NEUROTOXIC EFFECTS OF METAL OXIDE NANOPARTICLES ON THE SOMATOSENSORY SYSTEM OF RATS FOLLOWING SUBACUTE INTRATRACHEAL APPLICATION

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ABSTRACT: Metal-containing nanoparticles have newly been recognized as one of the major factors in occupational and other inhalation exposures. Fumes containing manganese, cadmium or lead are formed in high-temperature industrial processes. Metal oxide (MnO_2, CdO_2, PbO) nanoparticles were synthesized and instilled in aqueous suspension in the trachea of rats for 6 weeks, and functional changes in the somatosensory system were studied. From treated rats, as well as untreated and vehicle controls, spontaneous and stimulus-evoked activity of the primary somatosensory cortex, and compound action potential from the tail nerve, were recorded in urethane anaesthesia. In the spontaneous activity there was a shift to higher frequencies in all treated groups. The latency of the somatosensory evoked potential was typically lengthened and its dependence on the frequency of stimulation increased by high dose of Cd and Pb, and by high and low dose of Mn. On the tail nerve, Pb had the strongest effect. The similarity of the three metals' effects indicated common mechanisms of action, most likely disturbance of Ca-dependent neuronal phenomena and oxidative stress.

KEY WORDS: nanoparticles, manganese, cadmium, lead, neurotoxicity

INTRODUCTION

A number of procedures in the metal industry (mainly high-temperature operations such as casting, welding, flame cutting etc.) generate so-called metal fumes, an aerosol consisting of suspended metal and metal compound (mainly oxide) particles. Inhalation of metal fumes is a major route of occupational metal exposure and the direct cause of acute and chronic diseases like metal fume fever and COPD (Balmes, 2004).

Manganese is used in steel alloys, in manufacturing of dry cell batteries, in organo-Mn fungicides, and in the petrol additive methylcyclopentadienyl manganese

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tricarbonyl (MMT). Mn is an essential microelement necessary for several organs including the brain (mitochondria, astrocytic glutamine synthetase) (Normandin and Hazell, 2002). Excess Mn inactivates glutamine synthetase and hyperactivates superoxide dismutase, resulting in oxidative damage of dopaminergic structures. The final result is manganism (an occupational disease resembling Parkinson's disease) seen in workers exposed to airborne Mn (Bowler et al., 2006), and modelled in animals (Yu et al., 2003). The effect of inhaled Mn on the brain can also lead to epilepsy (Hernandez et al., 2003). In our earlier studies, behavioural and electrophysiological changes were found in rats after several weeks of oral Mn exposure (Vezér et al., 2005).

Cadmium has industrial applications in manufacturing of steel and other alloys, pigments, batteries, and in electroplating. Occupational exposure is mainly due to inhalation of metal fumes, and the exposure of smokers due to Cd content of tobacco leaves is similar (Elinder et al., 1983). Although the main target organs of Cd are the liver, kidneys and bones, and the respiratory and cardiovascular systems, Cd can also cause behavioural and neurological disorders. Reduced visuomotor performance, and difficulties of concentration and postural balance, were observed in occupational Cd exposure (Viaene et al., 2000). In children, a straight relationship was found between hair Cd content and visual or auditory evoked potential parameters (Thatcher et al., 1984). Analogous effects were observed in rats (Agar et al., 1990; Yargicoglu et al., 1996). Our previous works on Cd neurotoxicity revealed altered electrocorticogram (ECoG) power spectrum, and effects on cortical evoked potentials and peripheral nerve action potentials (Papp et al., 2003; Institóris et al., 2002).

