INVESTIGATION OF EFFECTS OF TWO ENVIRONMENTAL HEAVY METALS IN A COMBINED EXPOSURE MODEL ON THE NERVOUS SYSTEM IN RATS

A. Papp[a]*, E. Horváth[a], Zs. Máté[a], A. Szabó[a]

In the present study, the interaction of inhalational and oral exposure to manganese and lead was investigated. Young adult male Wistar rats (2 x 10 per group) were treated orally with $MnCl_2$ (15 and 60 mg/kg b.w.) or Pb acetate (80 and 320 mg/kg) for 3 or 6 weeks. Then, one half of the groups was further treated by intratracheal instillation of nanoparticulate MnO_2 (2.63 mg/kg) or PbO (2 mg/kg) for an equal period of time. Body weight gain and signs of general toxicity were regularly checked. Finally, the rats' motor behavior was tested in an open field box, and their spontaneous and evoked cortical electrical activity was recorded in urethane anesthesia. MnO_2 nanoparticles caused disproportionately strong reduction of body weight gain but with Pb the weight effect was more dependent on dose. In the open field test, Mn caused hypomotility, more strongly after 6 weeks oral plus 6 weeks intratracheal than after 6 weeks oral treatment. Pb-treated rats showed increased ambulation but less rearing and somewhat longer local activity. Spontaneous cortical activity was shifted to higher frequencies after oral Mn application, but this change was not intensified by subsequent nanoparticle application. Oral Pb had an opposite effect. Cortical evoked potentials showed latency lengthening. In several cases, the effect of Mn and Pb was about as strong after 3 weeks oral plus 3 weeks intratracheal as after 6 weeks oral administration, although the summed dose was ca. two times lower in the former case. There can be a more-than-additive interaction between the amounts of heavy metals entering the organism in different routes and chemical forms.

* Corresponding Author
  Fax: +36-62-545-120
  E-Mail: papp.andras@med.u-szeged.hu
[a]   Department of Public Health, University of Szeged Faculty of Medicine. 6720 Szeged, Dóm tér 10.

Introduction

Chemical risk, resulting from application of metal-based and other xenobiotics, is a major problem of our days. Numerous metals are regarded as xenobiotics 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.).

Exposure to heavy metals is mostly occupational. Metal-containing dusts and fumes are generated along the complete life cycle of metal products (e.g. during welding[1]), and can be found in the workplace atmosphere, causing exposure primarily via inhalation. The resulting internal dose is influenced by chemical form and size of the suspended particles. In the last 20 years nanoparticles (NPs) and their interaction with living organisms, raised health concerns[2]. Combustion processes and working on materials at high temperature generate NPs (particles which are smaller than 100 nm at least in one dimension), their composition being determined by the materials worked on or burnt. Likewise, manufactured nanomaterials contain at least one component with at least one dimension in the 1-100 nm range.

Inhaled NPs are either deposited in the nasopharynx or get down to the alveoli[3]. NPs are not held back by biological barriers like alveolar and capillary wall, are distributed throughout the body by blood circulation, and reach distant target organs including the central nervous system. 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 and pathogenetical activity[2].

Apart from inhalation, the second most important route of exposure to heavy metals is ingestion. Airborne particles can be ingested directly; dust can contaminate food or drink, etc. Metals from the sedimenting dust can be incorporated into edible parts of cultivated plants, either directly or via soil pollution (as it happened near the metal reprocessing plant Metallokémia in Budapest[4]). Ingested heavy metals are absorbed in the intestine mostly to ca. 10%. Common metal transport mechanisms, responsible for the uptake of essential metals, are involved, which explains that individuals with increased calcium or iron demand absorb more of the toxic metals. Toxic metals are transported as "free-riders" not only from the intestine to the blood but also from the blood to the central nervous system (CNS). Trivalent metal ions, like Mn^{3+} use transferrin to pass the blood-brain barrier.

Based on practical importance and on previous experiences of our research group[5,6,7], lead and manganese were chosen for the present study.

