PhD Thesis

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

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Introduction and Aims

Foreign materials in the air can be of both natural and artificial origin; and can be highly variable in chemical composition and other properties such as physical state, volatility, particle size etc. Airborne particles can be classified by their size as sedimenting dust (>10 μm), suspended or fine dust (100 nm-10 μm; often called PM10) and ultrafine dust or nanoparticles (NPs, <100 nm). The origin, environmental presence and health effects of NPs gained much attention in the last ca. 20 years due partly to substantial developments in methodology and instrumentation.

Environmental NPs are either natural or man-made. Within the latter, emission of NPs as pollutants, and synthesis of NPs as nanomaterials for novel technologies, are to be distinguished. Natural sources of NPs include forest and bush fires, volcanoes, sea spray, erosion of rocks and soil, etc. The main anthropogenic sources of NPs are combustion, other high temperature processes, and working on solid materials (although mechanical effects do not generate many particles much below the 100 nm limit). Aerosol concentration (which necessarily includes NPs) is today an important measure of ambient air quality; regulated by national and international (EU).

Nanotechnology, purposeful production and application of nano-sized particles and structures, is another potential source of emission of NPs. Manufactured nanomaterials contain at least one component that has at least one dimension in the 1 to 100 nm range, and appear in personal care products, electronics, fuel cells, and many consumers' goods. Application of NPs and nanofibres in consumers' products means that routes of uptake such as ingestion and dermal absorption, must be considered in addition to inhalation. Particles in the inhaled air are deposited at different sites within the airways, determined first of all by their size. NPs are either deposited in the nasopharynx or get down to the alveoli. Once deposited, NPs translocate readily to other body parts by different transfer routes and mechanisms. This involves transcytosis (by caveola formation) across epithelia of the respiratory tract into the interstitium. By crossing the alveolar and capillary membrane, NPs reach the blood circulation directly or via the lymph drainage. Extrapulmonary effects depend on particle size and solubility, site of deposition, and the integrity of the epithelial lining. Metal ions dissolved from the surface of metal-containing NPs also must be considered among the mechanisms of action. The primary olfactory neurons provide a direct pathway for NPs to the brain.

A major problem about the direct and indirect health effects of NPs is the lack of toxicity data. Substances in nano form can show profoundly different properties than in more conventional states. Due to their small size, high number concentration, and large specific surface area, NPs have greater biological activity than larger particles, including oxidative stress induction. Even relatively inert materials are more toxic and inflammatogenic in NP form than in more coarse particles. NPs also generate reactive oxygen species more intensely than larger particles, and can contribute to adverse health effects in the respiratory tract as

well as in extrapulmonary organs. Epidemiological evidence associates enhanced level of ambient NPs with adverse respiratory and cardiovascular effects.
High temperature procedures of the metal technology emit a lot of particles, so that exposure to metal-containing airborne NPs is primarily an occupational hygienic problem. For this thesis, three metals – Mn, Pb and Cd – were chosen; based on practical importance and on previous experiences at the Department.

Lead (Pb) has been a ubiquitous environmental pollutant with no known biological functions and toxicity even in low doses.
Both primary production from ore and reprocessing of Pb waste is based on smelting, a high-temperature process with substantial emission of metal fumes. Leaded petrol (still in use in several countries) results in fine and ultrafine particles of PbO_2 in the exhaust.
Pb causes disruption of the biosynthesis of haemoglobin. Its nervous systems effects can manifest in brain damage; diminished learning ability and behavioral problems (aggression, impulsive behavior and hyperactivity) in children; and peripheral neuropathy. Pb can also enter to the foetus through the placenta and cause serious damage to the nervous system before birth. 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. In adults occupationally exposed to Pb, alterations of various forms of central and peripheral evoked activity, like sensory evoked potentials and nerve conduction velocity, were described. In our earlier studies Pb, given orally to rats, altered cortical electrical activity and memory performance. At presynaptic endings, Pb blocks voltage-gated Ca-channels but is partly permeated to the intracellular space, where it acts as false activator of various Ca-dependent processes. Alterations in the dopaminergic, cholinergic and glutamatergic control of behaviour were observed. Pb may induce oxidative stress which in turn may be involved in toxic effects of Pb such as neurodegeneration and cognitive problems.