The main sources of airborne lead have been leaded petrol (still in use in some countries) as well as processing and reprocessing of Pb. The nervous system is an important target of Pb toxicity. Impaired IQ and different behavioural abnormalities were found in children with elevated body Pb level (Needleman and Gatsonis, 1990) together with electrophysiological abnormalities (Otto et al., 1985). In Pb-exposed workers, cortical sensory evoked potentials and nerve conduction velocity were affected (Lille et al,. 1988; Discalzi et al., 1993; Araki et al., 2000). Blood Pb of occupationally exposed persons was in good correlation with impaired neurobehavioural performance (Schwartz et al., 2001) and increased postural sway (Yokoyama et al., 2002). The nervous system effects of Pb exposure were successfully replicated in animal experiments (Nagymajtényi et al., 1997; Vezér et al., 2000).

In inhalational exposure, the size of the aerosol particles is a decisive factor. Particles of less than 1 μm aerodynamic diameter are routinely regarded as "ultrafine dust" or PM1 (EN 481). Their health importance has been recognized newly. Although these nanoparticles constitute a small fraction of the total dust in mass units, they are numerous, resulting in high specific surface area. Indeed, area proved to be more accurate dose measure in ultrafine particles toxicology than mass. (Oberdörster et al., 2005). The large surface combined with the chemical characteristics (high reactivity of metal oxides in the present case) may induce massive biological responses. Another typical characteristic of nanoparticles, relevant for toxic effects, is mobility within the organism (endo- and transcytosis, axonal transport etc.); theoretically predicted (Oberdörster et al., 2005) and experimentally verified (Elder et al., 2006; Roels et al., 1997).

Within the framework of the *Regional University Knowledge Centre for Environmental and Nanotechnology, Szeged, Hungary* a series of animal experiments has been carried out to see the nervous system effects of toxicologically relevant nanoparticles. Here, the effects on the somatosensory system seen after 6 weeks application of oxide nanoparticles of the mentioned three metals are described.

MATERIALS AND METHODS

Adult male Wistar rats (300–350 g body weight) were obtained at the university's breeding centre and were housed in standard conditions (22 °C, 12-h light/dark cycle with light on at 06:00, free access to tap water and standard rodent chow). The experiments with the three metal oxide nanosuspensions (see below) were carried out separately. For each, there was an untreated control group (Con), a vehicle control group (W), and a low dose (LD) and a high dose (HD) group; with 10 rats in each group at start. The doses applied are given in *Table 1*. They were based, on one hand, on data about the ventilation volume of rats (Strohl et al., 1997); and on the other hand, on published inhalation toxicity effects in rats (Cd: Takenaka et al., 2004; Pb: Pinon-Lataillade et al., 1993; Coffigny et al., 1994). In case of Mn, previous experience (Sárközi et al., 2007) could be relied upon.

TABLE 1. Treatment groups and doses

Group	Code	Substance	Dose (mg metal / kg b.w.)
Untreated control	Con	–	–
Vehicle control	W	Distilled water	–
Manganese low dose	LD-Mn	MnO_2 nanosuspension	2.63
Manganese high dose	HD-Mn		5.26
Cadmium low dose	LD-Cd	CdO_2 nanosuspension	0.04
Cadmium high dose	HD-Cd		0.4
Lead low dose	LD-Pb	PbO nanosuspension	2
Lead high dose	HD-Pb		4

The nanoparticulate metal oxides were synthesized at the Department of Applied Chemistry, University of Szeged. MnO_2 nanoparticles were made by a technique combining sonication and hydrothermal treatment. CdO_2 and PbO were produced in solid phase, by milling the base materials ($CdCl_2$ with Na_2CO_3; $Pb(Ac)_2$ with NaOH) and calcining the intermediate product carbonate or hydroxide. Particle size (20–30 nm) was determined by X-ray diffraction and transmission electron microscopy.