Lead (Pb) has been a ubiquitous environmental pollutant for centuries, and is toxic even in low concentrations[8]. Primary production and reprocessing of Pb causes substantial emission of metal fumes. Neurological effects were observed in workers with chronic Pb exposure: headache, lethargy, dizziness, diminished reaction time, worsened cognitive and visuomotor performance, and reduced nerve conduction velocity[9]. The general population is exposed mainly by contaminated drinking water or food. Pb is accumulated in the central nervous system, first in the cortex and hippocampus[10], and produces encephalopathy. Exposure to low concentrations of Pb has been associated with behavioral abnormalities, learning and hearing impairment and impaired cognitive functions in humans and in experimental animals[11]. Some approximations 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 level[12], such an effect was observed also in Hungary[13]. Pb can also pass the placenta and cause serious damage to the nervous system before birth.

Manganese (Mn) is an essential micronutrient, e.g. as cofactor in metallo-enzymes:. The human body contains about 10 mg Mn, stored mainly in the liver and kidneys. The daily demand is 2-3 mg[14]. Mn is used in many important alloys, so welding fumes and similar industrial emissions are also a source of Mn-containing NPs. Chronic inhalation of Mn-containing dust and fumes is the typical form of occupational exposure. The Mn-related chronic neurological disorder (manganism) starts with apathy, anorexia, headache, hypersomnia, weakness of the legs etc. and progresses to a Parkinson-like syndrome[15]. Such disorder was also observed in patients undergoing maintenance hemodialysis[16] or in inadvertent oral overdosing of Mn. Thus, other physicochemical forms of Mn and other routes of exposure are also relevant to the health of the CNS. In several regions of the USA with Mn-rich drinking water, loss of visual and verbal memory, typical for Mn-induced brain damage, was described[17]. The neurotoxic spectrum of Mn is variable and goes beyond the classical manganism. In shipyard workers, EEG and visual evoked potential alterations were observed and elevated blood Mn levels were measured[18].

The aim of this work was to model the complex (occupational and environmental) human exposure to Mn and Pb in rats as a model, with combined – oral and intratracheal – administration using dissolved and nanoparticulate form of the metals.

Methods

Animals and treatment

Young adult male Wistar rats (initial body weight 280–350 g) 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 pelleted feed and drinking water

Aqueous solution of Mn and Pb was given to the rats orally by gavage (per os, po.), while the suspension of the metals in NP form was instilled in the trachea (intratracheally, it.; for details see[6]).

Table 1 The substances used for treatment and the doses applied

Group code	Substance and dose (mg kg^{-1} b.w.)	Treatment time
MnC3	untreated	3 weeks
MnVC3	distilled water, per os	3 weeks
MnL3	MnCl$_2$·4H$_2$O 15 mg kg^{-1} b.w. po.	3 weeks
MnH3	MnCl$_2$ 60 mg kg^{-1} b.w. po.	3 weeks
MnC33	untreated*	6 weeks
MnVC33	distilled water, po. + HEC (hydroxyethyl cellulose) it.	3 weeks + 3 weeks
MnL33	MnCl$_2$ 15 mg kg^{-1} b.w. po. + MnO$_2$ NPs 2.63 mg kg^{-1} b.w. it.	3 weeks + 3 weeks
MnH33	MnCl$_2$, 60 mg kg^{-1} b.w. po. + MnO$_2$ NPs 2.63 mg kg^{-1} b.w. it.	3 weeks + 3 weeks
MnC6	untreated*	6 weeks
MnVC6	distilled water, po.	6 weeks
MnL6	MnCl$_2$ 15 mg kg^{-1} b.w. po.	6 weeks
MnH6	MnCl$_2$ 60 mg kg^{-1} b.w. po.	6 weeks
MnC66	untreated	12 weeks
MnVC66	distilled water, po. + HEC it.	6 weeks + 6 weeks
MnL66	MnCl$_2$, 15 mg kg^{-1} b.w. po. + MnO$_2$ NPs 2.63 mg kg^{-1} b.w. it.	6 weeks + 6 weeks
MnH66	MnCl$_2$, 60 mg kg^{-1} b.w. po. + MnO$_2$ NPs 2.63 mg kg^{-1} b.w. it.	6 weeks + 6 weeks

