NEUROTOXICITY OF A MODELLED COMPLEX ENVIRONMENTAL HEAVY METAL EXPOSURE IN RATS

Summary of PhD Thesis

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INTRODUCTION

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. 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 ancient countries e.g. in cosmetics and for food and drink containers The human health effect was not obvious at that time and the "food-preserving" effect of metal kitchenware, was appreciated. 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. Exploiting and processing the metals was on one hand a substantial technical achievement but on the other hand a source of human exposure and environmental pollution. The symptoms seen in lead-mine workers were documented by Hippocrates and are described the same way even today. Also the environmental effects of ancient mines and smelters can be detected today.

Chemical risk, resulting from application of xenobiotics including metal-based ones, is present also in today's life 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.

Exposure to heavy metals is mostly occupational. Metal-containing dusts and fumes are generated along the complete life cycle of metal articles 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 chemical form and particle size. Traditionally,

suspended dust (PM10) received especial attention because this “thoracic” fraction can reach the alveoli by inspiration. But as it became possible to investigate the origin and environmental presence of nanoparticles (NPs) and their interaction with living organisms, they turned out to have important health effects. Combustion processes and working on materials at high-temperature generate NP, their composition being determined by the materials worked on or burnt. Manufactured nanomaterials contain at least one component with at least one dimension in the 1-100 nm range. Application of NPs and nanofibres in consumers’ commodities means that routes of uptake such as ingestion and dermal absorption must be considered.

On inhalation, NPs are either deposited in the nasopharynx or get down to the alveoli. NPs are not held back by the usual biological barriers (alveolar and capillary wall) and reach other target organs by different transfer routes and mechanisms, including transcytosis across epithelia. NPs are distributed throughout the body by the circulation. 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, and they can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs. 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.

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. Environmental 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 (like cadmium in cereals, especially rice).
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.

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. Metal ions, interacting with ionized moieties of amino acids in the polypeptide chain, disrupt the non-covalent polar and ionic interactions which stabilize the

secondary (or higher order) structure. The covalent link provided by disulfide bridges is also attacked by numerous heavy metal ions (lead and cadmium have an especial affinity to sulfur).
Numerous cellular functions (muscle contraction, exocrine and endocrine secretion, etc.) are regulated by local changes in Ca^{2+} concentration. Increased intracellular Ca concentration is essential for transmission in chemical synapses. Ca^{2+} ions also carry inward current via selective voltage- and ligand-gated Ca channels, so finally neither side of a chemical synapse can function properly in case another metal interferes with Ca. Disturbed neurotransmission belongs to the toxic spectrum of certain neurotoxic heavy metals.

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.

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, or as NPs, 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?

MATERIALS AND METHODS

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 the table below. The doses were based on the results of previous studies.

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 were dissolved in distilled water to 1 ml/kg b.w. administration volume. The NPs were synthesized at the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics. MnO_2 NPs a diameter of 30.91±9.91 nm, CdO_2 NPs, 65.6±12.4 nm, and PbO NPs, 38.9±7.2 nm. 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.

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.

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.

The po. doses were given once daily, 5 days per week, for 3 and 6 weeks (Mn and Pb) or for 3 weeks (Cd), to 2 x 10 rats per group. 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. Body weight was measured before every treatment to determine the exact dose and to keep track of the weight gain.

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. From the EcoG, the relative spectral power by frequency bands (traditional human EEG bands, delta to gamma) was determined by the software automatically. 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. 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.

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 brain weight, were calculated.

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.

RESULTS AND CONCLUSION

Body weight gain was reduced by all three metals. With Mn and Cd, the effect of the NP form on body weight gain, applied after po. application of the dissolved form, 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. Cd NPs induced even a weight loss. 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, Mn caused hypomotility (reduced ambulation and rearing). These 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 and somewhat longer local activity. Also Cd caused reduced OF motility, most efficiently in the po.+it. scheme: ambulation speed was reduced and events of local activity or immobility became longer.

In the ECoG, 6 weeks of po. Mm application caused a shift to higher frequencies. This change was not made 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 ca. two times 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 could 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.

The most important effects of the three metals are summarized in the following tables (table symbols: ↑, increase; ⇑, significant increase vs. vehicle control; ↓, decrease; ⇓, significant decrease vs. vehicle control; Ø, no change).