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 6 weeks. The instilled volume was 1.0 ml/kg b.w.; vehicle controls (group W, water) had distilled water only. Intratracheal instillation

was performed in quick diethyl ether anaesthesia. The anaesthetised animal was suspended on a board, fixed at 60° to horizontal, by hanging its upper incisors in a wire loop which held the animal in place, and its mouth open. Focussed light was aimed transdermally on the trachea, the tongue was pulled forward with a pair of non-traumatic forceps, and a custom-made laryngoscope was used to gain access to the glottis. The nanosuspension (or distilled water for the controls) was instilled into the trachea by means of a syringe and 1.2 mm OD plastic tubing, inserted between the vocal chords.

One or two days after the last instillation, the animals were prepared for electrophysiological recording. In urethane anesthesia (1000 mg/kg b.w. ip.), the animal's head was fixed in a stereotaxic frame, and the left hemisphere was exposed by opening the bony skull. Lidocaine (10%) was sprayed on the wounds, and the exposed dura was protected by a thin layer of vaseline. After 30 minutes recovery, a silver recording electrode was placed on the primary somatosensory (SS) area. Electrocorticogram (ECoG) was taken for 6 minutes and the relative spectral power of the frequency bands (delta, theta, alpha, beta1, beta2, gamma; standard human EEG bands as described in Kandel and Schwartz, 1985) was determined. Then, somatosensory stimuli (square electric pulses: 3-4 V, 0.05 ms) were delivered to the contralateral whisker pad of the rat via a pair of needle electrodes. One train of 50 stimuli was applied at 1, 2 and 10 Hz frequency. After averaging, latency and duration of the evoked responses (EPs) was measured manually (for details, see Papp et al., 2003). To see the effects on peripheral nerves, compound action potential was recorded from the rat's tail nerve. Two stimulating needles (delivering 4–5 V, 0.05 ms pulses at 1, 20 and 50 Hz) were inserted into the tail base; and another two, for recording, 50 mm distally. From the records, the conduction velocity of the nerve was calculated. The change of the latency of the somatosensory EP, and latency and amplitude of the nerve action potential, with increasing stimulation frequency was also investigated as an indicator of the action of the treatment on the state of the nervous system (Papp et al., 2004). All electrophysiological recording and analysis were done by means of the Neurosys 1.11 software (Experimetria Ltd, Budapest, Hungary). Following electrophysiology, the rats were sacrificed by an overdose of urethane. All results were tested for significance with one-way ANOVA and the post hoc analysis was done by Scheffe's test. During the whole procedure, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed.

RESULTS

Spontaneous cortical activity

Six weeks exposure by the metal oxide nanoparticles caused decrease in the low and increase in the high frequency bands of the ECoG. In case of Mn and Cd, also the effect of the low dose was significant whereas in the LD-Pb group the change was negligible *(Fig. 1)*. The difference between the ECoG band spectrum of the Con

and W groups was minimal indicating that the changes seen in the treated groups were probably not caused by the repeated ether anaesthesia and instillation of distilled water.

