**PhD Thesis**

# GENERAL AND NERVOUS SYSTEM EFFECTS OF THE NEUROTOXIC METAL MANGANESE UNDER VARIOUS CIRCUMSTANCES OF APPLICATION

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# The Applicant's Relevant Publications

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**Abstracts**

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4. *Máté Zs*, Horváth E, Nagy V, Kovács K (2011) Hegesztési füstben előforduló fémek nanorészecskéinek hatásai patkányokban. Fiatal Higiénikusok VII. Fóruma, Esztergom. Egészségtudomány 55, 130-131.

5. Szabó A, Takács Sz, *Máté Zs*, Horváth E, Oszlánczi G, Papp A (2011) Neurotoxicity of nanosized manganese by subchronic exposure. 9th Göttingen Meeting of the German Neuroscience Society, Göttingen. Neuroforum 17 Suppl. 1. (T11-15B)

6. *Máté Zs*, Szabó A, Hermesz E, Jancsó Zs, Boros I (2010) Alterations in the central nervous system caused by intratracheal instillation of Mn, in aspects of neurophysiology and molecular biology. ISIRR 11th International Symposium Interdisciplinary Regional Research, Szeged. p. 100. ISBN: 978-963-508-600-9

7. Szabó A, *Máté Zs* (2010) Combined subchronic exposure of solute and nanosized manganese administered intratracheally to rats. 7th FENS Forum of European neuroscience, Amsterdam. FENS Abstracts Vol. 5, 164.51.

8. *Máté Zs*, Szabó A, Hermesz E, Jancsó Zs, Boros I (2010) Mangán intratracheális adagolásával előidézett funkcionális idegrendszeri eltérések patkányban. A MÉT LXXIV. Vándorgyűlése, Szeged. Acta Physiol Hung. 97, 456-457.

9. *Máté Zs* (2010) Oldott formájú és nanoszemcsés Mn szubkrónikus kombinációs kezelésének hatása a patkány idegrendszerére. Fiatal Higiénikusok VI. Fóruma, Debrecen. Egészségtudomány, 54, 113.

10. *Máté Zs*, Szabó A, Papp A (2010) Looking for parameters of functional neurophysiological alterations caused by manganese in rats. IBRO International Workshop, Pécs. Front Neurosci, doi: 10.3389/conf.fnins.2010.10.00050

11. *Máté Zs* (2009) A mangán akut és szubkrónikus neurotoxikus hatásának vizsgálata patkány szomatoszenzoros kérgén. XXIX. Országos Tudományos Diákköri Konferencia Biológia Szekció, Veszprém. Program és összefoglalók. pp. 213. ISBN: 978-963-9696-66-2

12. *Máté Zs*, Szabó A, Papp A (2009) Acute and chronic effect of manganese on the somatosensory system of rats investigated with double-pulse stimulation. A Magyar Idegtudományi Társaság (MITT) XII. Konferenciája, Budapest. Front Neurosci, doi: 10.3389/conf.neuro.01.2009.04.212

# Summary

The role of metals is essential in everyday life but they also present a source of environmental pollution and harmful human exposure. Heavy metals are dense elements, persist in the environment and are either completely useless or toxic for the human organism or essential micronutrients but toxic when overdosed. Emission of heavy metals into the environment can be of natural or anthropogenic origin. The most important man-made sources are ore processing and combustion processes (power generation or waste incineration) raising the concern of occupational health hazard. Due to the environmental transport of metals through air, water and food chain, distant areas can be polluted leading to exposure of the general population.

Manufacturing of metals usually requires high temperature operations, during which metal fumes are released into the air resulting in significant inhalational occupational exposure. One of the most common sources of such metal aerosols is welding. Welding fume is a complex mixture of metal oxides and materials of the electrode. The most common elements in steel that welders are primarily exposed to by inhalation are iron (Fe), manganese (Mn) and chromium (Cr). Airborne particles, released into the environment by welding, have major adverse health effects, causing among others neurological disorders and respiratory illnesses. Welders who are at excessive exposure may be at risk of developing early Parkinson's disease or manganism caused by overexposure to Mn. Occupational exposure to $Cr^{6+}$ and $Cr^{3+}$ compounds and their respiratory effects have been described several times, but information about the neurological effects is scarce, though it causes free radical formation and thus oxidative stress, reduces mitochondrial activity, and studies prove that Cr can also reach the brain. Concerning Fe, pulmonary toxicity of $Fe_3O_4$ inhalation was studied, but no neurological or behavioural tests were conducted, however Fe overload causes disruption of the mitochondrial energy production, and leads to generation of reactive oxygen species responsible for the toxic effect. The neurotoxicity of Mn has been described extensively but the role that other agents peculiar to welding may play in the causation of the above mentioned neurological disorders are still unknown.

The extent of inhalational metal exposure is influenced by numerous factors including chemical form in which the metal is present in the particles and the particle size. Much of the particles derived from the welding process have a diameter below 100 nm, i.e. they are nanoparticles (NPs). Inhaled NPs deposited in the nasopharynx can pass a healthy blood-brain

barrier (BBB) and reach the CNS via direct transport along the olfactory pathway. Particles that reach the alveoli are translocated directly to the blood and transported further. Pulmonary circulation continues to supply the brain with significant amounts of welding-related metals long after the exposure has been terminated.

Based on a long line of antecedent research at the Department on the neurotoxicity of Mn, and on interactions of neurotoxic metals, the goal of this work was modelling human heavy metal exposure more adequately, involving the combination of several industrially relevant metals, more than one physicochemical form of one metal, and variable ways and sites of administration. From all that, the aims of the present PhD work were specified as follows:

- To examine the effects on general toxicological, CNS electrophysiological and behavioural parameters exerted by nanoparticulate Mn, Fe and Cr in an identical experimental setup;
- To repeat these experiments with double and triple combinations of the mentioned metals, in order to reveal possible interactions and to have a better model of welding fumes and similar industrial emissions;
- To examine the relationship of external and internal doses in dependence of the physicochemical form and site of application of Mn, and on co-application of the other two metals;
- To test whether analysis of the second:first ratio, a method used previously to study the CNS effect of the mitochondrial toxin 3-nitropropionic acid and the acute effect of Mn, is applicable for detection of neuro-functional alterations induced by subacute application of Mn and other metals.

Young adult male Wistar rats were used. In Experiment I, 2.5 mg/ml $MnCl_2$ was dissolved in tap water, and given as drinking fluid to the animals for 6 weeks. Experiment II lasted for 5 weeks, the rats had intratracheal (it.) instillations of aqueous solution of $MnCl_2$ (0.5 mg/kg b. w.) once a day, five days per week. Instillation was carried out in brief diethyl ether anaesthesia. At the end of the treatment period in experiment I and II, after preparation for electrophysiological investigation, two recordings of somatosensory (SS) evoked potentials (EPs) elicited by double stimulation were made in urethane anaesthesia. From the EPs parameters of the amplitudes and latencies of the positive and negative peaks were

calculated. Second:first ratio of the corresponding parameters was determined to measure the dynamic interaction between the two excitation processes. For Experiment III animals were intratracheally instilled with a suspension of $MnO_2$, $Fe_3O_4$ or $Cr(OH)_3$ NPs (2 or 4 mg/kg b. w.). Treatment was performed once a day, 5 times per week, for 4 weeks. Experiment IV was carried out by treating the animals with the double and triple combinations of $MnO_2$, $Fe_3O_4$ or $Cr(OH)_3$ NPs. The treatment lasted for 4 weeks; it. instillation was performed once a day, 5 times per week. At the end of the treatment period in experiment III and IV, open field (OF) test and electrophysiological recording was conducted. For the latter, spontaneous electrical activity (electrocorticogram, ECoG) and EPs were recorded from three cortical areas (SS, visual [VIS], auditory [AUD]).

Body weight of the animals was regularly measured during the experiments, to follow-up weight gain and to determine the exact daily doses. Following electrophysiological recording, the animals were sacrificed by an overdose of urethane, were dissected, and blood was collected. Brain, lungs, heart, thymus, liver, kidneys, spleen and adrenal glands were removed and the organ weights were measured. From these data, relative weights were calculated by relating organ weights to brain weight or 1/100 of body weight. Metal level was determined from blood, brain and lung samples.

In Experiment I (subacute po. treatment with $MnCl_2$ solution) neither the body weight gain, nor relative organ weights were affected by the treatment. The calculated daily Mn intake of the treated rats was equivalent to 141 mg $MnCl_2$, which meant that the cumulative dose of Mn was 1633 mg/rat by the end of the 6th week. Upon $MnCl_2$ treatment, significant Mn deposition evolved in the treated animals' brain, but not in blood. Second:first ratio of the EPs was shifted to lower values in case of the peak-to-peak amplitudes, and the positive and negative peak latencies (the latter two being significant). The absolute latency values had no significant change which suggested that the ratio may be a more sensitive indicator.

In Experiment II (subacute it. treatment with $MnCl_2$ solution) no significant effect on the body weight gain in the *Treated* group was seen, however relative weight (related to 1/100 body weight) of certain organs such as heart and lungs was significantly increased. At the end of the treatment, calculated cumulative dose of Mn was merely ca. 1 mg/rat; all the same, Mn concentration in both brain and lungs – but not in blood – was significantly elevated. The second:first ratio of the peak-to-peak amplitude showed significant decrease in the *Treated* group (at 240 and 180 ms ISI) which change was basically similar to the alteration observed in po. treatment. As to the positive and negative peaks, the second:first ratio was lower at all

ISIs (significantly at 300 and 240, and 240 ms respectively). Significant correlation of brain Mn level with the second:first ratio of the negative peak amplitude was found at 240 ms ISI.

In Experiment III and IV (subacute it. treatment with Mn, Cr or Fe NPs and with their combinations) body weight gain of the animals treated with Mn and Cr NPs (or their combination) was significantly reduced, and so was the weight gain in case of Fe+Cr combination (*FC)*, however, Fe-only treatment lessened the negative effect of the treatment procedure. As for the organ weights, the most prominent alteration was the increase of the lungs' relative weight (related to both calculation bases) upon treatment with Mn and Cr NPs, and each of the metal NP combinations. In the OF test, local activity and immobility was increased and vertical activity was decreased by Cr and Mn and by Cr-containing combinations. The effect of Fe NP treatment was opposite. In the ECoG, application of metal NPs caused a shift to higher frequencies, and this effect of Mn and Cr on the SS ECoG was significant, but the alterations caused by the metal NP combinations were more pronounced. The observed alterations on the VIS and AUD ECoG were similar to those of the SS ECoG. The SS EPs showed significantly increased latency on the action of Mn and Cr NPs (and their combination, *MC*), and of *FC*. In Fe NP treated groups, however, latency was decreased significantly. VIS and AUD latency were increased by both doses of Mn, high dose of Cr, and the combinations of *MC* and *FC*. In case of SS EPs elicited by double pulse stimulation, peak-to-peak amplitudes were increased significantly by Mn at almost each ISI. The effect of Cr treatment was opposite to Mn, and Fe NP treatment had no noteworthy effect alone. Generally, in case of combined metal NP treatment, no noteworthy change was found in the peak-to-peak amplitudes, however, second:first ratio of them showed significant increase in *FC* and the triple combination (*FMC*) groups. The second:first ratio of the peak-to-peak amplitudes was significantly decreased by Mn-only treatment, and the opposite effect of Cr was also seen here. In combination, the effect of *FC* and *FMC* was similar to that of Cr. Mn content of the brain, lungs, and also blood was significantly increased by Mn and Cr NPs, but the effect of Fe was only seen on increased lung Fe levels. In combinations, brain and blood levels of Mn were similar to that of caused by Mn alone in low dose; Cr levels were, however, influenced by the other metals applied. The correlation between brain Mn levels and SS and VIS ECoG index and SS and VIS latency was significant in Mn-only treated groups and also in combination with Cr in *MC* group. Brain Mn levels also correlated with the second:first ratio of the peak-to-peak amplitude.