Group code	Substance and dose (mg/kg b.w.)	Treatment time
PbC3	untreated	3 weeks
PbVC3	distilled water, po.	3 weeks
PbL3	Pb(CH$_3$COO)$_2$, 80 mg kg^{-1} b.w. po.	3 weeks
PbH3	Pb(CH$_3$COO)$_2$, 320 mg kg^{-1} b.w. po.	3 weeks
PbC33	untreated*	6 weeks
PbVC33	distilled water, po. + HEC it.	3 weeks + 3 weeks
PbL33	Pb(CH$_3$COO)$_2$, 80 mg kg^{-1} b.w. po. + PbO NPs 2 mg kg^{-1} b.w. it.	3 weeks + 3 weeks
PbH33	Pb(CH$_3$COO)$_2$, 320 mg kg^{-1} 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$_3$COO)$_2$, 80 mg kg^{-1} b.w. po.	6 weeks
PbH6	Pb(CH$_3$COO)$_2$, 320 mg kg^{-1} b.w. po.	6 weeks
PbC66	untreated	12 weeks
PbVC66	distilled water, per os + HEC it.	6 weeks + 6 weeks
PbL66	Pb(CH$_3$COO)$_2$, 80 mg kg^{-1} b.w. po. + PbO NPs 2 mg kg^{-1} b.w. it.	6 weeks + 6 weeks
PbH66	Pb(CH$_3$COO)$_2$, 320 mg kg^{-1} b.w. po. +PbO NPs, 2 mg kg^{-1} b.w. it.	6 weeks + 6 weeks

* C33 and C6 were always two different groups of rats.

Chemical identity and dose of the substances applied to the rats, along with treatment times, are given in Table 1. For intratracheal administration, the NPs were suspended in 1% hydroxyethyl cellulose dissolved in PBS (pH 7.4). This vehicle was physiologically neutral and slowed the aggregation of the NPs. There was one administration every workday (that is, 5 times a week); body weight was measured before every administration to determine the exact daily doses and to follow weight gain.

Behavioral investigation by the open field method

At the end of the treatment periods, open field (OF) test was done, in one 10-min session per rat, to assess their spontaneous locomotor activity. The test was performed between 8 and 11 hours in the morning, after 30 min adaptation to the dimly lighted test room. The instrument recorded the animal's horizontal and vertical motor activity, from which counts, time and run length of the activity forms (ambulation, local activity, immobility, rearing) were automatically calculated (for technical details and suitability, see[7]).

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, and the left hemisphere was exposed by opening the bony skull. For recording, the rat was placed into the stereotaxic frame of the electrophysiological setup. Body temperature was maintained by the thermostated (+36.5°C) support plate. To record spontaneous and evoked cortical activity, a ball-tipped silver recording electrode was positioned on the dura over the primary somatosensory (SS) area. SS stimulation was done by a pair of needles inserted into the whiskery part of the nasal skin, delivering square electric pulses. The recording session started with six minutes recording of spontaneous activity (electrocorticogram, ECoG) first. From the ECoG records, the relative spectral power of the frequency bands. Then evoked potentials (EPs) from the same cortical site were recorded. Electrical stimulation of the whiskers was done by delivering rectangular electric stimuli (3-4 V, 0.05 ms). Trains of 50 stimuli were applied, one each with 1, 2 and 10 Hz frequency. Individual EPs were automatically averaged, and their latency and duration was measured manually.

From the general toxicological, behavioral and electrophysiological data, group mean values (±SD) were calculated. All results were checked for normality by means of the Kolmogorov-Smirnov test, then were tested for significance using one-way ANOVA with post hoc LSD 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 authority competent in animal welfare issues licensed the methods used in the experiments under No. XXI./02039/001/2006.