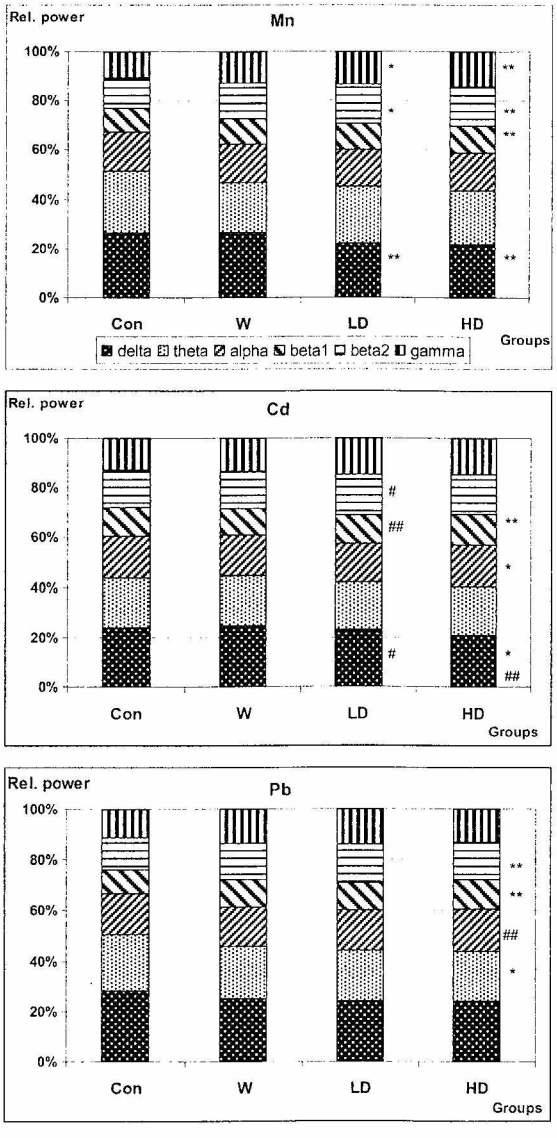

Fig. 1. Power spectrum of the spontaneous activity of the rats' somatosensory cortex after 6 weeks treatment with Mn, Cd and Pb (see Table 1 *for doses and group codes). Abscissa: groups. Ordinate: relative power of the ECoG bands (see insert in the upper graph).*
**, **: p<0.05, 0.01 vs. Con; #, ##: p<0.05, 0.01 vs. W, always between identical bands).*

Cortical evoked potentials

In the untreated controls, the latency of the somatosensory EP was between 7.5 and 8 ms at low (1 Hz) frequency stimulation. In the W groups, the values were about the same in each experiment. In the Mn experiment, lengthening of the latency was seen with both doses *(Fig. 2)* and the differences vs. control and HD vs. LD suggested a dose-dependent effect. Reduced frequency-following ability of the cortex was indicated by the significant latency increase at 10 Hz vs. 1 Hz stimulation in the treated groups. The duration of the EPs was more variable and was significantly reduced only in the HD-Mn group.

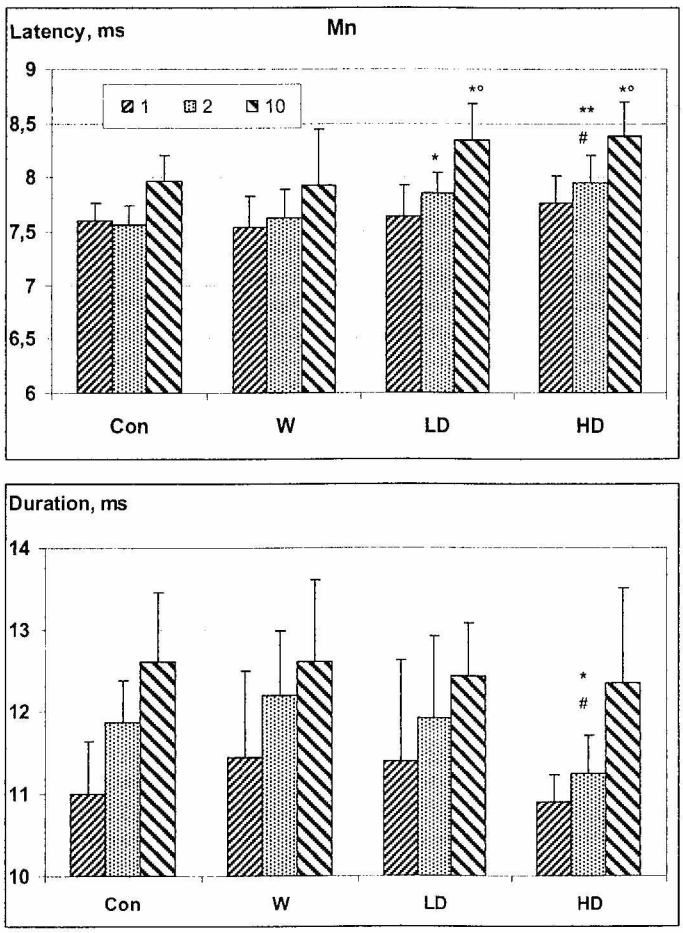

Fig. 2. Latency (left) and duration (right) of the somatosensory evoked potential obtained with 1, 2 and 10 Hz stimulation (see insert in upper graph) of the whiskers in control and Mn-nanoparticle treated rats. Mean+SD, n=10.
**, **: p<0.05, 0.01 vs. Con; #: p<0.05 vs. W; °: p<0.05 vs. 1 Hz stimulation within the same group.*