Summary of the effects of manganese

Parameters	3 weeks		3+3 weeks		6 weeks		6+6 weeks	
	MnL	**MnH**	**MnL**	**MnH**	**MnL**	**MnH**	**MnL**	**MnH**
Body weight gain	Ø	Ø	↓	⇓	↓	⇓	⇓	⇓
Lung rel. w.	Ø	↑	⇑	⇑	Ø	Ø	⇑	⇑
Liver rel. w.	Ø	Ø	Ø	⇓	Ø	Ø	⇑	Ø
OF amb. time	Ø	Ø	Ø	Ø	⇓	⇓	↓	⇓
local time	Ø	Ø	Ø	Ø	Ø	Ø	⇑	⇑
immob. time	Ø	Ø	Ø	Ø	↑	Ø	⇑	⇑
rearing time	Ø	Ø	Ø	Ø	↑	↑	↓	⇓
SS EP lat. 1 Hz	Ø	⇑	↑	↑	↑	⇑	⇑	⇑
VIS EP lat.	⇑	⇑	⇑	⇑	⇑	⇑	⇑	⇑
AUD EP lat.	⇑	⇑	↑	⇑	⇑	⇑	⇑	⇑
Nerve cond. vel.	⇓	⇓	⇓	⇓	⇓	⇓	↓	⇓

Summary of the effects of lead

Parameters	3 weeks		3+3 weeks		6 weeks		6+6 weeks	
	PbL	**PbH**	**PbL**	**PbH**	**PbL**	**PbH**	**PbL**	**PbH**
Body weight gain	Ø	⇓	↓	↓	↓	⇓	⇓	⇓
Lung rel. w.	Ø	Ø	⇑	⇑	Ø	Ø	⇑	⇑
Liver rel. w.	Ø	Ø	⇑	Ø	Ø	Ø	↑	↑
OF amb. time	Ø	Ø	Ø	Ø	↑	⇑	↑	⇑
local time	Ø	Ø	Ø	Ø	Ø	↑	↑	↑
immob. time	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
rearing time	Ø	Ø	Ø	Ø	Ø	⇓	↓	⇓
SS EP lat. 1 Hz	Ø	↑	Ø	↑	↑	↑	↑	↑
VIS EP lat.	⇑	⇑	⇑	⇑	⇑	⇑	⇑	⇑
AUD EP lat.	⇑	⇑	↑	⇑	⇑	⇑	⇑	⇑
Nerve cond. vel.	⇓	⇓	↓	⇓	⇓	⇓	↓	⇓

Summary of the effects of cadmiumk

Parameters	3 weeks **Cd3**	3+1 weeks **Cd31**	1+3 weeks **Cd13**
Body weight gain	⇓	⇓	⇓
Lung rel. w.	Ø	⇑	⇑
Liver rel. w.	↓	⇓	↓
OF amb. time	↓	⇓	↓
local time	↑	⇑	↑
immob. time	Ø	⇑	↑
rearing time	↓	⇓	Ø
SS EP lat. 1 Hz	Ø	↑	↑
VIS EP lat.	⇑	⇑	↑
AUD EP lat.	⇑	⇑	↑
Nerve cond. vel.	⇓	⇓	⇓

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.

THE APPLICANT'S RELEVANT PUBLICATIONS

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ACKNOWLEDGEMENT

I would like to thank to Prof. Dr. László Nagymajtényi, Head of the Department of Public Health, for the opportunity to carry out this PhD work, and that he always provided the appropriate conditions.

I am especially grateful to my supervisor Dr. András Papp who helped to acquire the methodological background, to whom I could turn at any circumstances if I had any questions. Without his help and advice this study could not have been realized.

Many thanks to my colleagues, Dr. Andrea Szabó, Dr. Gábor Oszlánczi, Zsuzsanna Máté, Szabolcs Takács, and Viktória Nagy for supporting me and helping me with the daily work.

Many thanks to Dr. Zoltán Kónya Head of the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics, and to Dr. András Sápi and Péter Pusztai, for providing me the necessary nanomaterials.

And last but not the least, I would like to thank Anita Balázs, Gyuláné Kiss, János Kis and Mihályné Németh for their assistance in working with animals, and finally to Imre Gera and Lászlóné Szalai for their technical help.