Cd has been used for industrial purposes (electroplating, pigments, coatings, Cd-based semiconductors, Ni-Cd batteries). The applications have been decreasing in the last two decades, because Cd is one of the most toxic environmental and industrial pollutants due to its ability to damage, among other, the lungs, liver, kidney, testis and the placenta. Significant inhalation of Cd can occur from tobacco smoke and in occupational settings. Extrapyramidal damage, amyotrophic lateral sclerosis, optic nerve damage, striatal damage and peripheral polyneuropathy were observed as long term neurotoxic consequences of Cd. In children, a straight relationship between hair Cd and altered visual or auditory evoked potential parameters was found. Cd can block the presynaptic influx of Ca which may result in altered transmitter release so that Cd possibly affects the balance of excitation/inhibition. It also significantly increases malondialdehyde level and glutathione peroxidase activity, and causes oxidative stress indirectly by displacing Fe and Cu from metalloenzymes.

Mn is, in contrast to Pb and Cd, an essential micronutrient. Within the organism, it is mainly found in tissues rich in mitochondria. Mn-containing metalloenzymes include superoxide dismutase and glutamine synthetase. Overexposure to Mn has traditionally been an occupational risk factor in, e.g., mining and the metal industry. In steel, manganese improves hardness, strength, and wear resistance. Welding rods are frequently coated with a Mn-containing layer to reduce the oxidation of the steel parts to be jointed at the temperature of welding. Regarding the composition of welding fumes and similar industrial emissions, workplace exposure to Mn-containing NPs is very likely. A novel use of Mn is in the production of semiconductor nanocrystals and nanoflowers. Chronic inhalation of manganese compounds is known to cause severe neurologic disorders. The illness, called manganism, progresses in three stages from apathy, anorexia, asthenia, hypersomnia, weakness of the legs and irritability, through psychomotor and psychic disturbances to the final Parkinson-like syndrome. Disorders with electrophysiological signs after Mn exposure include myoclonus in welders and epileptic activity in over-exposed children. EEG and visual evoked potential alterations were observed in persons with elevated blood Mn. Oxidative stress, including mitochondrial toxicity and abnormal activity of Mn-SOD and glutathione peroxidase, is apparently important in the neurotoxicity of Mn.

Exposure to airborne metal particles is an important issue in occupational, and possibly environmental, hygiene. In previous experiments of the Department it was found that recording and analysis of electrophysiological signals from the brain and from a peripheral nerve is a sufficiently sensitive method to detect the effects of orally applied lead, cadmium and manganese on the nervous system of rats. In the present work, a new model with more realistic chemical form of the metals (oxide NPs) and more realistic way of application (instillation into the trachea) was introduced and the electrophysiological measurements were supplemented with behavioral and chemical ones.
The questions, to be answered on the basis of the results of this work, are as follows:

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

Materials and Methods

Young adult male Wistar rats (280–350 g body weight at start) were used for the experiments, in groups of 8-10 animals per treatment dose and time. The animals were housed under standard conditions (22–24 °C, 12 h light/dark cycle with light starting at 6:00 a.m., up to four rats in one cage) with free access to conventional standard rodent chow and drinking water.

Metal nanoparticles were synthesized at the Department of Applied Chemistry, University of Szeged, Faculty of Science and Informatics. MnO_2 nanoparticles were made by a technique combining sonication and hydrothermal treatment. An appropriate amount of aqueous $KMnO_4$ solution was mixed with ethylene glycol and sonicated with an ultrasound device. The resulting dark suspension was loaded to a Teflon-lined stainless steel autoclave, and heated at 200°C for 16 h, then allowed to cool to room temperature. The brownish precipitate formed was filtered and washed with 80°C preheated distilled water, and finally dried at 100°C for 1 h. CdO_2 and PbO were produced in solid phase, by milling the base materials ($CdCl_2$ with Na_2CO_3; $Pb(CH_3COO)_2$ with NaOH) and calcining the intermediate product carbonate or hydroxide.