Application of CdO_2 nanoparticles caused significant latency lengthening only in the HD-Cd group *(Fig. 3)*. In this group, also the duration of EP was significantly lengthened, at all stimulation frequencies, vs. Con and vs. W. Also noteworthy is the latency increase at 10 vs. 1 Hz stimulation in the HD-Cd group.

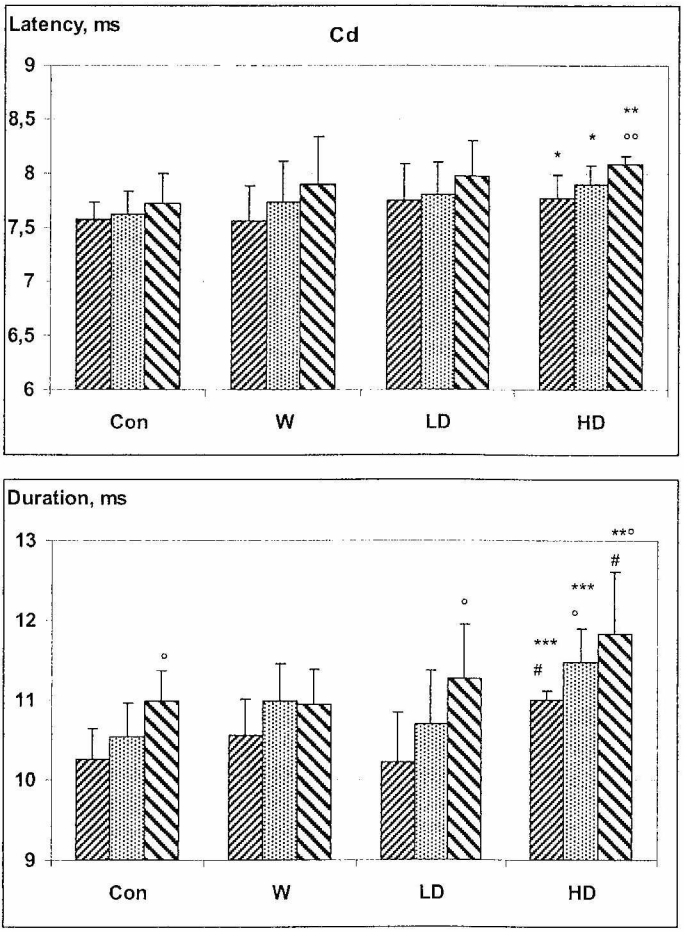

Fig. 3. Latency (left) and duration (right) of the somatosensory evoked potential obtained with 1, 2 and 10 Hz stimulation of the whiskers in control and Cd-nanoparticle treated rats. Mean+SD, n=10.
*, **, ***: *p<0.05, 0.01, 0.001 vs. Con;* #: *p<0.05 vs. W;* °: *p<0.05 vs. 1 Hz stimulation within the same group.*

The effect of instillation of PbO nanoparticles had significant effect on the EP latency only in the high dose group *(Fig. 4)*. In this group, besides the lengthening itself, also the frequency dependent increase of the latency was significant, in con-

trast to the controls and the LD-Pb group. Increased frequency dependence was seen also on the duration of EPs.