Based on these results, the questions formulated in the aims can now be answered as follows:

- The general and nervous system effects of Mn were approximately identical to those found in previous experiments. The effects of Cr were, under identical conditions, partly similar to those of Mn. This similarity could be due to shared mechanisms of action. Data on neurotoxic effects of Cr are scarce in the literature so the findings described in this thesis may be novel. The effects of Fe were partly minimal, partly opposite to those of Mn.
- The interactions of the three metal studied were dissimilar on various parameters. The effect of Mn on body weight gain, and on electrophysiological and some OF parameters, were counteracted by Fe, but Cr was apparently not involved in such interaction. In the tissue metal levels, Fe acted on Cr but not on Mn. All that indicated that measured internal dose is not the sole determinant of the functional alterations and so, not an ideal biomarker of effect.
- Even when Mn was applied alone, the physicochemical form and site of application greatly influenced the resulting internal dose which may be of importance in case of various forms of human exposure.
- Electrophysiological tests, including double pulse stimulation and second:first ratio calculation, may well be suitable for detection and follow-up of functional damages in the nervous system but the particular form applied in the present work turned out not to be optimal.

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# Abbreviations

| | |
|---|---|
| ANOVA | analysis of variance |
| AUD | auditory |
| BBB | blood-brain barrier |
| CNS | central nervous system |
| Cont | untreated control |
| DMT-1 | divalent metal transporter 1 |
| ECoG | electrocorticogram |
| EEG | electroencephalogram |
| EP | evoked potential |
| GABA | γ-aminobutyric acid |
| GLP | good laboratory practice |
| HD | high dose |
| ip | intraperitoneal |
| ISI | interstimulus interval |
| it | intratracheal |
| LD | low dose |
| LSD | least significant difference |
| MMT | methylcyclopentadienyl manganese tricarbonyl |
| Mn-SOD | manganese-superoxide dismutase |
| MRI | magnetic resonance imaging |
| MS | Microsoft |
| NADH | redukált nikotinamid-adenin-dinukleotid |
| NP | nanoparticle |
| OF | open field |
| PAA | polyacrylic acid |
| po | per os |
| ROS | reactive oxygen species |
| SS | somatosensory |
| Tf | transferrin |
| Tf/TfR | transferrin/transferrin receptor |
| VC | vehicle control |
| VIS | visual |

# 1. Introduction

## 1.1. Metals: general physical and chemical properties. Heavy metals

The role of metals in the development of human culture and technology became significant around 4000 B.C., when people discovered how they can extract copper from its ores and manufacture objects of everyday use, and the copper era began. With changing way of life, humans started to use other easily extractable metals from the environment, such as tin or lead. As time went by, metals became vital for everyday life but they also presented a source of environmental pollution and harmful human exposure.

At present, more than 80 metals are known, representing 75% of the elements in the periodic table. There, the place of metallic elements is determined by their similarities in electron orbital arrangements (see below) and the resulting chemical properties. Group I in the periodic table includes alkali metals, while alkaline-earth metals, such as magnesium or calcium (Ca), are found in Group II. Elements in Group III-XII are called transition metals, the name coming from being placed between the most reactive elements on the left and the non-metals on the right. Metals that are not otherwise categorized (e.g. aluminium or lead) are the post-transitional metals. Lanthanides and actinides (the inner transition metals) constitute series of Group III. Metalloids, elements with properties intermediate between metals and non-metals, occur in the periodic table along the diagonal border between these. These categories are basically of theoretical chemical nature but have been newly included in the definition of heavy metals, a concept with eminent toxicological aspects.

Metal atoms usually have 1-3 electrons in their outermost (valence) shell, and with their tendency to lose electrons, are able to form cations. Also in biological systems, metals fulfil their functions mostly in ionic form. Metal atoms with more than one oxidation state, e.g. iron (Fe) and manganese (Mn), tend to be involved in redox interactions which, under protoplasmic conditions, may result in the generation of oxidative free radicals. Compounds formed by transition metals of different valence states play an important role in metal toxicity (Appenroth, 2010).

Several terms are used for the classification of metals, such as light or heavy metals, essential, beneficial, toxic and trace metals (Phipps, 1981). It has been customary to group metals into light and heavy ones based on their density. At first, metals with densities over 7 $g/cm^3$ were defined as “heavy”. Over the years, no consistency was achieved in this

definition, but most scientists accepted that metals of density over 4.5 or 5 g/cm$^3$ should be regarded as heavy metals. With the realized unimportance of density in the prediction of biological effects of metallic elements, other properties like atomic weight, atomic number, different chemical properties or toxicity have emerged as criteria of "heavy metals", resulting in confusion (Duffus, 2002). Appenroth (2010) suggested that heavy metals should be rather defined based on their position in the periodic table, because this position refers to the chemical properties and hence the possible toxic effect. According to this classification, transition elements (chromium [Cr], Mn, Fe, etc.), rare earth metals (lanthanides and actinides), post-transition metals and metalloids should be considered as heavy metals.

### *1.1.1. Biological functions and effects of metals, metal toxicity*

A directive of the European Union dealing with dangerous substances (Directive 76/464/EEC) uses the terms toxicity, persistence and bioaccumulation to define dangerous chemicals. Heavy metals as elements do persist in the environment. A lot of metals are xenobiotics, substances which can be neither utilized nor neutralized by the organism (Lyman et al., 1982). This is so because they used to have minimal presence (and, hence, bioavailability) during the evolution of life, before anthropogenic emission into the environment had begun; and because they are either completely useless or toxic for the human organism (e.g. mercury, lead or cadmium) or essential as micronutrients but toxic when overdosed (Mn, Cr, Cu, etc.).

Emission of heavy metals into the environment can be of natural or anthropogenic origin. The most important man-made sources are ore processing operations, such as mining or smelting, and combustion processes (power generation or waste incineration). The consequences of ore mining operations can persist for hundreds of years, long after mining activities had ceased (Breitenlechner et al., 2010) and the emitted metals remain persistent in the environment. Due to the environmental transport of metals through air and water, not just the areas of primary emission are polluted. Transported by the rivers, and stored in river bed sediments, heavy metals can reach underground waters (however, pollution decreases with the distance from the site of emission). Certain metals tend to be accumulated by plants from contaminated agricultural soils, and transferred to herbivorous animals (or directly from contaminated water to animals bred in polluted aquatic environment), and through the food chain, into humans. This was the case around the metal reprocessing plant "Metallokémia" in Budapest (Hungary), where smelting operations ceased in 1990, but by-products (slag

containing heavy metals, such as lead or cadmium) were stored on the plant's premises further on (Pápay and Horváth, 1992). Cadmium and lead content of the soil and groundwater was highly elevated (Szabó, 1991). Plants (leafy vegetables and various fruits) cultivated in the local residents' gardens absorbed high amounts of these heavy metals from the soil and water, and caused increased lead level in the blood of local children consuming home-grown fruits or vegetables (Groszmann et al., 1992). In Brazil, mercury emission from low-tech gold mining resulted in high methyl mercury content of river fish, and foodborne exposure of the local population with neurophysiological and neurobehavioral consequences (Mergler, 2002).

The concentration of essential elements in human and animal organism is under homeostatic control, and the uptake from the environment (via food and/or drinking water) is regulated by the nutritional demand. Disturbances in the regulatory mechanisms can result in either insufficient (deficiency) or excess (toxicity) metal uptake.

Ingested heavy metals are absorbed from the intestine to around 10%. Common metal transport mechanisms, responsible for the uptake of essential metals, are involved in that, which explains why individuals with Fe or Ca deficiency absorb more of the toxic metals (e.g. excess Mn absorption in anaemic individuals, or lead absorption in children requiring more Ca for bone growth). Trivalent ions of toxic metals like Mn or Cr use transferrin (Tf) for transport from the blood, to pass the blood-brain barrier (BBB) and to reach the central nervous system (CNS) (Takeda, 2003; Vincent, 2001).

Inhalational exposure occurs predominantly in the occupational environment. Miners and metal workers are exposed to airborne metal particles first of all, but inhabitants around industrial sites are also at risk. Metal-containing dusts and fumes are generated in the whole life cycle of metals, from ore mining through smelting and final product manufacturing to waste management and recycling, and are found in the workplace atmosphere sometimes at hazardous concentrations. The extent of metal exposure is influenced by numerous factors including the type (nasal/oral), volume and intensity of breathing, chemical form in which the metal is present in the particles (solubility, etc.) and the particle size (see 1.2.).

Human exposure to heavy metals, such as mercury, cadmium or lead, is also possible trough consumers' products (Duruibe et al., 2007), mainly by dermal exposure.

One of the general damaging effects of heavy metals is denaturation of proteins. By the interaction with ionized moieties of amino acids in the polypeptide chain, metal ions disrupt the non-covalent polar and ionic interactions which stabilize the secondary (or higher order) structure of the protein. Some of the toxic heavy metals (including Fe, Cr or Mn) are capable of redox cycling while others (e.g. cadmium) affect cellular antioxidant defence

(Valko et al., 2005). This explains that disturbed redox balance and oxidative stress is a frequent consequence of heavy metal exposure.

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

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

### *1.1.2. Sources, properties and biological effects of metal containing aerosols*

Manufacturing of metals from ores, and processing them to final products, requires high temperature operations, during which metal fumes – containing both microscopic and submicroscopic particles – are released into the air. One of the most common sources of such metal aerosols is welding.

Welding fume – a typical industrial aerosol – is a complex mixture of metal oxides and materials of the welding rod (Lockey et al., 1988; Yu et al., 2000). The most common elements in steel that welders are primarily exposed to by inhalation are Fe, Mn and Cr, followed by nickel, silicon, aluminium, etc. (Jenkins, 2003). Airborne particles, released into the environment by welding, have major adverse health effects. Among welders who are permanently exposed to high concentrations of metal-enriched welding fumes, higher incidence of bronchitis, pneumonitis and metal fume fever was reported (Sferlazza and Beckett, 1991).

Beyond changes in lung function, there is an increasing interest towards the neurotoxic effects of welding fume metals. Epidemiological studies revealed that welders, suffering excessive exposure, may be at risk of developing early Parkinson's disease (Gorell et al., 1999) or manganism (see 1.3.), caused by overexposure to Mn (Discalzi et al., 2000; Sadek et al., 2003) which raises the concern of occupational health hazard. Occupational exposure to hexavalent ($Cr^{6+}$) and trivalent ($Cr^{3+}$) compounds and their respiratory effects have been described several times (Suzuki et al., 1984; Derelanko et al., 1999), but information about the neurological effects in humans or animals is scarce. In 1941, Lieberman (1941) described workers with symptoms of dizziness, headache and weakness after excessive inhalation of Cr aerosols; and Repetto et al. (1996) reported $Cr^{3+}$ toxicity on neuroblastoma cell line in vitro. Concerning Fe, inhalational studies with $Fe_3O_4$ were performed by Pauluhn (2009) and Szalay et al. (2011), but beside studies on pulmonary toxicity, no neurological or behavioural tests were conducted. Neurotoxicity of Mn, however, has been described extensively (ATSDR, 2008a), and the applicant's results on the electrophysiological effects of oral and inhalational exposure have also been published (Máté et al., 2011a, 2011b). The role that other agents (except Mn), peculiar to welding, may play in the causation of the above mentioned neurological disorders are still unknown (Flynn, 2009).

It is not possible to assess the overall toxic effects of welding fumes based on individual data of the components. Since welding fumes contain complex particles of the applied metals (mostly as metal oxides) and materials of the electrode coating, the fate of a

possibly non-toxic metal can be affected by a toxic one, and vice versa (Jenkins, 2003). Mn and Fe use the same transport mechanisms in the body (as described above), and therefore, it is possible that by blocking the transport of Mn trough the BBB, Fe is able to decrease neurological damage caused by Mn. All the same, it is necessary to examine the welding metals' individual ability to cause alterations in the CNS to obtain a basis for comparison of the individual and combined effects of the metals.