Results

Body weight gain

Oral Mn treatment had minimal effect on body weight; but as soon as it, administration of Mn NPs started, the weight gain in the treated groups got substantially slower than either in the vehicle-treated (**MnVC;** Fig. 1) or untreated (**MnC**, not shown) control. Oral Pb had visible effect on body weight gain from the 4th week on (Fig. 1) but switching to it, administration on the 6th week caused also here a marked drop.
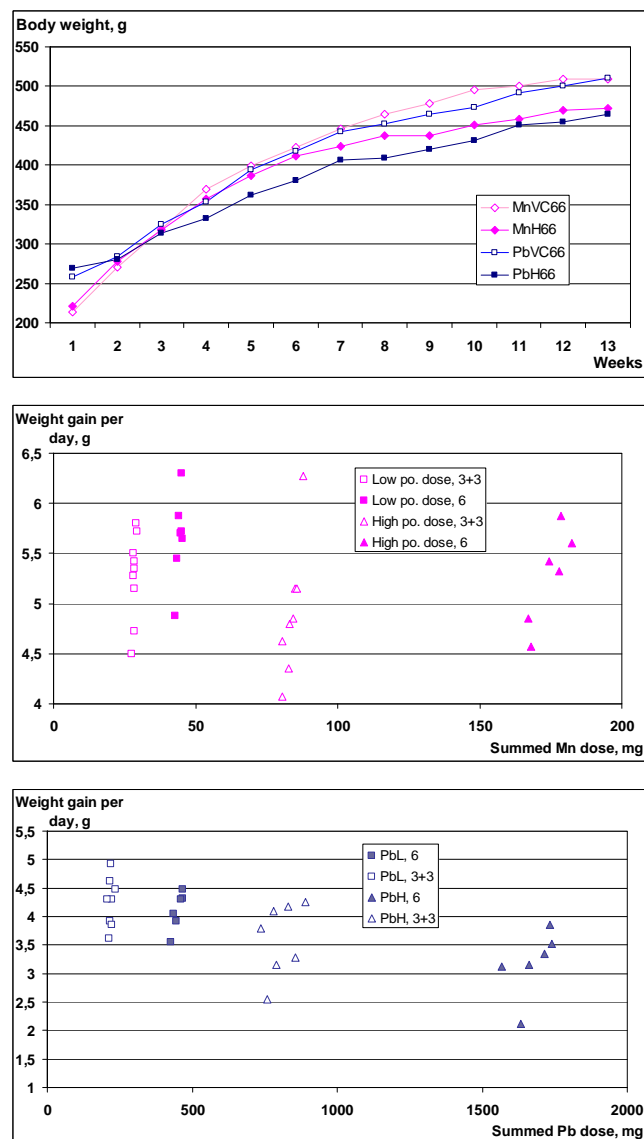

Figure 1. Top: body weight curves of high dose Mn and Pb treated rats and the corresponding controls. Middle and bottom: relationship of metal dose and weight gain. See Table 1 for group coding and doses.

The relationship between weight gain and summed dose was dissimilar: for Mn it was more influenced by the form (NP vs. dissolved) but for Pb, more by the summed dose.

Open field motility

Noteworthy changes in OF motility were seen only after 6 and 6+6 weeks of metal exposure (Fig. 2). Mn decreased ambulation and rearing (the latter, only after 6+6 weeks) and increased local activity and immobility. Pb caused increased ambulation but decreased rearing.
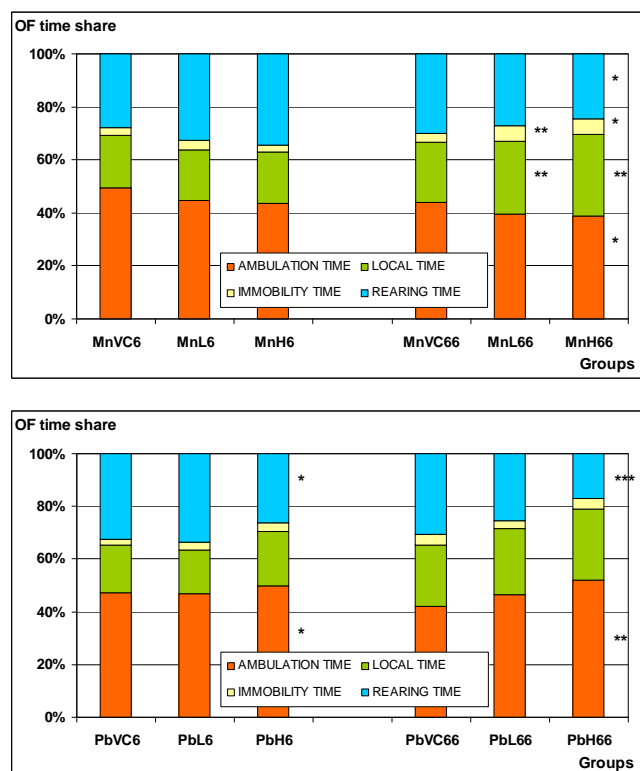

Figure 2. Time share of the four open field activity forms in the total session time (top, Mn treated; bottom, Pb treated rats).
*, **: p<0.05, 0.01 vs. **VC**.

Cortical electrical activity

The general trend in the electrocorticogram (ECoG) band spectra on Mn effect was 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. 3). After 6 weeks oral Pb treatment, increased slow and decreased fast ECoG activity was seen, compared to the control **PbVC6**. However, in the rats receiving 6+6 weeks Pb exposure, this frequency shift disappeared.