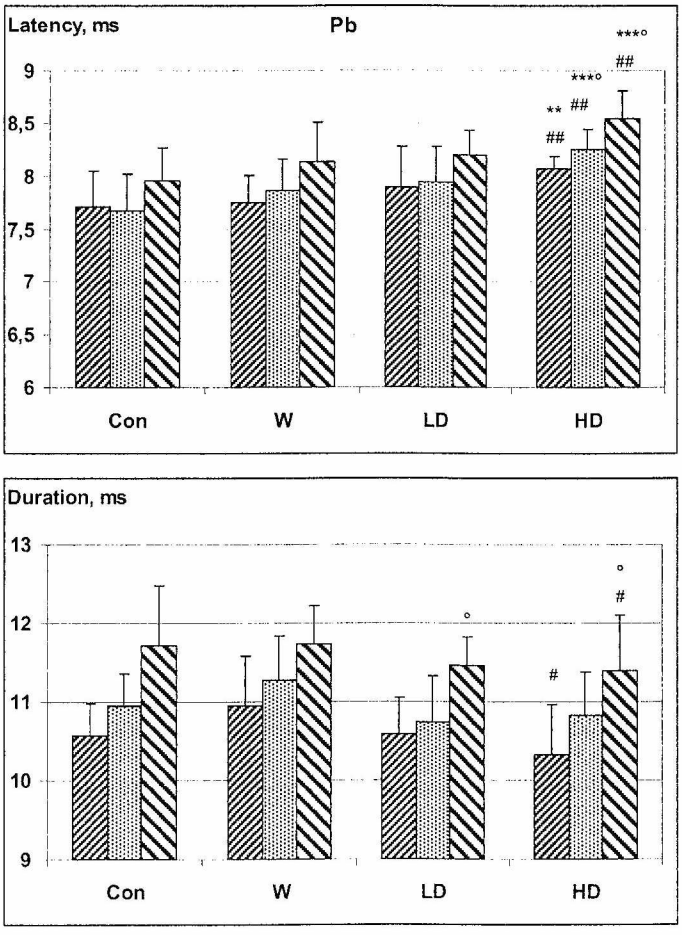

Fig. 4. Latency (left) and duration (right) of the somatosensory evoked potential obtained with 1, 2 and 10 Hz stimulation of the whiskers in control and Pb-nanoparticle treated rats. Mean+SD, n=10.
**, **, ***: p<0.05, 0.01, 0.001 vs. Con; #, ##: p<0.05, 0.01 vs. W; °: p<0.05 vs. 1 Hz stimulation within the same group.*

Tail nerve action potential

Only Mn and Pb had a noteworthy effect on the tail nerve conduction velocity. The decrease caused by both metals seemed to be dose-dependent *(Fig. 5)*. The relative increase of the latency and decrease of the amplitude of the tail nerve action potential on 20 and 50 Hz stimulation vs. slow (1 Hz) stimulation was present also in the

Con and W groups. These dynamic parameters were not much altered by MnO_2 nanoparticles; the effect of CdO_2 and PbO was, in contrast, significant *(Fig. 6)*. In the HD-Cd group, mostly latency was affected, while in the HD-Pb group frequency-dependent latency increase and amplitude decrease were both more intense than in the controls.