Beside chemical composition, another crucial property of aerosol from welding or other metal industry processes is particle size. This will determine, among others, where along the human airways different particles will be deposited (ICRP, 1994).

Particles deposited in the tracheo-bronchial region (typically, around 10 μm diameter) are rapidly removed by the mucociliary escalator and excreted from the body through the gastrointestinal system (where the absorption of eventual metal content is low, see above) thus having a short half-life in the body. Deposition in the nasopharynx is also possible (Tjalve and Henriksson, 1999) from where molecules, or particles if sufficiently small, can reach the CNS via transport along the olfactory pathway (Elder et al., 2006; Oszlánczi et al., 2010a). Particles that reach the alveoli are translocated directly to the blood by alveolar cells, and transported further by the bloodstream (Antonini et al., 2006). Pulmonary circulation continues to supply the brain with significant amounts of welding-related metals (e.g. Mn) long after the exposure has been terminated (Kalliomaki et al., 1983). Mn can easily cross the BBB and reach the brain through receptor-mediated endocytosis (bound to Tf receptor), and can be carried directly into the cells via the divalent metal transporter 1 (DMT-1) or via glutamate activated ion channels (Takeda, 2003; Quintanar, 2008). The transferrin/transferrin receptor (Tf/TfR) pathway is, normally, the main route of Fe transport through the BBB (Malecki et al., 1999), but is loaded to ca. 30% with Fe in humans and therefore with its free capacity can also be involved in Cr transport (Vincent, 2001). Elevated levels of Cr were found in brain tissue after intraperitoneal (ip.) or subcutaneous administration of $CrCl_3$ by Sipowicz et al. (1997), proving that Cr can also reach the brain.

In a review on welding aerosols, Antonini et al. (2003) indicated that much of the particles derived from the welding process have a diameter below 100 nm, i.e. they are nanoparticles (NPs).

### 1.2. Nanoparticles: properties and health effects

NPs of natural origin have been present all the time. With the development of technology, smelting, casting, welding, etc. of metals (Antonini et al., 2003) or plastics (e.g. teflon: Seidel et al., 1991) emerged as sources of NPs in industrial settings, but various combustion processes are not less important NP sources. Engineered nanomaterials are becoming, unobservedly, part of everyday life: several products are commercially available from cosmetics and suntan lotions to tennis balls, paints and stain-resistant clothing (Kahru and Dubourguier, 2010). As soon as the methods to investigate the origin and environmental presence of NPs, and their interaction with living systems, became available, they were recognized to have important health effects on their own, and nanotoxicology became a rapidly developing field (Suh et al., 2009).

The potential health impact is influenced by the site of deposition (for NPs, mostly nasopharyngeal and alveolar: ICRP, 1994). Also, the surface-to-mass ratio of these particles is extremely high, which means a small mass fraction within a complete aerosol sample, but with a high number of particles and a very high and reactive overall surface (Oberdörster et al., 2005).

NPs are emitted as primary particles or are generated from gaseous precursors in an atmospheric chemical process called nucleation. Their composition is determined by the composition of the materials worked with or burnt. Also for the general population, inhalation may be a significant source of NP exposure, e.g. from car exhaust because most of the metals leaving the engine with the exhaust gases are in form of microscopic and submicroscopic oxide particles. Extensive use of NPs means that besides inhalation, routes of dermal and oral uptake should be also considered.

Inhaled NPs have various interactions in a living system and can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs (Oberdörster et al., 2005). A healthy BBB is supposed to prevent foreign particles from entering the brain; NPs of various compositions were, however, detected in the brain of rats after application through the airways (Kreyling et al., 2006). Extrapulmonary effects of NPs depend on several factors including particle solubility, particle or aggregate size, the site of deposition, and the integrity of the alveolar epithelial lining (Elder et al., 2006). Results of Horváth et al. (2011) with cadmium exposure have supported that the BBB may be weakened by ions of toxic metals and so will be more permeable for NPs.

In vivo and in vitro toxicological studies showed that NP forms of relatively inert materials can become toxic: nanosized $TiO_2$ (ca. 20 nm diameter) caused more severe inflammation than the same compound in pigment grade (ca. 250 nm) grains (Oberdörster, 2000). This phenomenon is general, concerning both nanotechnological products and nano-sized pollutants (such as welding fumes). The non-toxic $Fe_3O_4$ may gain toxic properties in NP form as well (Szalay et al., 2011).

### 1.3. Manganese: properties and toxicity

Mn is a silvery-grey, hard and brittle transition metal (atomic number 25). Although Mn is an essential micronutrient (the daily demand being 2-3 mg for adults; ATSDR, 2008b) chronic exposure to excess Mn causes dysfunctions in the CNS (see below). Symptoms caused by Mn deficiency have been described in animals (in chicken, pigs, etc.; Klasing et al., 2005), including abnormal bone formation (due to disturbed functioning of Mn-activated enzymes, such as glycosyl- and xylosyl-transferases), abnormal lipid and carbohydrate metabolism, growth retardation, reproductive failure and skin problems. In rats, high levels of Ca and phosphorus in the diet was found to worsen Mn deficiency (Wachtel et al., 1943). Another factor influencing Mn absorption and transport is Fe. Increased intestinal absorption of Mn was found in Fe-deficient rats (Flanagan et al., 1980), and Fe-supplementation was reported to decrease tissue Mn levels in rats and mice (Davis et al., 1990; Hurley et al., 1983).

Mn is a component of several metalloenzymes, such as Mn-superoxide dismutase (Mn-SOD; playing important role in counteracting oxidative stress; Law et al., 1998), glutamine synthetase, alkaline phosphatase, arginase, and pyruvate carboxylase (Quintanar, 2007). Glutamine synthetase (a glia-specific Mn-protein) catalyzes the glutamic acid to glutamine conversion in the CNS, thereby inactivating the transmitter. Excess amounts of Mn inhibit this enzyme, which is one of the neurotoxic mechanisms of Mn (Normandin and Hazell, 2001).

Absorption of Mn from the gastrointestinal tract has been estimated to be 1-5% in humans (and also in rats; Davis et al., 1993). Excretion is mainly biliary and rapid, Mn occurs in the bile after 1 hour of ingestion (Malecki et al., 1996); while urinary excretion is not sensitive to dietary Mn intake (Davis and Greger, 1992). The usual biomarkers of Mn exposure are metal levels in blood, urine, faeces and hair. In certain studies (Lucchini et al., 1999) positive correlation of Mn level in blood and urine with exposure levels was found while others reported that these are not reliable indicators of Mn exposure (Järvasilo et al.,

1992; Roels et al., 1992; Smyth et al., 1973). At present, neither biochemical nor neurological indicators are available to detect the early nervous system effects of Mn intoxication, although in animal studies altered levels of certain neurotransmitters have already been used for evaluation of CNS effects of Mn (Bonilla et al., 1984; Eriksson et al., 1987). For human cases, Greger (1998) suggested the use of neuro-functional tests.

One of the most important applications of Mn is steelmaking because Mn increases corrosion resistance and toughness (e.g. in railway rails). Mn is also found in the coating of welding rods (for protection of the glowing hot welded metal parts from oxidation). In the production of dry cells, $MnO_2$ is used. Mn is also present in nanotechnological applications; in semiconductor nanocrystals (Yang et al., 2005) and in $ZnS:Mn^{2+}$ nanoflowers (three-dimensional synthetic nanostructures, growing in a flower- or tree-like shape; Chen et al., 2005). Maneb and Mancozeb (Ferraz et al., 1988) represent the use of Mn in agricultural fungicides; while the contrast agent trisodium mangafodipir (Mn-DPDP, Teslascan) is used in magnetic resonance imaging for diagnostic purposes (Rofsky and Earls, 1996).

Most of the inhalable Mn found in (both workplace and residential) environment is of anthropogenic origin, and the typical form of occupational Mn exposure is chronic inhalation (usually but not exclusively as $MnO_2$). Among ore miners, Couper (1837) has reported "manganese madness" in the $19^{th}$ century. Nowadays, this Mn-related chronic neurological disorder is called manganism, and it usually progresses in three stages (Calne et al., 1994). The initial symptoms are non-specific: apathy, anorexia, asthenia, headache, hypersomnia, spasms, arthralgia, weakness of the legs, impotence and irritability may occur. In the second stage, psychomotor and psychic disturbances dominate, such as dysarthria, excess salivation, and difficulty in walking. The third stage represents a Parkinson-like syndrome with its associated symptoms, such as cock gait (Bowler et al., 2006). In spite of the similar symptoms, however, the site of damage in manganism and in Parkinson's disease is different. While in Parkinson's disease the mesencephalic and dopaminergic neurons are damaged primarily, the targets of Mn are the striatal neurons (Erikson and Aschner, 2003). Unfortunately, the extrapyramidal symptoms are irreversible and unresponsive to L-Dopa.

In the proximity of industrial sources, air levels of 220 to 330 $ng/m^3$ Mn were reported, while in rural areas (or in urban areas without industries) Mn levels were around 10-70 $ng/m^3$ (Barceloux, 1999b). In areas where methylcyclopentadienyl manganese tricarbonyl (MMT) is added to petrol as octane booster, higher ambient Mn levels were found compared to areas where MMT is not used (Lynam et al., 1999). This results in inhalational Mn exposure for the general public; but for them, ingestion may be equally important.

Abnormally high Mn levels in the drinking water were reported by Kawamura et al. (1941) in Japan (resulting from improper disposal of used dry cells), and by Kondakis et al. (1989) in Greece (due to geological reasons), causing CNS symptoms in the residents of the polluted areas. In regions of the USA where the Mn content of drinking water is high, loss of visual and verbal memory, typical for Mn-induced brain damage, was described (Woolf et al., 2002). Cases of Mn overexposure in babies fed on cow milk- or soybean-based formulas were reported by Marlowe and Bliss (1993). Among patients receiving long term, Mn-supplemented parenteral nutrition, hypermanganesaemia (Crook, 2001) and altered magnetic resonance imaging (MRI) scans (similar to those observed in case of manganism; Ono et al., 1995) were reported. In a child, epileptic activity was observed following accidental inhalational exposure to Mn (Hernandez et al., 2003), and in a young girl receiving parenteral nutrition, tremors and seizures (Komaki et al., 1999). Alterations in the EEG and evoked potentials (EPs) after occupational Mn exposure were reported by Sinczuk-Walczak et al. (2001) and Halatek et al. (2005).

### 1.4. Iron: properties and toxicity

Fe, a transition metal with the atomic number of 26, is lustrous metallic with a greyish tinge. Due to its advantageous technical properties, Fe is used in numerous alloys, first of all in steels. Fe is of great value in biology also: it is essential for both plant and animal life, e.g. by carrying oxygen and thus enabling vital functions. Ancient Greeks and Egyptians treated weakness and digestive problems (such as diarrhoea or constipation) with Fe. Later, in the $17^{th}$ century, Fe was prescribed for chlorosis, now known as iron-deficiency anaemia; and since then it became apparent that iron-deficiency is one of the most common mineral deficiencies (Baynes, 1994). The largest part of Fe within the human organism is found in haemoglobin and myoglobin (Morris, 1987). Beyond oxygen delivery to the tissues, Fe is a cofactor of many enzymes of energy metabolism and thermoregulation (Fairbanks, 1994). Mitochondrial energy production includes enzymes containing Fe in their active centre, such as aconitase, succinate dehydrogenase or the cytochromes (Wigglesworth and Baum, 1988).