The chemical purity of the nanoparticles was checked by X-ray diffraction, and their particle size, by X-ray diffraction and transmission electron microscopy (TEM). The mean diameter of MnO_2 particles was ca. 23 nm, of PbO was ca. 19 nm, of CdO_2 was ca. 20 nm. For administration to the rats, the nanoparticles were suspended in distilled water. The suspension was sonicated to prevent aggregation, and was instilled into the rats' trachea 5 days a week, for 9 weeks (in case of Mn) or 6 weeks (in case of Pb and Cd). The instilled volume was 1.0 ml/kg b.w.; vehicle controls had distilled water only. The treatment scheme was as follows:

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

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

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

At the end of the treatment period the rats' spontaneous locomotor activity was measured in an open field (OF) box of 48x48x40 cm size, equipped with two arrays of infrared movement detectors at floor level and in 12 cm height. The animals were placed into the centre of the box for one 10-min session, and the instrument recorded their horizontal and vertical motor activity. From these data, counts, time and run length of the activity forms (ambulation, local activity, immobility, rearing) were automatically calculated.

On the day following the OF test, the animals were anaesthetized by intraperitoneal injection of 1000 mg/kg b.w. urethane. The head of the rats was fixed in a holder, the skin was opened by a mid-sagittal cut and the muscles and connective tissues adhering to the skull were removed. The left hemisphere was exposed by removing most part of the temporal bone. Wounds were sprayed with 10 % lidocaine and the exposed cortex was protected with a thin layer of petroleum jelly.

After 30 minutes recovery, the rat was laid into the stereotaxic frame of the recording setup, and silver electrodes were placed on the primary somatosensory (SS), visual (VIS) and auditory (AUD) areas. The recording sequence started with 6 minutes of ECoG. Then, sensory EPs were recorded by applying the sensory stimuli in trains of 50. For somatosensory stimulation, 2 needles were inserted into the contralateral whisker pad to deliver square electric pulses (3-4 V, 0.05 ms, 1-10 Hz). Visual stimulation was produced by a high-luminance white LED aimed directly at the rat's right eye, driven by 0.2 ms pulses. The acoustic stimuli were clicks (40 dB) of a small earphone guided into the animal's right ear via the hollow ear bar. The frequency of stimulation was 1 Hz in all modalities, plus 2 and 10 Hz for SS stimulation in order to observe frequency-dependent changes. Finally, compound action potential form the rat's tail nerve was recorded. Two stimulating needle electrodes (delivering 4 -5 V, 0.05 ms, 1 Hz pulses) were inserted into the tail base; and another two, for recording, 50 mm distally. Ten stimuli were applied, at 1 Hz rate to determine action potential latency; and at higher rates to see the frequency dependence of latency (and amplitude). From the ECoG records, the relative band powers (standard human EEG bands: delta, theta, alpha, beta1, beta2, and gamma) ware obtained automatically. The cortical EPs and tail nerve potential were averaged, and latency and duration was measured manually. From the tail nerve latency data, conduction velocity was obtained.

Right after finishing electrophysiological investigation, the animals were sacrificed by an overdose of urethane, and were dissected. Organs were removed and weighed, and the relative organ weight of the brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals, related to 1/100 of body weight or to the brain weight, was calculated. Blood, brain, lung and liver samples were collected and stored at -22°C. From the samples, protein content, SOD and Mn-SOD activity, and GSH content was determined.

For GSH assay, the SH groups bound to nonproteins were quantified by Ellmann reagent (DTNB) from protein-free samples. SOD assay was carried out by a method based on the

effect that this enzyme in alkaline medium can inhibit the spontaneous adrenaline-adrenochrome transition depending on concentration. For metal level determination, ca. 1 g samples were digested in 5 ml 65 % HNO_3 at 90°C for 90 min. Metal level was determined by inductively coupled plasma mass spectrometry (at the laboratory of the MOL Hungarian Oil and Gas Company).

