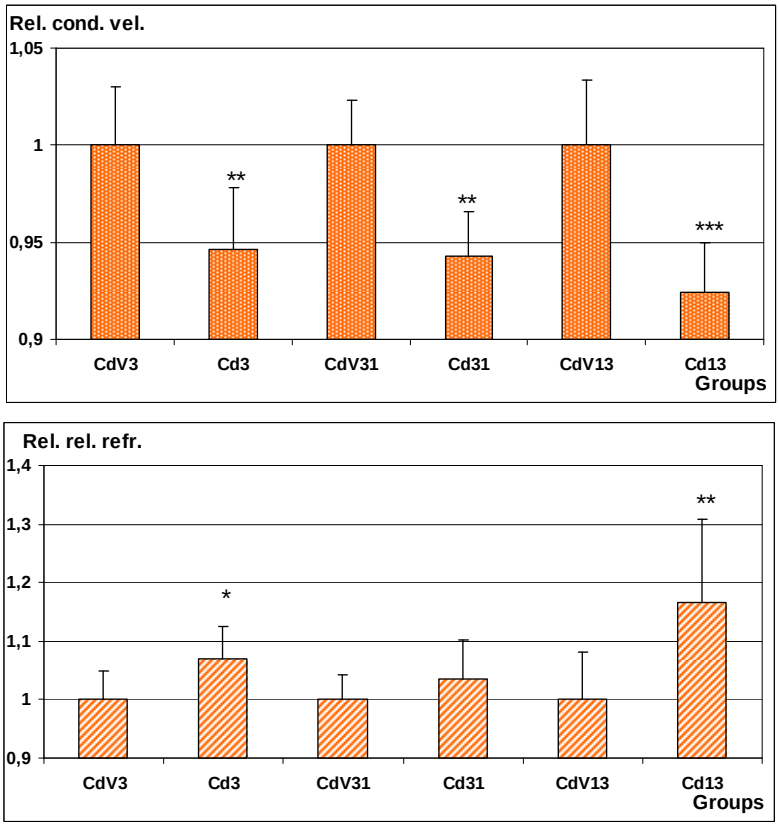

Figure 17 Conduction velocity (top) and relative refractory period (bottom) in rats treated with Mn according to the various schemes. Mean+SD, n=8. Relative values were normalized to value measured in the corresponding **VC** group.
*,**,***: $p<0.05$, 0.01, 0.001 vs. corresponding **VC**.

4. DISCUSSION

In evaluation of toxicological results obtained in an animal model, one of the main questions is to what extent the doses used correspond to those observed in cases of human exposure.

Reported high Mn levels in drinking water were ca. 50 μg/L in Greece (Kondakis et al., 1989) or even above 1000 μg/L in Bangla Desh (Wassermann et al., 2006). From the latter data and 2 L of water consumed a day, the daily dose for an adult would be ca. 0.03 mg/kg Mn. Our doses were in fact much higher, but the resulting blood level in a previous experiment with the same doses (Vezér et al., 2005) was ca. 36 μg/L blood, in the same order of magnitude with Mn blood levels of heavily exposed workers (Halatek et al., 2005).

If a daily ventilation volume of 0.5 m^3/kg b.w. is supposed for the rats (based on physiological data by Strohl et al., 1997) the it. dose of 2.63 mg/kg MnO_2 NPs corresponds to ca. 1.7 mg/m^3 in continuous exposure (or three times that for 8 hours per day). In heavily polluted workplace atmospheres, ca. 0.8 mg/m^3 (Bader et al., 1999) was described as human exposure.

The dose for it. application of Pb was originally derived from papers by Coffigny et al. (1994) and Pinon-Lataillade et al. (1993) who obtained 500-700 μg/L 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 again. So, 2 mg/kg dose was chosen, which previously resulted in ca. 1700 μg/L blood levels (Oszlánczi et al., 2011). In actual industrial exposures, airborne Pb levels lower than 5 mg/m^3 also caused similar blood levels: 1.06 mg/m^3 Pb in fumes (probably including NPs) in a lead battery workshop and 635 μg/L Pb in the exposed workers' blood (controls: 87 μg/L; Jiang et al., 2008); or 0.6 mg/m^3 Pb in another lead battery plant, and up to 500 μg/L Pb in the exposed workers' blood (Lormphongs et al., 2004). The po. Pb doses are hard to bring into direct human analogy because oral exposure to Pb, if any, typically comes from several sources. Anyway, in an earlier study with 10 weeks oral exposure of rats to 500 mg/kg b.w. Pb-acetate, 2500 μg/L blood level was obtained (Vezér, unpublished). The lower doses used in the present work (80 and 320 mg/kg) probably resulted in blood levels nearer to those found in exposed humans.