Fe homeostasis in the organism is determined by bodily needs because the body has a limited capacity of Fe excretion (except for blood loss). Fe can be excreted in faeces and urine, in small amounts in sweat, hair or nails (McDowell, 2003). The primary site of Fe absorption from the gastrointestinal system is the duodenum, but after inhalational exposure, Fe can also get into the blood circulation. Once in the blood, Fe is transported primarily by

DMT-1 (which carries non-heme Fe; Bressler et al., 2004) or Tf, binding $Fe^{3+}$ with a very high affinity (Aisen and Listowsky, 1980). Fe transport through the BBB (and also neuronal Fe uptake) is dependent on Tf-TfR binding (Malecki et al., 1999; Thomspson et al., 2001). On cellular uptake (i.e. endocytosis), Fe is released into the cytoplasm from the endosomes and either utilised in enzymes or stored in labile Fe "pools" in the form of ferritin (Williams, 1982). Cellular uptake of Fe is regulated at the level of Tf and TfR synthesis, influenced by Fe load. The bioavailability of Fe is influenced by certain factors, such as ascorbic acid, the dietary intake of which enhances the absorption of non-hem Fe, while phytate, polyphenols and Ca act as inhibitors (Klasing et al., 2005). Interactions between trace minerals are known to affect absorption in case the metals compete for the same transporter, e.g. Mn and Fe for DMT-1 or Tf. The recommended dietary Fe allowance varies between age groups and genders (especially in pre- and postmenopausal women), the median being 8 mg/day for men and ca. 18 mg/day for women (Schümann et al., 2007).

In case of overload, Fe can deposit in certain mitochondria rich organs, such as the heart, liver and pancreas. Fe overload causes disruption of the mitochondrial energy production by decreasing enzyme activity (Bacon et al., 1983), and leads to generation of reactive oxygen species (ROS) responsible for the toxic effect (Eaton and Qian, 2002). Another possible mechanism of Fe toxicity involves Fe deposition in the cellular lysosomal compartment, causing rupture of lysosomes and release of digestive enzymes into the cytoplasm, which may end in cell death (Eaton and Qian, 2002).

The brain requires high Fe supply for myelogenesis and myelin maintenance (Thompson et al., 2001). Fe deficiency may result in hypomyelination (Larkin and Rao, 1990), or to delayed onset of auditory (AUD) evoked potentials as a consequence of disrupted myelin sheath (Roncagliolo et al., 1998).

For normal brain function, undisturbed production of neurotransmitters is essential. Dopamine, norepinephrine and serotonin require Fe for their synthesis (Thompson et al., 2001). Presence of Fe was observed at sites of GABAergic innervations in the brain (Hill, 1985). Certain neurodegenerative diseases, like Parkinson's or Alzheimer's disease, multiple sclerosis, but even epilepsy were reported to be in close relationship with disrupted Fe homeostasis (Thompson et al., 2001). In case of Friedrich's ataxia, e.g., the defect of mitochondria and the associated Fe accumulation results in the formation of free radicals. In Parkinson's or Alzheimer's disease, accumulation of Fe in the basal ganglia and induction of oxidative stress is held responsible (Sayre et al., 1999).

Zhu et al. (2011) reported increased microvascular permeability and prolonged blood coagulation after intratracheal (it.) instillation of $Fe_2O_3$ NPs, and, in in vitro experiment, generation of oxidative stress by ROS formation, mitochondrial swelling and disruption of the mitochondrial inner structure by both $Fe_3O_4$ and $Fe_2O_3$ NPs. Szalay et al. (2008) also suggested that $Fe_3O_4$ NPs caused membrane injury in epithelial cells and a moderate inflammatory response accompanied by fibrosis.

Fe oxide NPs are, on the other hand, of considerable interest for biomedical applications: targeted drug delivery, cancer diagnosis and therapy, magnetic resonance imaging etc. (Zhu et al., 2011; Naqvi et al., 2010) which is yet another reason for examining their toxicity.

### 1.5. Chromium: properties and toxicity

Cr is a silvery metallic element which has the atomic number 24. Its name comes from the Greek "chroma", meaning colour. Cr occurs naturally in rocks, soils, animals and plants (ATSDR, 2008a) in any of the oxidation states from -2 to +6 (but not zero; Klasing et al., 2005). Cr was first used in the production of pigments and in leather tanning, but along with the advances in technology, Cr was used more for metallurgic applications and alloy formation. Further applications of Cr include wood preservatives, fungicides in the agriculture, algicides, porcelain and glassmaking, stainless steel cookware or tattoos (Cohen and Costa, 2007). Cr particles arising from metallurgy, the primary source of ambient Cr, are mainly in the form of ferrochrome, a Fe-Cr alloy containing Cr between 50-70%.

The main forms of Cr are the trivalent ($Cr^{3+}$) and the hexavalent ($Cr^{6+}$) state. $Cr^{3+}$ is a component of the glucose tolerance factor, thus regulating glucose, protein and fat metabolism (Mertz, 1969; Klasing et al., 2005). For this, the adequate intake for Cr for adults is 0.025-0.035 mg/day (Goldhaber, 2003). Environmental $Cr^{6+}$ originates almost totally from human activity, such as metallurgical processes, manufacturing of Portland cement, sewer sludge and waste incineration, etc. (Cohen and Costa, 2007). It is more toxic than $Cr^{3+}$, is a strong oxidant, carcinogen, allergen and acute irritant both in humans and animals (Barceloux, 1999a). The natural sources of Cr in the environment are weathering of rocks and leaching from soils, but exposure of the general population is negligible compared to occupational exposure. Besides dermal contact, one of the most likely ways of being exposed to Cr, ingestion and inhalation are also of high importance.

Cr absorption and metabolism is strongly dependent on the valence state. Once in the organism, the vast majority of $Cr^{6+}$ is reduced by the acidic environment of the stomach (or by the epithelium lining fluids in the lung) to $Cr^{3+}$. This reduction may have a causative role in Cr toxicity, because during this process free radical formation may occur, leading to oxidative stress (Bagchi et al., 2002). The remaining $Cr^{6+}$, not reduced by the gastric juices, is absorbed in the intestine and transported to the liver. While the absorption of $Cr^{3+}$ is poor, $Cr^{6+}$ is able to enter into the cells to a greater extent by means of non-specific anion carriers (Klasing et al., 2005). Ascorbic acid, certain amino acids and oxalate increase, while zinc may reduce, absorption of Cr (Offenbacher et al., 1997). In NP form, trivalent Cr was transported more efficiently in an in vitro experiment and as possible explanations, particle size, surface charge and hydrophobicity were mentioned (Zha et al., 2008).

Similarly to Fe and Mn, Tf is involved in the transport of Cr (Vincent, 2001). Most of the $^{51}CrCl_3$ given by gavage to rats was present in blood, and 80% was bound to Tf (Hopkins and Schwarz, 1964). According to Offenbacher et al. (1997) Fe overload caused alterations in Cr transport by saturation of Tf, limiting the amount of Cr being transported. In case of high concentration of Cr in the blood, $Cr^{3+}$ may bind to serum albumin or globulin.

The main sites of Cr deposition after exposure are the liver, kidney and spleen, whereas the lung, brain or the heart accumulates less (Cohen and Costa, 2007). Blood Cr levels do not represent tissue concentrations and body stores. The main route of Cr excretion is the urine.

While inhalation of soluble Cr compounds (mists or dust, mainly in occupational settings) results in damaged nasal mucosa and perforated nasal septum, exposure to insoluble (more exactly less soluble) compounds cause damage to the lower respiratory tract (ATSDR, 2008a). Workers acutely exposed to $Cr^{6+}$ compounds may develop asthma and other signs of respiratory distress (Olaguibel and Basomba, 1989). High concentration of $CrO_3$ fumes were reported by Lieberman (1941) to cause respiratory symptoms, such as sneezing, nasal discharge, laboured breathing, and nasal septum ulceration and perforation in workers in a chrome plating plant after 2-3 months of exposure, but dizziness, headache and weakness was also found in the same exposed group. Chronic exposure to $Cr^{6+}$ may increase the risk of evolving lung cancer or other (non-cancer) respiratory disease and may lead to death. Respiratory symptoms were also described after exposure of workers to chromite ore (containing $Cr^{3+}$), and increase in the number of complaints and clinical signs was reported in parallel with the increased number of respirable $Cr^{3+}$ and $Cr^{6+}$ particles (ATSDR, 2008a). Although ingested $Cr^{3+}$ is considered non-toxic, hypoactivity, mydriasis, lacrimation and body

weigh loss was reported as signs of acute Cr toxicity (ATSDR, 2008a). Based on recent findings, Levina and Lay (2008) suggested to pay more attention to the toxic effect of trivalent Cr compounds.

$Cr_2O_3$ is a trivalent Cr oxide compound, often originating from industrial process. Horie et al. (2011) reported that cytotoxicity of nanoparticulate (60 nm) $Cr_2O_3$ was higher compared to fine particles, and that $Cr_2O_3$ NPs reduced the mitochondrial activity and released hexavalent Cr in the medium. Exposure of human carcinoma or keratinocyte cells to Cr NPs increased intracellular ROS level, and caused oxidative stress. The substance used in the present work, nanoparticulate $Cr(OH)_3$, is the hydrated form of $Cr_2O_3$.

### 1.6. Aims

This thesis is the continuation of a long line of antecedent research at the Department on the neurotoxicity of Mn, in dissolved (Vezér et al., 2005) and more recently in nanoparticulate form (Oszlácnzi et al., 2010a,b), and on interactions of neurotoxic metals (Pecze et al., 2005). The overall goal, modelling human heavy metal exposure – which is often inhalational but can also be oral – more adequately, led to more elaborate experimental designs, one involving the combination of several industrially relevant metals, and the other involving more than one physicochemical form of one metal. Varying the way and site of administration was included also. From all that, the aims of the present PhD work were specified as follows:

- To examine the effects on general toxicological, CNS electrophysiological and behavioural parameters exerted by nanoparticulate Mn, Fe and Cr in an identical experimental setup;
- To repeat these experiments with double and triple combinations of the mentioned metals, in order to reveal possible interactions and to have a better model of welding fumes and similar industrial emissions;
- To examine the relationship of external and internal doses in dependence of the physicochemical form and site of application of Mn, and on co-application of the other two metals;
- To test whether analysis of the second:first ratio, a method used previously to study the CNS effect of the mitochondrial toxin 3-nitropropionic acid (Szabó et al., 2005) and

the acute effect of Mn (Máté et al., 2008) is applicable for detection of neuro-functional alterations induced by subacute application of Mn and other metals.

# 2. Materials and methods

## 2.1. Experimental animals and substances

Young adult male Wistar rats were obtained from the university's breeding centre. The different experiments were started with different initial body weights, see Table 1. The animals were housed, with up to four rats in one cage, under GLP-equivalent conditions (22±1 ºC, 30-60% relative humidity, 12-h light/dark cycle with light on at 06:00), and had free access to tap water and standard rodent chow (Bioplan, Isaszeg).

The rats were exposed to Mn by per os (po.) and it. administration. In po. administration, aqueous solution of $MnCl_2$ was applied, while in it. administration the rats were instilled either with aqueous solution of $MnCl_2$, or with a suspension of $MnO_2$, $Fe_3O_4$ or $Cr(OH)_3$ NPs or their combinations (the treatment schemes are given in Table 1.)

$MnCl_2$ was of >99% purity, available commercially (Sigma-Aldrich Hungary). The $MnO_2$, $Fe_3O_4$ or $Cr(OH)_3$ NPs for Experiment III and IV were synthesized at the Department of Physical Chemistry and Materials Science, University of Szeged, Faculty of Science and Informatics.

For $MnO_2$ NPs, an aqueous alkaline medium containing polyacrylic acid (PAA; MW 5000) and ethanol was prepared and stoichiometric amount of $KMnO_4$ solution was dripped into it under stirring. A sol containing $MnO_2$ NPs of 10-20 nm diameter was generated, the concentration and pH of which was adjusted as desired with high purity water and HCl. For vehicle control, the starting medium was completed with KOH and NaOH, and pH was set to ca. 7.

$Fe_3O_4$ NPs were generated in a concentrated solution of ferrous and ferric chloride by adding NaOH in 10% excess slowly, then suddenly under vigorous stirring. The resulting black suspension was washed from residual alkali, then strongly acidified to induce peptization. $Fe_3O_4$ NPs were stabilized with PAA, and NaCl, HCl and NaOH solutions were used to adjust pH 6.5. Diameter of the NPs was 10-20 nm.