Statistical evaluation of all data was done by one-way ANOVA after Kolmogorov-Smirnov normality check. The relationship between the bodily metal load and the measured neuro- and general toxicological parameters was tested by linear regression. During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed.

Results and Discussion

Effects of Mn NP exposure

Intratracheal instillation of MnO_2 NPs significantly reduced the rats' body weight gain. From the 6^{th} week on, there was no weight gain in the *Mn-LD* group, and the rats in *Mn-HD* showed even some weight loss. The relative weight of the lungs showed massive increase, while the relative liver weight decreased. In the *Mn-HD* group, increased weight of the adrenals was probably due to stress.

The OF motility decreased dose- and time-dependently. The time spent with ambulation decreased significantly already after 3 weeks treatment. The corresponding increase in the time share of local activity and immobility, and the decrease in rearing, became significant by the 9^{th} week. Moreover, not only the time of ambulation but also the distance covered, and the speed of walking, were reduced in the Mn-treated rats, and these were apparently reluctant to change to ambulation once they were only locally active or not active at all.

In the ECoG there was a shift towards higher frequencies, gradually developing and reaching significance by the 6^{th} and 9^{th} week (after 9 weeks, not only in the *Mn-HD* but partly also in the *Mn-LD* group). There was no qualitative difference between the spectrum changes seen in the recorded cortical areas.

From the data of the cortical EPs, onset latency was the most sensitive to the Mn NP exposure. Both doses caused latency lengthening of the SS EP after 9 weeks exposure but the effect of the high dose was stronger. On the VIS and AUD EP latency, only the higher dose caused significant lengthening. The conduction velocity of the tail nerve was significantly decreased after 9 weeks exposure but the dependence of this parameter on the frequency of stimulation was not altered.

Significant Mn deposition was detected in the treated rats' brains after 9 weeks exposure, which was apparently saturating as the difference between *Mn-HD* and *Mn-LD* groups was little. Blood levels were more proportional to the dose.

In the total SOD and Mn-SOD activity of the brain samples, the Mn NP exposure caused an increase with some dose-dependence. Only the increase of total SOD was significant. The

level of reduced glutathione, another indicator of the local redox conditions, was moderately decreased in the treated rats' brains.

The correlation of Mn load and the toxicological parameters studied was significant only for the ECoG index and the nerve conduction velocity with the brain Mn level. ECoG index was also correlated with brain GSH level.

Effects of Pb NP exposure

Intratracheal treatment by Pb NPs also caused significant retardation in the rats' body weight gain, from the 2nd treatment week on.

The lungs showed significant increase of relative weight after 6 weeks treatment, and had in the *Pb-HD* group a strongly emphysematous appearance. The decrease in the relative weight of the liver, and increase in that of the kidneys, remained below significance.

After 6 weeks Pb NP exposure, rats in both treated groups spent significantly more time with ambulation than the controls. The decrease in rearing, and even more the increase in local activity, was less characteristic and was significant only vs. *Con*.

In the ECoG activity, a weak trend of decreased intensity in the low and increase in the high frequency bands was seen, but this was significant mostly in the *Pb-HD* group only, and not in every recorded area. The latency of the SS EP was hardly different in the *Con* and *W* groups, and noteworthy frequency-dependent increase was seen only with 10 Hz stimulation. In the *Pb-LD* group, there was minor lengthening of latency but the frequency-dependent increase at 10 Hz stimulation (vs. 1 Hz) became significant. In the *Pb-HD* group, significant latency increase was seen and the frequency-dependent increase was also more pronounced.

The VIS and AUD EP also had increased latency in the *Pb-HD* group In line with the lengthened cortical latencies, the conduction velocity of the tail nerve was reduced in the treated groups. In the frequency dependence of the conduction velocity there was, however, no significant alteration.