Latency of the SS EP was increased by Mn (Fig. 4). This increase was seen at all stimulation frequencies but the slope of frequency dependence of the latency (a possible indicator of metal-induced cortical fatigability) was not much altered.

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, in accordance with the relationship of summed Mn dose and body weight (Fig. 1).
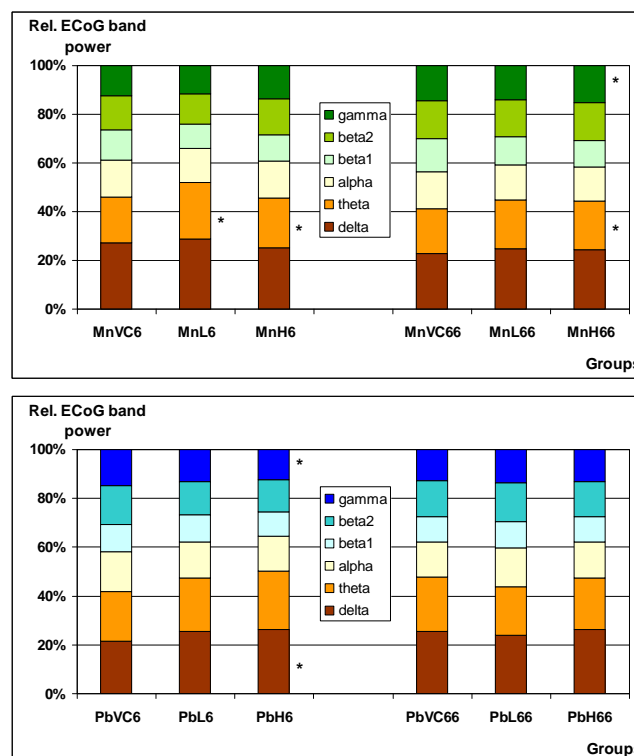

Figure 3. Band spectrum of the spontaneous activity recorded from the somatosensory cortex of rats after 6 and 6+6 weeks of Mn (top) and Pb (bottom) exposure.
*: p<0.051 vs. **VC**

Latency of the SS EP was increased in the Pb-treated rats (Fig. 5). 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** somewhat bigger than in **PbL6**; indicating that the NP form could have disproportionately strong effect on the nervous system (but not on body weight, see Fig. 1) also in case of Pb. In **PbH33** and **PbH66**, the frequency dependent lengthening of latency became also more intense.

Discussion

General and neuro-functional parameters indicated repeatedly that there could be a non-additive interaction between the amounts of heavy metals given by po. and it, application.