$Cr(OH)_3$ NPs were also generated by alkali treatment, this time of $CrCl_3$ solution at boiling temperature. After cooling, pH was set to 4.11, and the solution was reheated for aging the precipitate 6 hours long. The precipitate was repeatedly washed by centrifuging and resuspending in ultra pure water until peptization took place. For administration, pH was set to 6.0 and concentration was adjusted.

The chemical purity of the nanoparticles was checked by X-ray diffraction, and their particle size, by X-ray diffraction and transmission electron microscopy.

### 2.2. Experiments and treatment schemes

Four experiments (numbered I to IV) were performed in this study. The data on methods and times of treatment, on doses, and on investigations performed, are summarized in Table 1.

#### *2.2.1. Experiment I: Subacute per os treatment with $MnCl_2$ solution*

For po. administration, 2.5 mg/ml $MnCl_2$ was dissolved in tap water, and given as drinking fluid to the 12 animals of the treated group for 6 weeks. This amount of Mn was, according to literature data, a small, but at the same time relevant dose (Morello et al., 2008; Elbetieha et al., 2001), considering possible Mn intoxication of environmental origin. Animals of the control group (n=8) consumed clear tap water during the exposure period. Mn content of the tap water used was negligible (0.03 μg/ml; Szegedi Vízmű, 2010), therefore it was disregarded at the calculation of cumulative dose. In order to prevent Mn precipitation from tap water, 0.25 mg/ml citric acid was added. The pH of the drinking fluid, containing $MnCl_2$ and citric acid in the above mentioned concentrations, was 6.7. Based on the water consumption of the animals, the solution had no unpleasant taste.

During the treatment period, body weight and water consumption of the animals was measured daily (for the sake of the latter, the animals were housed alone). From the data, cumulative Mn dose (per kg body weight) could be calculated. At the end of the 6-week treatment, after preparation for electrophysiological investigation (see 2.4.1.) two recordings of somatosensory (SS) evoked potentials (EPs) per animal (see 2.4.2.) were made in urethane anaesthesia.

**Table 1** Treatment schemes of the experiments.

| | **Experiment I** ***(Subacute per os)*** | **Experiment II** ***(Subacute intratracheal)*** | **Experiment III** ***(Subacute intratracheal)*** | **Experiment IV** ***(Subacute combined intratracheal)*** |
|---|---|---|---|---|
| **Duration** | 6 weeks | 5 weeks | 4 weeks | 4 weeks |
| **Groups (with code) and number of animals per group** | *Control*: 8<br>*Treated*: 12 | *Control* (untreated): 6<br>*Vehicle control*: 12<br>*Treated*: 12 | Untreated control (*Cont*): 6<br>Vehicle control for Fe and Mn treatment (*FMVC*): 8<br>Low dose $Fe_3O_4$ (*FeLD*): 8<br>High dose $Fe_3O_4$ (*FeHD*): 8<br>Low dose $MnO_2$ (*MnLD*): 8<br>High dose $MnO_2$ (*MnHD*): 8<br>Vehicle control for Cr treatment (*CrVC*): 8<br>Low dose $Cr(OH)_3$ (*CrLD*): 8<br>High dose $Cr(OH)_3$ (*CrHD*): 8 | Vehicle control (*VC*): 10<br>$Fe_3O_4$+ $MnO_2$ (*FM*): 10<br>$Fe_3O_4$+ $Cr(OH)_3$ (*FC*): 10<br>$MnO_2$+ $Cr(OH)_3$ (*MC*): 10<br>$Fe_3O_4$+ $MnO_2$ +$Cr(OH)_3$ (*FMC*): 10 |
| **Body weight at start** | 200±20 g | 200±20 g | 260-280 g | 260-280 g |
| **Substances, doses, and way of administration** | $MnCl_2$<br>2.5 mg/ml<br>po. via drinking water | $MnCl_2$<br>0.5 mg/kg b. w.<br>it. | *FeLD*: 2 mg/kg b. w.<br>*FeHD*: 4 mg/kg b. w<br>*MnLD*: 2 mg/kg b. w.<br>*MnHD*: 4 mg/kg b. w.<br>*CrLD*: 2 mg/kg b. w.<br>*CrHD*: 4 mg/kg b. w.<br>it. | *FM*: 2 mg/kg b. w.<br>*FC*: 2 mg/kg b. w.<br>*MC*: 2 mg/kg b. w.<br>*FMC*: 2 mg/kg b. w.<br>it. |
| **Vehicle** | Tap water | Distilled water | PAA (FMVC), normal saline (CrVC) | Normal saline and PAA |
| **Investigation** | Electrophysiology<br>Dissection<br>Organ weighing<br>Tissue metal level determination | Electrophysiology<br>Dissection<br>Organ weighing<br>Tissue metal level determination | OF activity<br>Electrophysiology<br>Dissection<br>Organ weighing<br>Tissue metal level determination | OF activity<br>Electrophysiology<br>Dissection<br>Organ weighing<br>Tissue metal level determination |

### *2.2.2. Experiment II: Subacute intratracheal treatment with $MnCl_2$ solution*

In this experiment the rats had it. instillations of $MnCl_2$ (0.5 mg/kg b. w., instillation volume: 1 ml/kg b. w.), once a day, five days per week, for 5 weeks. The applied dose was based on previous experience and on literature data (Roels et al., 1997; Sriram et al., 2010). Instillation was carried out in brief diethyl ether anaesthesia. The rats were put on an oblique board (60° to horizontal) hanging with their upper incisors in a wire loop, and the larynx was illuminated transdermally by means of a fibre optic light guide placed against the animal's neck. The larynx and trachea was visualized using a custom-made laryngoscope. The tongue was pulled forward with a pair of non-traumatic forceps, and the $MnCl_2$ solution (or distilled water for the controls) was instilled into the trachea by means of a 1 ml syringe connected to a thin plastic tube (1.2 mm OD) inserted between the vocal chords (the method of instillation was based on Oka et al., 2006). The recording at the end of the 5-week treatment was identical to that done in Experiment I.

### *2.2.3. Experiment III: Subacute intratracheal treatment with NPs containing Mn, Cr and Fe*

The rats, divided into 10 groups, were intratracheally instilled with a suspension of $MnO_2$, $Fe_3O_4$ or $Cr(OH)_3$ NPs (2 or 4 mg/kg b. w.; for details see Table 1). Treatment was performed once a day, 5 times per week, for 4 weeks. This short period was chosen because experience showed that the nanoparticulate form of Mn (and other metals) tends to be more toxic than other (dissolved etc.) forms (Horváth et al., 2012). $MnO_2$ and $Fe_3O_4$ NPs were suspended in PAA, the vehicle also used to treat the *FMVC* vehicle control group. $Cr(OH)_3$ NPs were suspended in normal saline, so the *CrVC* control group was saline treated. An untreated control (*Cont*) group was also used because of the expectable own effects of the treatment procedure. It. treatment was performed as described in 2.2.3. At the end of the treatment, open field (OF) test (see 2.3.) and electrophysiological recording (see 2.4.) was conducted.

### *2.2.4. Experiment IV: Subacute intratracheal treatment with combinations of Mn, Cr and Fe NPs*

In this experiment, animals were treated with the double and triple combinations of $MnO_2$, $Fe_3O_4$ or $Cr(OH)_3$ NPs. The treatment lasted for 4 weeks; it. instillation was performed

once a day, 5 times per week (it was supposed that during 4 weeks the effects will develop but animal loss due to excessive toxicity will be minimal). Five groups of animals were set up as detailed in Table 1. The vehicle control (*VC*) group was treated with PAA and normal saline with several hours delay. Fe and Mn NPs were both suspended in the PAA medium, and could be administered together. Administration of NPs in different vehicles (combinations containing Cr: FC, MC and FMC) was separated in time by several hours in order to decrease burden put on the lungs by the instillation (that is, to avoid the administration of double volume). It. treatment was performed as described in 2.2.3. At the end of the treatment, OF test (see 2.3.) and electrophysiological recording (see 2.4.) was conducted.

### 2.3. Behavioural investigation in the open field

OF test was applied on the day following the last instillation. The rats were put into an OF apparatus to test their spontaneous horizontal and vertical motor activity in one 10 min session per rat.

An OF box of 48x48x40 cm size was used, equipped with two arrays of infrared movement detectors at floor level and in 12 cm height (Conducta 1.0 System, Experimetria Ltd, Budapest, Hungary). The test was performed between 8 and 11 hours in the morning. Just before the test, the animals were left in the dimly lit test room for 30 minutes for acclimatisation. One by one, the animals were placed into the centre of the box. The instrument recorded the animal's horizontal and vertical motor activity based on the interruptions of the infrared beams. From these data, counts, time and run length of the activity forms (ambulation, local activity, immobility, rearing) were automatically calculated. More than 40 mm shift in the location of interrupted beams at the floor level during a time unit of 1 s was interpreted as ambulation (i.e. walking, horizontal activity), less shift, as local activity (motions without changing the place, e.g. grooming), and no shift at all, as immobility. Rearing was recorded if beams at floor level and at the higher level were interrupted simultaneously. It was known form previous experience (e.g., Vezér et al., 2005) that OF test was suitable for investigating the impairment of higher nervous functions caused by heavy metals.

## 2.4. Electrophysiological investigation

### *2.4.1. Anaesthesia and preparation*

Preparation and electrophysiological measurement was done after anaesthetizing the animals with urethane (Reanal, Hungary). Urethane has been used in animal experimentation for a long time, among others because – despite its long-term effect – it has minimal depressive effect on the cardiovascular and respiratory system, or on the reflexes (Maggi and Meli, 1986). The effect of urethane is long lasting (more than 24 hours) therefore it can only be used for terminal narcosis, in experiments where recovery or awakening is not necessary. For anaesthesia, 1000 mg/kg b. w. urethane (Mook, 2006) was injected intraperitoneally. The depth of the anaesthesia was checked by pinching the hind foot toes of the animal. If there was no reaction, the rat was ready for preparation.

The head of the rats was fixed in a holder frame, the skin was opened by a mid-sagittal cut, and the muscles and connective tissues attached to the skull were removed. Wounds were sprayed with 10% Lidocaine, then the left temporal bone was cut along its inner circumference by a dental drill bit attached to a mini drill, the bone was lifted, and the left hemisphere was thus exposed. The exposed dura surface was covered with a thin layer of petroleum jelly in order to protect it from dehydration. The prepared animals were wrapped in a warm cloth to maintain body temperature and were put aside for at least 30 min for recovery.

### *2.4.2. Electrophysiological recording and evaluation*

After the recovery period, the rat was placed into the stereotaxic frame of the electrophysiological setup. To stabilize body temperature, a thermostated (+36.5°C) base plate was used to support the rat's underside during the recording procedure.

For recording spontaneous and evoked cortical activity, ball-tipped silver recording electrodes were placed on the dura over the primary SS projection area of the whiskery pad (barrel field), and over the primary visual (VIS) and auditory (AUD) focuses. These regions were determined on the basis of a somatotopic map (Zilles, 1984), and the fine positioning of the electrodes was done by searching for the punctum maximum of the evoked response. A stainless steel clamp was attached to the cut skin edge as indifferent electrode.

Stimulus-evoked activity (sensory EPs) was recorded in each of the experiments. SS stimulation was done by electric pulses given trough a pair of needles inserted into the whiskery skin (3-4 V; 0.05 ms). VIS stimulation was performed by flashes delivered by a high-luminescence white LED directly into the contralateral eye of the rat. For AUD stimulation, clicks (ca. 40 dB) were applied into the contralateral ear of the rat from a mini earphone through the hollow ear bar of the stereotaxic frame.