In the case of Cd exposure, our intratracheal dose (0.04 mg/kg b.w.) is somewhat above those found in industrial settings; based on the daily ventilation volume of rats mentioned above. Measured airborne levels were ca. 30 μg/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). However, in a rat inhalation experiment 70 and 550 μg/m^3 were used (Takenaka et al., 2004).

Both the general and functional neurotoxicological parameters indicated several times during the experiments described in this thesis that there can be a non-additive interaction between the amounts of heavy metals given by po. and it. application.

The toxic effects of Cd were mostly stronger in the combination groups (**Cd13** and **Cd31**) than in the group treated with oral Cd only (**Cd3**); exemplified both by body weight gain and SS EP latency (Fig. 15,16). In **Cd31**, where the rats received 3 weeks oral then 1 week intratracheal exposure, the amount of Cd given it. was much less than that given po. previously, yet the final effect suggested potentiating interaction. During the 3 weeks of oral Cd treatment, the blood-brain barrier (BBB) may have been weakened (as described by Shukla et al., 1996), so the Cd-containing NPs applied afterwards had higher chance to enter the brain. A potentiation-like effect was also seen in the time course of the body weight, and in the relative liver weight. In an experiment with 6 weeks intratracheal application of CdO_2 NPs, a tenfold dose (0.4 mg/kg) was needed to achieve significant CNS effects (Papp et al., 2009) which is another argument for the potentiating interaction.

Pb is another metal known to affect BBB (Goldstein et al., 1974), which may explain that, among the Pb-treated rats, lengthening of SS EP latency was nearly equal in **PbH33** and **PbH6,** despite the ca. twice higher summed dose in the 6 weeks po. Pb administration.

In the experiment with Mn and with Cd (but not with Pb), the disproportionately strong effect of NP exposure was seen also on the body weight gain, a general parameter entirely independent of the state of the BBB. Metal level data from previous experiments with the same doses and routes of application as used here (Mn: Oszlánczi et al., 2010b; Vezér et al., 2005) favor the reasoning that inhaled NPs cause internal exposure more efficiently than ingested, dissolved metals. In the works referred to above, 9 weeks it. application of 5.26 mg/kg b.w. of MnO_2 NPs resulted in ca. 7000 μg/kg Mn in the treated rats' brain while 60 mg/kg $MnCl_2$ applied orally for 10 weeks gave only ca. 290 μg/kg. It is an open question, to be settled in further experiments (by electron microscopy), whether NPs themselves appear in the brain and in other organs – due to their extreme mobility within the organism – or their metal content is dissolved and absorbed, inducing the known general and organ-specific toxic effects.

When discussing toxicity of NPs, oxidative stress must not be forgotten. Lungs are affected the most directly, and welding fumes containing Mn and other metals were reported to cause oxidative stress and inflammation in the airways, with depletion of glutathione (McNeilly et

al., 2004). 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). Generation of reactive oxygen species was detected in the brain in case of all three metals, (Mn: Zhang et al., 2009; 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 changes of fluidity and other membrane properties which, in turn, disturbs all membrane- and receptor-bound phenomena, such as synaptic transmission, and these are likely to be reflected e.g. in various forms of cortical activity.

Cortical evoked potentials reflect alterations in synaptic transmission most directly. The increased latency observed in the rats treated with each of the metals 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 (blocked membrane Ca-channels and increased intracellular Ca release at the same time) was reported by Kita et al. (1981). In rats exposed to traffic-generated dust containing Pb and Cd, short-term memory was impaired (Zanchi et al., 2010), and memory formation and consolidation is known to depend on Ca-regulated neuronal events.

The main excitatory transmitter in the CNS is glutamate. After being released into the synaptic cleft and having activated the receptors, glutamate is taken up and converted to glutamine by the astrocytes. Mn^{2+} decreases both the uptake (Erikson and Aschner, 2003) and the transformation to glutamine (Normandin and Hazell, 2001). 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, resulting in smaller/slower cortical evoked responses, and may also exert excitotoxicity (Coyle and Puttfarcken, 1993).