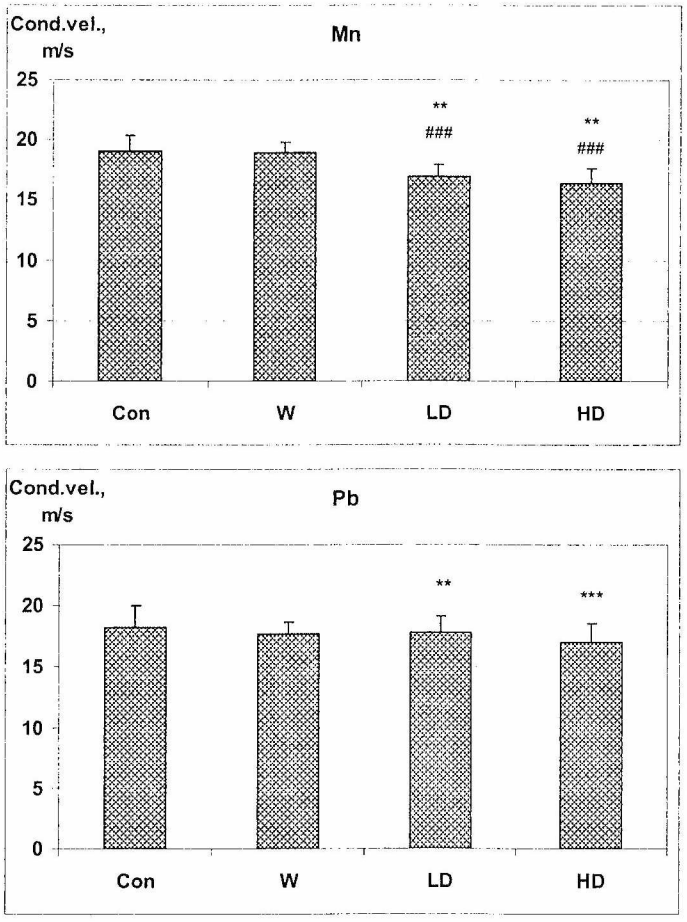

Fig. 5. Conduction velocity of the rats' tail nerve in the controls and after Mn-nanoparticle (upper) and Pb-nanoparticle (lower) treatment. Mean+SD, n=10.
***, ***: p<0.01, 0.001 vs. Con; # ##: p<0.001 vs. W.*