In Experiment I-IV, only SS EPs were recorded using double stimulation. In that, the first stimulus was followed by the second one after 300, 240, 180, 120 and 60 ms interstimulus intervals (ISIs). A complete record involved 20 "runs"; and in one run each ISI occurred once in decreasing order (5 "sweeps"). Recording of 20 "runs" (a complete record) took 6 min. Such complete recordings followed in 20 min intervals thus the animals had enough time (14 min) for recovery. This stimulation scheme was applied, and found sensitive for neurotoxic influences, in earlier experiments of the Department (Szabó et al., 2005; Lukács et al., 2007). Stimulation, recording of EPs, and analysis of the records (described below) was governed by the software Clampex 8.0 (Axon Instruments Inc., USA).

The SS EPs were amplified ($10^4$ AC, 5 Hz and 1 kHz boundaries, EXT-MC biological amplifier by Experimetria Ltd., Hungary) and fed through a Digidata 1320A interface (Axon Instruments, USA) into the PC. The EPs were averaged automatically by the software from the recorded 20 "runs", for each double stimulation. On playing them back, cursors were positioned manually on the positive and negative peaks of the EPs, generated by the first and second stimuli (Fig. 1A). The cursor positions defined numerical data which were put into an Excel table to calculate peak-to-peak amplitudes (B – C vertical difference in Fig. 1B) and the latency (measured horizontally between A – B and A – C in Fig. 1B) and amplitude of the positive (first; zero line – B in Fig. 1B) and negative (second; zero line – C in Fig. 1B) peak of the SS EP. In Experiment I-IV, two recordings per rat were made at the end of the treatment period. Data from these two records were averaged, then group means were calculated and compared.

A novel and important part of the evaluation was the calculation of second:first ratio. To do that, each parameter of the second EP was divided by the corresponding parameter of the first EP. Second:first ratio calculation was performed in case of each animal one by one (in acute experiments, after standardisation) and from these data, group means were calculated. The second:first ratio was interpreted as a measure of dynamic interaction between the two excitation processes – which themselves depend on both energy supply and on

undisturbed release and removal of transmitters. The procedure proved to be informative in earlier experiments (Szabó et al., 2005).

In Experiment III and IV, the software NEUROSYS 1.11 (Experimetria Ltd., Hungary) was also used for recording cortical activity. In these experiments, spontaneous electrical activity (electrocorticogram, ECoG) was first recorded from the three cortical areas mentioned above, simultaneously for 6 min. EPs were also recorded from each cortical area (SS, VIS, AUD) in Experiment III and IV. Fifty stimuli of each modality per rat were applied. The recorded biological signals were amplified and filtered as above, and fed into the digitizer interface of the PC-based recording setup (sampling frequency: 2 kHz for spontaneous and 8 kHz for evoked activity).

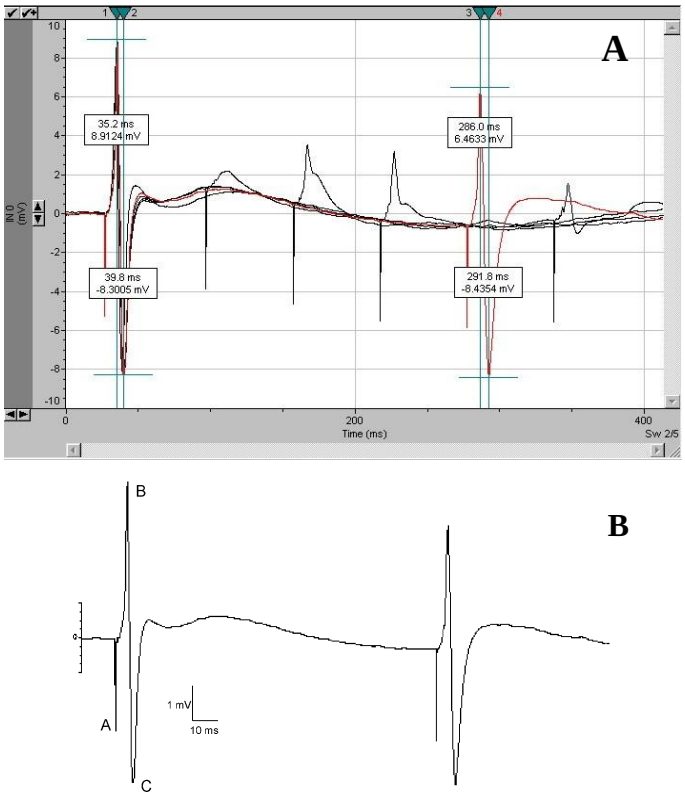


**Figure 1** Somatosensory evoked potentials induced by double-pulse stimulation. **A**: Screenshot from Clampex 8.0. Cursors measuring amplitude and latency are shown. **B**: Example trace. Specific measuring points are marked on the first evoked potential (see text for details).

From the ECoG records, relative spectral power of the frequency bands (delta, 0.5-4 Hz; theta, 4-7 Hz; alpha, 8-13 Hz; beta1, 13-20 Hz; beta2, 20-30 Hz; gamma, 30-50 Hz; Kandel and Schwartz, 1985) was determined. From the relative band power data, the so-called "ECoG index" was calculated with the formula ([delta]+[theta])/([beta1]+[beta2]). This proved to be a handy, albeit simplifying, descriptor of the ECoG spectrum in earlier works

(Dési and Nagymajtényi, 1999). EPs were automatically averaged off-line, and their parameters were measured manually by means of screen cursors of the software. Exemplified on the SS EP, onset latency was measured between the stimulus artefact (designated 0 in Fig. 2) and onset of the first wave (A in Fig. 2). Duration of the EP was calculated as the difference of the 0 - D and 0 - A times. In case of the VIS and AUD EPs, onset latency and duration was measured in the same way.

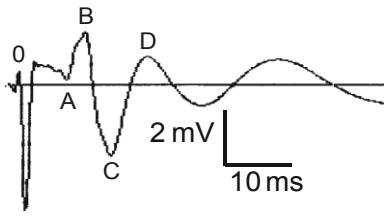


**Figure 2** An example of the SS EPs. Specific measuring points are marked 0 and A - D. See text for details.

Previous studies (Papp et al., 2001, 2004) demonstrated that by varying the frequency of stimulation the dynamic interaction of successive excitation processes in the sensory system can be assessed, which in turn reflects the actual state of the CNS. In theoretical basis and final aim, stimulating with varied frequencies was akin to double-pulse stimulation used in the present experiments. Frequency dependence in the parameters of the SS EPs was determined by delivering stimuli to the whisker pad also with 2 and 10 Hz frequency, beyond the standard 1 Hz.

### 2.5. General toxicological investigations

Body weight of the animals was regularly measured during the experiments, to follow-up weight gain and to determine the exact daily doses in Experiment II, III and IV. Following electrophysiological recording, the animals were sacrificed by an overdose of urethane, were dissected, and blood was collected from the abdominal vein by means of a heparinized syringe into 5 ml plastic tubes. Brain, lungs, heart, thymus, liver, kidneys, spleen and adrenal glands were removed and the organ weights were measured. From these data, relative weights (indicators of the general toxicity; Schärer, 1977) were calculated by relating organ weights to brain weight or 1/100 of body weight. Blood, brain, lung and liver samples were stored at -22°C.

For metal level determination, ca. 1 g samples were dried at 80°C to constant weight and were digested in 5 ml 65% $HNO_3$ at 90°C for 90 min. The digested matter was washed

quantitatively into 100 ml measuring flasks, and these samples were transferred to the Laboratory of the MOL Hungarian Oil and Gas Company for metal determination by inductively coupled plasma mass spectrometry.

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.

### 2.6. Data analysis

Statistical analysis in acute experiments was made by paired sample t-test. In the further experiments, general toxicological, behavioural and electrophysiological data were tested for significance with one-way ANOVA with post-hoc LSD test. Linear regression calculations between tissue metal levels and general- and neurotoxicological parameters were done by the “linear fit” function of MS Excel. The level of significance was set to $p<0.05$.

# 3. Results

## 3.1. Experiment I: Subacute per os treatment with $MnCl_2$ solution

During this 6 week treatment, water consumption of the treated animals was measured daily to calculate total Mn intake (it was, therefore, not measured it in the *Control* group). The average daily water consumption was 56 ml/rat, within normal range for rats of similar body weight, based on literature data (Olfert et al., 1993). The calculated daily Mn intake of the treated rats was equivalent to 141 mg $MnCl_2$/rat. This amount meant that, by the end of the 6th week, the cumulative dose of Mn was 1633 mg/rat.

Body weight gain of the animals was continuous and steady during the whole treatment, with only a slight difference between the *Control* and *Treated* group (Fig. 3).

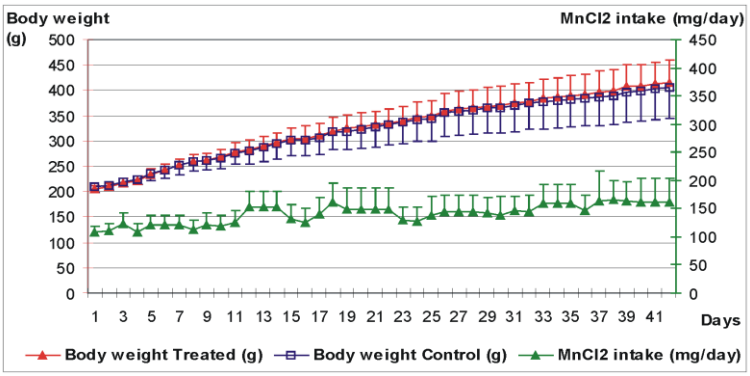


**Figure 3** Experiment I: Body weight gain and daily $MnCl_2$ intake of the rats during the 6 week (42 day) treatment period. Mean+/-SD, n=8.

There were no significant changes in the organ weights after the oral Mn treatment (Table 2), when related either to 1/100 body weight or to brain weight (the calculation base was itself unchanged, see Fig. 3).

During po. $MnCl_2$ exposure (and during it. application: Experiment II, below), significant Mn deposition evolved in the brain of the treated animals (Table 3); blood Mn levels were, however, not higher than in the corresponding controls.

**Table 2** Relative organ weights in Experiment I and II.

**A.** ***Relative organ weights (related to 1/100 of body weight)***

| | **Exp. I: Per os treatment** | | **Exp. II: Intratracheal treatment** | | |
|---|---|---|---|---|---|
| | *Control* | *Treated* | *Control* | *Vehicle control* | *Treated* |
| **Brain** | 0.518±0.074 | 0.472±0.037 | 0.486±0.027 | 0.527±0.033 | 0.531±0.037$^{*}$ |
| **Heart** | 0.263±0.014 | 0.257±0.013 | 0.264±0.020 | 0.257±0.017 | 0.276±0.011$^{\#\#}$ |
| **Thymus** | 0.120±0.018 | 0.121±0.021 | 0.089±0.009 | 0.110±0.028$^{*}$ | 0.118±0.021$^{**}$ |
| **Lungs** | 0.376±0.030 | 0.352±0.032 | 0.343±0.029 | 0.372±0.023 | 0.405±0.041$^{**\#}$ |
| **Liver** | 3.263±0.336 | 3.461±0.303 | 3.236±0.251 | 2.967±0.160 | 3.151±0.304 |
| **Spleen** | 0.174±0.026 | 0.171±0.023 | 0.172±0.012 | 0.160±0.028 | 0.178±0.026 |
| **Kidney** | 0.693±0.058 | 0.691±0.061 | 0.672±0.014 | 0.691±0.063 | 0.648±0.039 |
| **Adrenals** | 0.015±0.003 | 0.014±0.004 | 0.014±0.003 | 0.016±0.002 | 0.015±0.003 |

**B.** ***Relative organ weights (related to brain weight)***

| | **Exp. I: Per os treatment** | | **Exp. II: Intratracheal treatment** | | |
|---|---|---|---|---|---|
| | *Control* | *Treated* | *Control* | *Vehicle control* | *Treated* |
| **Heart** | 0.515±0.056 | 0.546±0.036 | 0.544±0.047 | 0.489±0.045 | 0.523±0.044 |
| **Thymus** | 0.236±0.051 | 0.259±0.056 | 0.184±0.018 | 0.209±0.056 | 0.224±0.047 |
| **Lungs** | 0.734±0.078 | 0.746±0.054 | 0.708±0.064 | 0.707±0.053 | 0.765±0.092 |
| **Liver** | 6.471±1.432 | 7.406±1.121 | 6.691±0.755 | 5.653±0.532 | 5.976±0.861 |
| **Spleen** | 0.346±0.089 | 0.364±0.066 | 0.354±0.035 | 0.306±0.067 | 0.338±0.055 |
| **Kidney** | 1.358±0.181 | 1.476±0.202 | 1.386±0.079 | 1.310±0.095 | 1.226±0.109*## |
| **Adrenals** | 0.0299 ± 0.0055 | 0.0295 ± 0.0085 | 0.0279 ± 0.0058 | 0.0305 ± 0.0031 | 0.0283 ± 0.0062 |

Mean±SD, n=6, *,** p<0.05, 0.01 vs. *Control*; #,## p<0.05, 0.01 vs. *Vehicle control*.