The decrease of conduction velocity in the tail nerve 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).

Energetic shortage could have a slowing effect on the basal cortical activity (ECoG) – similarly to cases of mitochondrial encephalopathy (Smith and Harding, 1993). Glutamatergic overactivity could have the opposite effect: glutamatergic collaterals from the specific afferents to the ascending reticular formation could increase cortical activity by cholinergic modulation (Metherate et al., 1992). The resulting net change may be (and in fact was) different depending on the relative strength of the two opposite influences.

The changes in open field motor behavior may partly result from the general CNS effects of the metals/NPs but the dopaminergic system plays here the crucial role. 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 Mn, Pb and Cd to induce oxidative stress is well known and was mentioned above as a common point in their effects on the nervous system. Of the activity forms measured, vertical motility is an specially sensitive indicator of striatal dopaminergic activity (Sedelis et al., 2001), and diminished rearing was observed in the rats exposed to Mn (Table 5) Pb (Table 8) and Cd (Table 11). Diminished horizontal movements after high dose Mn (**MnH66**) fits well in the 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 may be 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 more frequently among children with elevated blood Pb (Needleman and Gatsonis, 1990). Another

symptom reflecting impaired central motor control, postural sway, was also seen in Pb-exposed workers (Yokoyama et al., 2002).

Based on the results described and evaluated above, it can be stated that the attempt to model the complex exposure, coming both from environmental (food/waterborne) and occupational (inhalational) sources, was successful. In a world where the large-scale use of various metals is daily reality, where occupational exposure still endangers the workers' health, and where emissions from present and past times can be found in the environment and in commodities of environmental origin – notably, drinking water and food – the health effects in general, and in particular the effects on sensitive systems like the nervous system, are of primary concern. The occurrence of metals in nanoparticulate form, let it be nanotechnological materials or unwanted pollutants, adds a new feature to the old problem.

Especially when speaking of neurotoxicity, the study of functional alterations is important because by classical biomarkers – by detecting the levels of toxic metals in available human biological samples (blood or urine) – the damage to central or peripheral parts of the nervous system cannot be characterized adequately. This problem has been repeatedly raised in the literature (e.g. Manzo et al., 1996; Myers et al., 2003). Animal model experiments can contribute to the development of neuro-functional biomarkers which may be better suited for this purpose.

The questions specified in Aims (1.6.) can finally be answered as follows:

- The treatment schemes and doses used previously for oral and intratracheal application could be adapted without significant change in case of Mn and Pb treatment. In case of Cd, strong general toxicity required the development of a new scheme with shorter it. exposures. The doses, although caused comparable alterations in the electrophysiological (and partly in the behavioral) parameters, were at the systemic level not "equitoxic".
- In quantitative aspect it became clear that the NP form of the metals, applied after weeks of oral exposure to the dissolved form, had disproportionately strong effect on the body weight gain (Mn, Cd), as well as on some of the open field behavioral parameters and on parameters of evoked electrophysiological responses (all three metals). Qualitative difference was seen mainly in the electrocorticograms, which possibly reflected the interference of NP-specific (oxidative stress) and metal-specific (altered synaptic transmission etc.) actions. Comparison with earlier intratracheal-only

results also indicated that lower NP dose was enough to evoke the same effects when applied after oral treatment.

- The differences in dose-response relationship could be examined only in terms of the external dose. Measurements of internal dose (tissue metal levels) constitute the necessary next step of the studies. In case of oral application of the metals in aqueous solution, the effects were of similar kind and magnitude as seen earlier. It was known from the preceding intratracheal NP exposures that in this form much lower per kg doses are sufficient in terms of internal dose and functional effects. Comparison of the results from the present study to those mentioned above indicated that a lower NP dose was enough to evoke the same effects when applied after oral treatment.

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7. APPENDIX

IV. Papp, A., Sárközi, L., *Horváth, E.*, Horváth, E., Sápi, A., Kozma, G., Kónya, Z. Effects in the central nervous system of rats after six weeks exposure to metal oxide nanoparticles through the airways. In: Szilágyi M, Szentmihályi K (Eds) **Trace Elements in the Food Chain Vol. 3,** Deficiency or Excess of trace Elements In Environment as a Risk of Health. Hungarian Academy of Sciences, Budapest, 442-446. (2009)

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