In Mn exposure, the disproportionately strong effect of NPs was seen on the body weight gain, a general toxic parameter. Metal level data from previous comparable experiments[6,7] suggest that inhaled NPs cause internal exposure more efficiently than ingested, dissolved metals. Pb is known to affect BBB[19], 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.
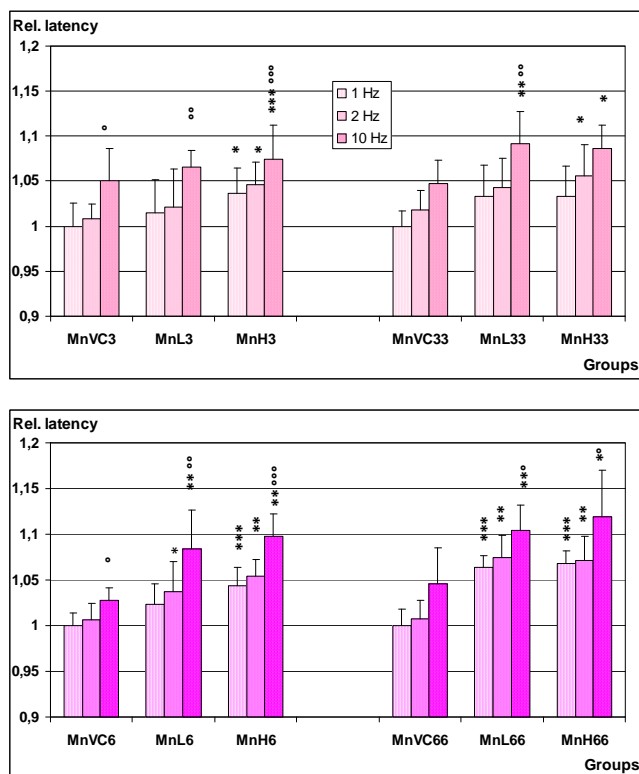
Figure 4. Latency of the somatosensory evoked potential in Mn treated rats (relative values normalized to **VC**, 1 Hz).
*, **, ***: $p < 0.05$, 0.01, 0.001 vs. **VC**
°, °°, °°°: $p < 0.05$, 0.01, 0.001 vs. 1 Hz stimulation within the same group.
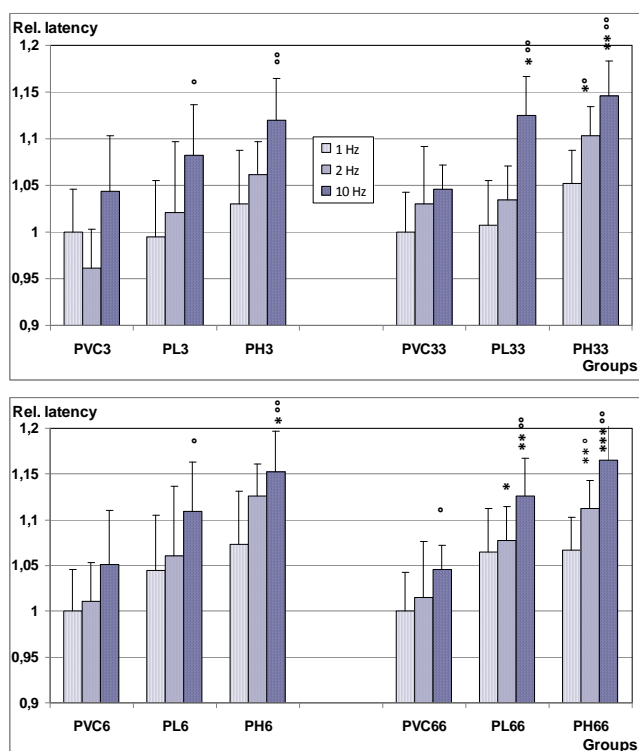

Figure 5. Latency of the somatosensory evoked potential in Pb treated rats (relative values normalized to **VC**, 1 Hz).
*, **, ***: $p < 0.05$, 0.01, 0.001 vs. **VC**
°, °°: $p < 0.05$, 0.01 vs. 1 Hz stimulation within the same group.

Metal NPs are always likely to cause oxidative stress. Metabolites indicating oxidative damage upon Pb exposure were detected in humans[20]. Generation of reactive oxygen species was detected in the brain in case of both Mn and Pb, resulting in membrane lipid peroxidation[20,21], leading to changes of membrane functions, such as synaptic transmission, which may be reflected in various forms of cortical activity.

Increased latency in the rats treated with both metals might be, at least partly, due to decreased synaptic efficiency. Pb^{2+} and Mn^{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, both 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[22,23].

Glutamate, the main excitatory transmitter in the CNS, is neutralized by uptake in astrocytes and conversion to glutamine; and this uptake is decreased by Mn[24] and Pb[25]. Excess glutamate may desensitize the postsynaptic receptors leading finally to smaller/slower cortical evoked responses. Altered glutamatergic excitation, and energetic shortage from mitochondrial effect of the metals, might have opposite effect on the ECoG spectrum. The resulting net change was in fact different depending on the relative strength of the two opposite influences.

The changes in open field motor behavior may result from the general CNS effects, but even more from the dopaminergic effects, of the metals or NPs. Motivation, determining OF activity, is regulated by mesolimbic/mesocortical dopaminergic structures. 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[26] and the metals investigated are known to cause oxidative stress as mentioned above. Decreased horizontal movements in-group **MnH66** is a feature of general hypomotility, possibly analogous to the Mn-exposed welders' syndrome[27]. Increased locomotion of the Pb-treated rats may be analogous to the human "attention deficit hyperactivity disease" found more frequently among children with elevated blood Pb[28].

Conclusion

The attempt to model a complex metal exposure, coming both from environmental (food/waterborne) and occupational (inhalational) sources, was apparently successful. With respect to the general use and ubiquitous presence of metals, their health effects in general, and in particular the nervous system and other sensitive systems, are of primary concern. Occurrence of metals in nanoparticulate form, let it be nanotechnological materials or unwanted pollutants, adds a new feature to the old problem.

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