**Table 3** Mn levels in tissue samples from Experiment I and II.

| | **Exp. I: Per os treatment** | | **Exp. II: Intratracheal treatment** | |
|---|---|---|---|---|
| | *Control* | *Treated* | *Vehicle control* | *Treated* |
| **Blood** | 222.3±49.4 | 222.1±41.1 | 253.8±133.9 | 249.1±44.6 |
| **Brain** | 1584.7±232.6 | 2531.9±256.9* | 1812.5±176.3 | 3084.8±472.9$^{\#\#}$ |
| **Lungs** | - | - | 1177.5±160.3 | 62022.15±16605.47$^{\#\#\#}$ |

Mean±SD, n=5; * p<0.05 vs. *Control*; ##, ### p<0.01; 0.001 vs. *Vehicle control*.

In this experiment, nervous system effect of Mn was characterized by recording SS EPs from the cortex and calculating the second:first ratio (see Methods, 2.4.2.). On subacute effect of Mn, this ratio shifted typically to lower values. Second:first ratio of the peak-to-peak amplitude was lower than that of the *Control* in case of each ISI, although without

significance (Fig. 4); the decrease being due mostly to diminished positive peak amplitude of the second EP. The second:first ratio of positive peak latencies was also significantly lower (*Treated* vs. *Control*) at nearly each ISI (Fig. 5A) while an identical change of the negative peak latencies was significant only at 300 and 60 ms ISIs (Fig. 5B). The absolute latency values had no significant change which suggested that the ratio may be a more sensitive indicator.

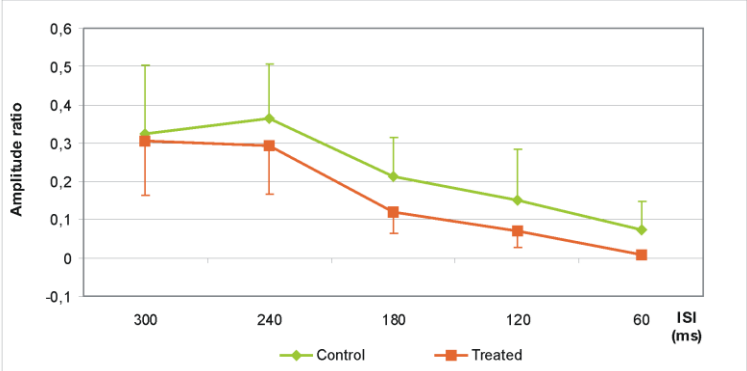


**Figure 4** Experiment I: Second:first ratio of the peak-to-peak amplitudes of the first and second EPs at different ISIs. Mean+/-SD, n=8.

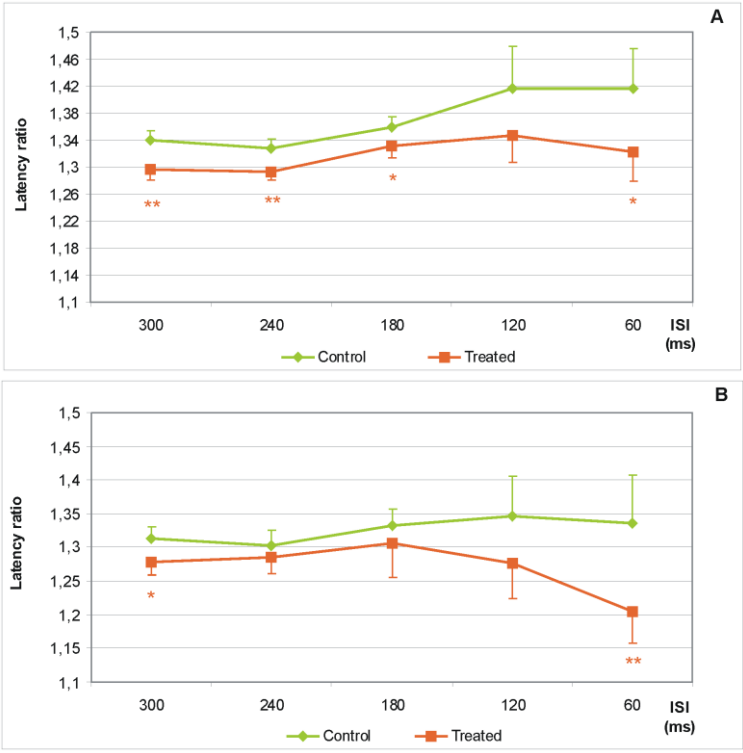


**Figure 5** Experiment I: Second:first ratio of the positive (A) and negative (B) peak latencies of the first and second EPs at different ISIs.

Mean+/-SD, n=8, *, ** $p<0.05$, 0.01 vs. *Control*.

### 3.2. Experiment II: Subacute intratracheal treatment with $MnCl_2$ solution

It. instillation of $MnCl_2$ had no significant effect on the body weight gain in the *Treated* group compared either to *Control* or to *Vehicle control* (Fig. 6). There was a slight difference between *Control* and *Vehicle control* also, but not significant. However, Mn had a clear effect on the relative weight (related to 1/100 body weight) of certain organs such as brain, lungs, heart and thymus. The increase of these organs' relative weights was significant (Table 2). By that time, calculated cumulative dose of Mn was merely ca. 1 mg/rat; all the same, Mn concentration in both brain and lungs – but not in blood – was significantly elevated in *Treated* compared to *Vehicle control* group (Table 3).

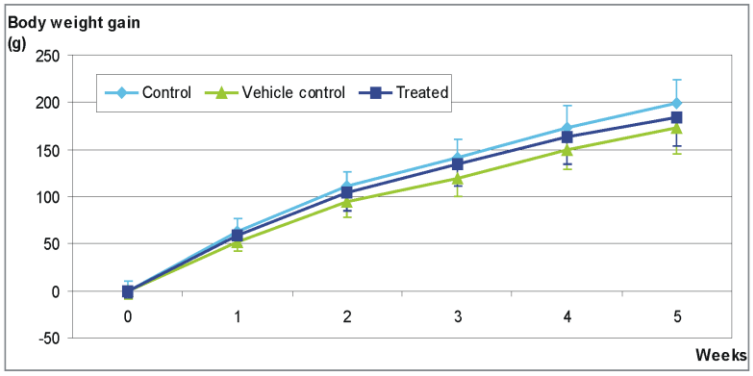


**Figure 6** Experiment II: The effect of the 5 week treatment on the body weight gain of the animals. Mean+/-SD, n=6.

The second:first ratio of the peak-to-peak amplitude showed significant decrease in the *Treated* group (Fig. 7) compared to *Control* (240 ms ISI) and to *Vehicle control* (240 and 180 ms ISIs), which change was basically similar to the alteration observed in po. treatment. As to the positive and negative peaks, the second:first ratio was lower at all ISIs compared to *Control* and *Vehicle control* groups and showed significance at 300 and 240 ms ISIs (Fig. 8A, B). The positive peak latency of the second EP increased vs. both controls, and the change was significant at the most ISIs (data not shown). This increase, however, was not reflected in the calculated second:first ratio.

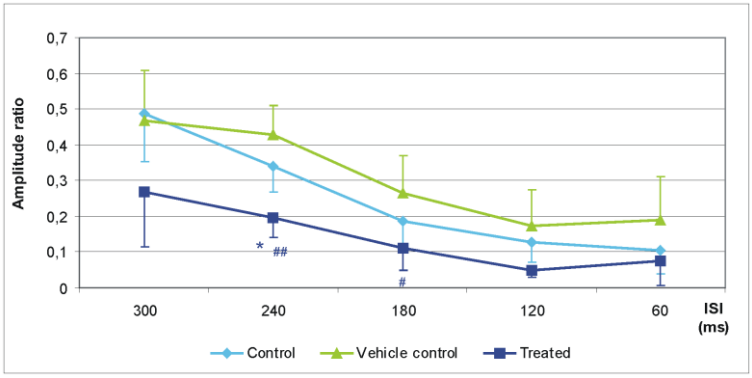


**Figure 7** Experiment II: Second:first ratio of the peak-to-peak amplitudes of the first and second EPs at different ISIs.
Mean+/-SD, n=6. * p<0.05 vs. *Control*; #, ## p<0.05, 0.01 vs. *Vehicle control*.

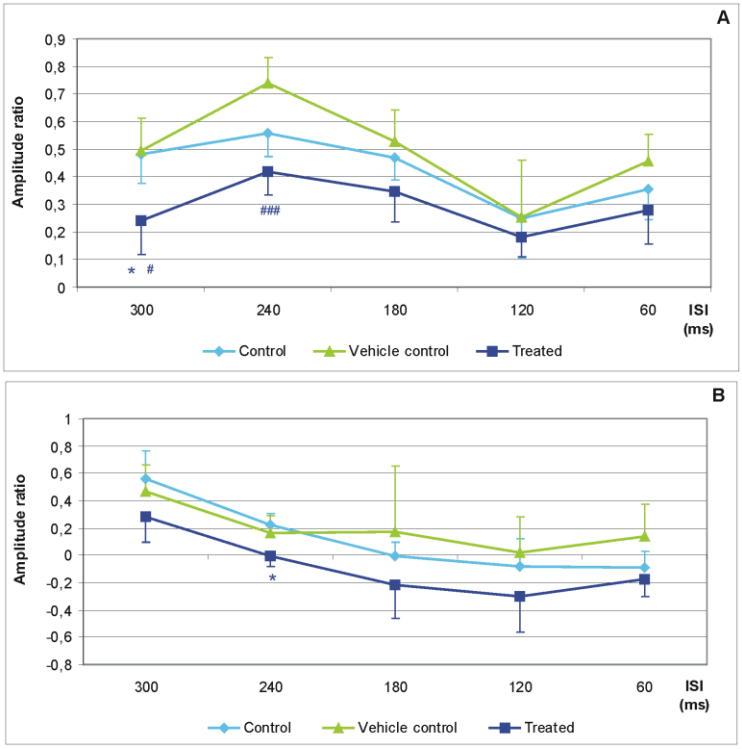


**Figure 8** Experiment II: Second:first ratio of the positive (A) and negative (B) peak amplitudes of the first and second EPs at different ISIs.

Mean+/-SD, n=6. * p<0.05 vs. *Control*; #, ### p<0.05, 0.001 vs. *Vehicle control*.

Significant correlation of brain Mn level with the second:first ratio of the negative peak amplitude was found at 240 ms ISI. The correlation between brain Mn level and the second:first ratio of the positive peak amplitude was weaker, but obvious (Fig. 9).