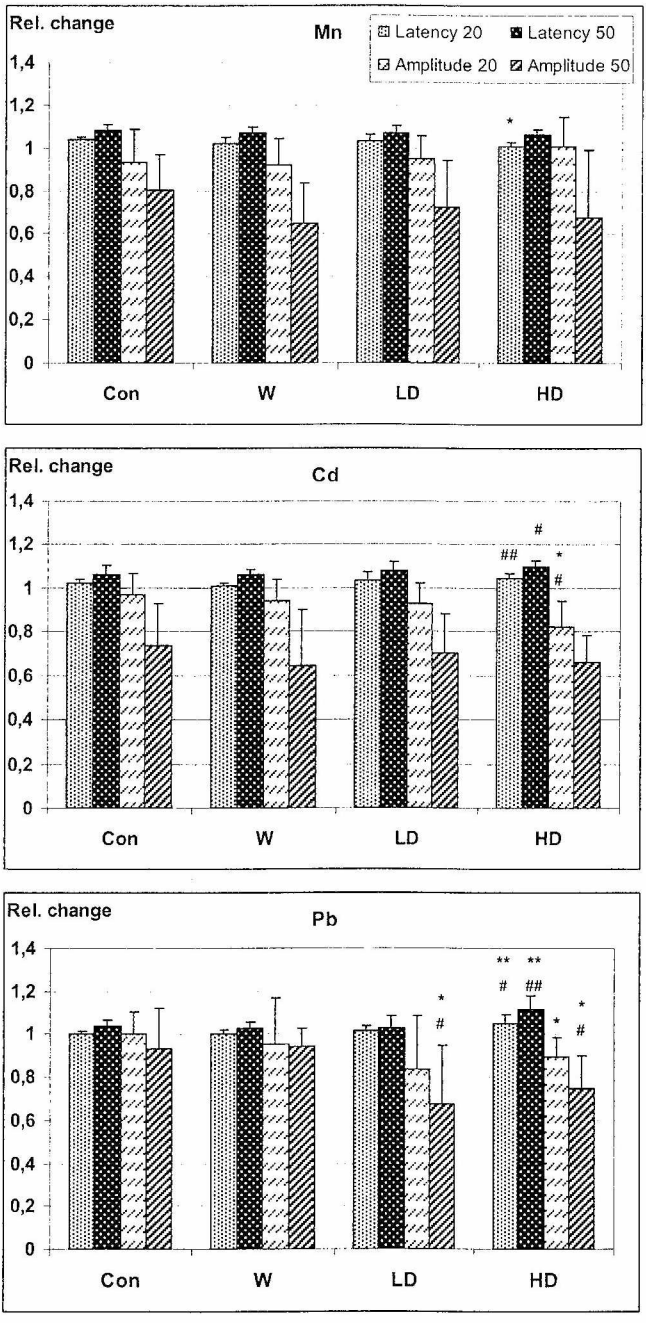

*Fig. 6. Relative change of the tail nerve action potential latency and amplitude at 20 and 50 Hz vs. 1 Hz stimulation frequency in rats treated with the three metal oxide nanoparticles. Mean+SD, n=10. **, ***: p<0.01, 0.001 vs. Con; # ##: p<0.001 vs. W.*

DISCUSSION

The observed changes in the electrophysiological parameters showed that the metal content of the nanoparticles could most probably reach the nervous system. The similarity of the present results to those obtained earlier with the same metals in other chemical forms and other ways of application support this first of all. Oral treatment of rats with $MnCl_2$ solution for 5 and 10 weeks induced lengthened EP latencies and reduced open field activity (Vezér et al., 2005); and the Mn levels found in brain and other tissue samples were proportional to the functional change. Administration of Pb-acetate – orally for 4–12 weeks – resulted in higher electrocorticogram mean frequency and slower evoked responses (Nagymajtényi et al., 1997). The effect of oral $CdCl_2$ was similar (Papp et al., 2003).

The qualitative similarity of effects of the three metals suggest that one or more common mechanisms of action may have played a role. A likely candidate is the effect of heavy metal ions on Ca-dependent processes. A number of heavy metal ions are known to block or affect some other way the Ca homeostasis (Büsselberg, 1995; Nelson, 1986). Due to the role of Ca channels in transmitter release (Mn: Takeda, 2003; Cd: Soliakov and Wonnacott 1996; Pb: Sandhir and Gill, 1993) synaptic transmission can be slowed and/or weakened which may explain the lengthening of EP latency and decrease of tail nerve conduction velocity. The frequency shift in the spontaneous cortical activity may be explained through changes in the cholinergic modulation (Cd: Carageorgiu et al., 2004; Pb: Suszkiw et al., 1984) or increased activity of the ascending reticular fibres due to decreased inactivation of glutamate by the Mn-dependent astrocytic glutamine synthetase (Normandin and Hazell, 2001).

Another mechanism common to fine and ultrafine metal particles is induction of oxidative stress. Signs of chronic airway inflammation and massive emphysema of the lungs were observed in HD-Mn and HD-Cd animals (Sárközi et al., 2007; Papp and Sárközi, 2008). The large and chemically reactive surface of the nanoparticles (Oberdörster et al., 2005) most likely played a role in these reactions. The inflammatory effect of welding fumes (McNeilly et al., 2004) and oxidative cell damage by Cd-containing quantum dots (Zhang et al., 2007) has been described, as well as the destructive effect of Pb on membrane lipids (Adonaylo and Oteiza, 1999).

Whether complete nanoparticles or metal ions dissolved from their surface were absorbed from the airways is, so far, an open question. In vitro, in distilled water as medium for dilution and application, the metal oxides applied have minimal solubility. After being phagocyted by alveolar macrophages, the particles are exposed to acidic pH (ca. 4.5) of the phagolysosomes (Lundborg et al., 1985) and surface dissolution will be likely (Handy et al., 2008).

The presence of nanoparticles in occupational, residential and other environmental settings is ubiquitous. Beyond high-temperature metal processing, metal-containing nanoparticles can, for example, be generated by incineration of unselected waste or emitted by worn-out internal combustion engines. Their contribution to health damage is considerable and not yet fully understood, justifying further studies.

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