The 6-weeks application of PbO NPs resulted in massive increase of the measurable Pb content of the tested organs, and the metal levels were roughly proportional to the dose.

The treatment diminished the SOD activity in each organ included in the biochemical analysis but the change must have happened in the Mn-independent SOD fraction because the Mn-SOD activity had no similar trend. GSH level significantly increased in liver and lung, but not brain, samples. The correlation test of Pb load and toxicological parameters showed that body weight gain, ECoG index and nerve conduction velocity were in quite strong and significant correlation with the Pb level in the blood or the brain. Biochemical parameters showed much weaker correlation.

Effects of Cd NP exposure

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

end of the treatment period. Increase of lung and thymus weight, and decrease of spleen weight was seen in the treated rats.
After 3 weeks Cd NP exposure, there was no clear change in the band spectrum of the treated rats' ECoG. After 6 weeks, a shift to higher frequencies was seen, which was significant in the *Cd-HD* group. The latency of the SS EP was significantly lengthened after 6 weeks in the same group and in this group the frequency dependence was also stronger than in the controls. The VIS and AUD EPs also had lengthened latency, in VIS in both treated groups vs. *Con*.
In the tail nerve the Cd NP exposure had no significant effect; and there was no noteworthy change in the rats' open field behavior either.
No Cd was detected in the brain and blood samples; the massive increase of lung and liver Cd levels indicated all the same that the treatment was technically correct and effective. Mn-SOD levels decreased in the samples from the Cd-treated rats but mostly only moderately. GSH increased in the brain and liver, but decreased in the lungs.

Discussion

Investigation of the nervous system effects of NPs in rats using subacute intratracheal instillation and a set of neuro-functional tests, supplemented with chemical ones, apparently constitutes a model of human exposure and its consequences to which no direct parallel was found in the literature. In terms of internal exposure, the model proved adequate; that is, the intratracheal application of metal NPs for several weeks caused significant increase of the Mn, Pb and Cd level in the tissue samples of the treated rats. And, compared to the results of earlier works of the Department using oral administration of heavy metals, it seemed that intratracheal instillation of NPs into the trachea was – at least in case of Mn and Pb – especially efficient in inducing increased metal levels in tissue samples.
A comparison to human exposure is possible only on the basis of blood metal levels. Application of Pb NPs gave rise to internal doses comparable to those found in exposed workers. Blood levels in the Mn-treated rats were considerably higher than in the known human cases but the difference between exposed and unexposed was similar. The lack of detectable blood and brain levels in the Cd-treated rats was most probably due to the specific deposition sites of this metal.
The qualitative similarity of the majority of neuro-functional alterations seen in rats treated with the three different metal NPs suggest that common mechanisms of action exist. These can be first of all oxidative stress, interference with Ca-dependent phenomena, and effects on transmitter systems. Increased levels of oxidative stress indicators, and ROS generation, has been reported with each of the metals. A likely consequence, membrane lipid peroxidation, changes membrane fluidity. This will then disturb all receptor-bound phenomena, such as synaptic transmission, which is possibly reflected in the cortical activity. Alterations in Ca-dependent presynaptic transmitter release, and in the efficacy of glutamatergic exitatory transmission, had influence on evoked activity, while changes of the ECoG reflected also changes of the ascending cholinergic activation. Slowed conduction velocity could be

explained by the effect of the metals on ion channels and/or on mitochondrial energy production.
The changes in open field motor behavior, observed in the Mn- and Pb-treated rats, indicted effects on the dopaminergic control. Dopaminergic neurons are especially vulnerable to oxidative stress which is a likely common effect of the three metals. Reduced horizontal motility after Mn NP exposure may be analogous to the welders' Parkinson-like syndrome, and increased locomotion of the Pb-treated rats, to "attention deficit hyperactivity disease" found to be more frequent among children with elevated blood Pb.

Conclusion

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

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

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The Applicant's Relevant Publications

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