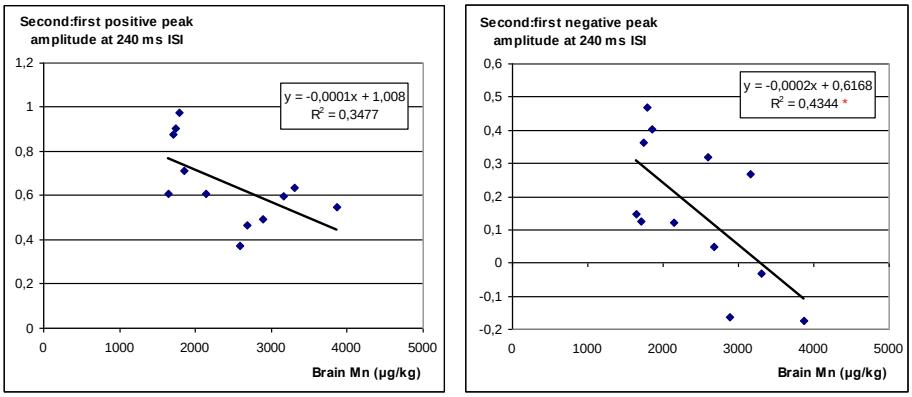


**Figure 9** Experiment II: Correlation of the brain Mn level to the second:first ratio of the positive and negative peak amplitudes at 240 ms ISI.
* $p<0.05$ for the linear fit.

## 3.3. Experiment III and IV: Subacute intratracheal treatment with NPs containing Mn, Cr or Fe, and with their combinations

### *3.3.1. Body and organ weights*

Body weight gain of the animals treated with metal NPs was significantly reduced (Fig. 10) compared to the untreated control (*Cont*). Decreased body weight gain was also seen in vehicle control groups (*FMVC* and *CrVC* was significant vs. *Cont*), reflecting the effect of the treatment procedure (mainly the repeated diethyl-ether anaesthesia) itself, but the effect of metal NPs was significantly different from that of the vehicle controls (Table 4).

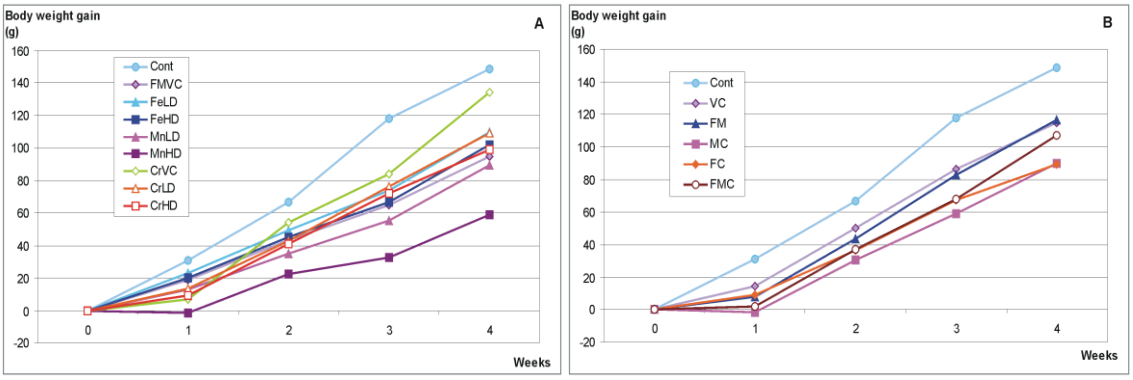


**Figure 10** Experiment III-IV: Time course of the control and treated rats' body weight gain over the 4 weeks of metal NP exposure. Mean values, n=8. Error bars are omitted for clarity.

Cr treatment caused dose-dependent significant decrease in body weight gain (vs. CrVC). Body weight effect of Mn administration was also dose related, but was significant only for MnHD (vs. FMVC and MnLD). Fe treatment, in contrast, lessened the negative effect of the treatment procedure. In case of treatment with metal NP combinations, reduction in the weight gain was more expressed after treatment with Mn and Cr NPs (MC) than after the three-metal combination (FMC). The effect on body weight gain commenced in the treated groups mostly on the 1st week already.

By plotting the calculated daily weight gain against the total applied Mn amount (calculated summed dose in Table 4) for each animal in groups where Mn NP treatment was applied (in single or combined form) one can see that the effect of the same Mn dose on the body weight gain was influenced by the other metal applied (Fig. 11). In Fe containing groups (*FM* and *FMC*) the body weight gain was less reduced than in Mn-only and *MC* groups. The same phenomenon was also seen on the weight gain curves presented in Fig. 10. To show the effect of another factor, then physicochemical form of the metal, data from a previous study (Horváth et al., 2012) are also included in Fig. 11. In that work, two groups received Mn for equally long treatment periods but in different chemical form and amount. (*MnL3+3***:** $MnCl_2$ po. for 3 weeks then $MnO_2$ NP it. for another 3, calculated summed dose 28.46±0.55 mg/rat; *MnL6*: $MnCl_2$ po. for 6 weeks, calculated summed dose 44.55±1.00 mg/rat). The weight gain of *MnL3+3* rats was mostly less than in the *MnL6* group, though the total amount of Mn administered was lower (note that the data from each experiment are to be compared to its own control).

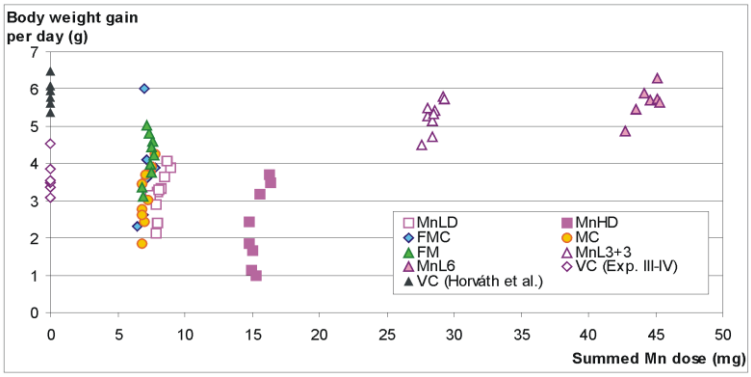


**Figure 11** Experiment III-IV: Calculated daily weight gain (total weight gain divided by the length of treatment period) of the individual rats plotted against their calculated summed Mn dose.
*MnL3+3*: 3 weeks $MnCl_2$ 15 mg/kg b. w. po. + 3 weeks $MnO_2$ NPs 2.63 mg/kg b. w. it.
*MnL6*: 6 weeks $MnCl_2$ 15 mg/kg b. w. po.

**Table 4** Body weight gain over the 4 weeks of metal NP exposure, and summed metal dose calculated for the same period.

| | **Body weight gain (g)** | **Calculated summed dose (mg/rat)** | | |
|---|---|---|---|---|
| **Groups** | | **Fe** | **Mn** | **Cr** |
| ***Cont*** | 148.8±17.51 | -- | -- | -- |
| ***FMVC*** | 94.63±12.73*** | -- | -- | -- |
| ***FeLD*** | 109.78±17.73** | 3.14±0.39 | -- | -- |
| ***FeHD*** | 101.78±19.63*** | 6.71±0.38 | -- | -- |
| ***MnLD*** | 89.7±17.02*** | -- | 8.15±0.45 | -- |
| ***MnHD*** | 59.14±27.03***##° | -- | 14.96±1.24 | -- |
| ***CrVC*** | 134±22.17 | -- | -- | -- |
| ***CrLD*** | 108.89±20.83**# | -- | -- | 6.62±0.8 |
| ***CrHD*** | 98.78±11.37***## | -- | -- | 13.18±0.74 |
| ***VC*** | 114.75±17.3*** | -- | -- | -- |
| ***FM*** | 116.57±14.29*** | 2.76±0.17 | 7.22±0.45 | -- |
| ***MC*** | 90±16.09***# | -- | 7.15±0.35 | 5.71±0.28 |
| ***FC*** | 89.29±11.69***## | 2.49±0.49 | -- | 5.21±1.03 |
| ***FMC*** | 107±5.34*** | 2.48±0.65 | 6.48±1.7 | 5.18±1.35 |

Mean±SD, n=8. **, *** $p<0.01$, 0.001 vs. *Cont*; #, ## $p<0.05$, 0.01 vs. the corresponding vehicle controls; ° $p<0.05$ vs. *MnLD*.
The summed dose was calculated by adding individual daily doses which were calculated on the basis of daily body weights.

It. instillation caused significant changes in the relative organ weights. When organ weights were related to 1/100 body weight, a general increase was observed compared to *Cont*, but in case of the lung, the increase was also significant compared to the corresponding vehicle controls at each metal NP (alone or in combination) treatment (Table 5). With the absolute brain weight as calculation basis (Table 6), relative weight of the heart, thymus and liver decreased significantly (vs. *Cont*), while relative lung weights showed significant increase in the metal NP treated groups compared to the corresponding vehicle controls. No other organ-specific effect of the metal NP treatment was found (decreased relative organ weights in *FMVC* or *CrVC* vs. *Cont* reflected also here the effect of the anaesthetic procedure). The general condition of the lungs after instillation of metal NPs was emphysematic, and dark spots of metal deposition were seen on dissection.

**Table 5** Relative organ weights of rats treated with metal NPs and the corresponding controls (calculation basis: body weight).

| | ***Relative organ weights (related to 1/100 of body weight)*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Groups** | ***Brain*** | ***Heart*** | ***Thymus*** | ***Lungs*** | ***Liver*** | ***Spleen*** | ***Kidney*** | ***Adrenals*** |
| ***Cont*** | 0.438 ± 0.033 | 0.266 ± 0.021 | 0.102 ± 0.008 | 0.311 ± 0.016 | 3.325 ± 0.283 | 0.169 ± 0.015 | 0.693 ± 0.057 | 0.0126 ± 0.0025 |
| ***FMVC*** | 0.526 ± 0.042*** | 0.267 ± 0.020 | 0.097 ± 0.026 | 0.539 ± 0.034*** | 3.265 ± 0.356 | 0.201 ± 0.052 | 0.681 ± 0.062 | 0.0159 ± 0.0025* |
| ***FeLD*** | 0.516 ± 0.020*** | 0.256 ± 0.009 | 0.095 ± 0.010 | 0.470 ± 0.032***### | 3.270 ± 0.251 | 0.176 ± 0.015 | 0.705 ± 0.064 | 0.0138 ± 0.0025 |
| ***FeHD*** | 0.504 ± 0.016*** | 0.254 ± 0.016 | 0.098 ± 0.024 | 0.520 ± 0.036***°° | 3.229 ± 0.189 | 0.187 ± 0.015* | 0.688 ± 0.054 | 0.0147 ± 0.0026 |
| ***MnLD*** | 0.549 ± 0.035*** | 0.285 ± 0.030 | 0.110 ± 0.012 | 0.701 ± 0.082***### | 3.430 ± 0.194 | 0.198 ± 0.023* | 0.741 ± 0.076 | 0.0185 ± 0.0027*** # |
| ***MnHD*** | 0.558 ± 0.024*** | 0.278 ± 0.024 | 0.110 ± 0.014 | 0.795 ± 0.082***###° | 3.705 ± 0.246*#° | 0.200 ± 0.020 | 0.712 ± 0.030 | 0.0191 ± 0.0026*** # |
| ***CrVC*** | 0.485 ± 0.050 | 0.245 ± 0.013 | 0.087 ± 0.022 | 0.325 ± 0.020 | 2.974 ± 0.194* | 0.162 ± 0.034 | 0.643 ± 0.021 | 0.0129 ± 0.0027 |
| ***CrLD*** | 0.536 ± 0.062** | 0.283 ± 0.043 | 0.113 ± 0.028 | 0.914 ± 0.073***### | 3.166 ± 0.572 | 0.178 ± 0.031 | 0.720 ± 0.083# | 0.0153 ± 0.0019*# |
| ***CrHD*** | 0.536 ± 0.028***# | 0.263 ± 0.021 | 0.097 ± 0.019 | 1.266 ± 0.097***###°°° | 3.156 ± 0.245 | 0.177 ± 0.020 | 0.863 ± 0.317 | 0.0147 ± 0.0018 |
| ***VC*** | 0.555 ± 0.059 | 0.310 ± 0.027 | 0.164 ± 0.037 | 0.724 ± 0.117* | 3.641 ± 0.509* | 0.204 ± 0.034 | 0.741 ± 0.077* | 0.0164 ± 0.0023** |
| ***FM*** | 0.577 ± 0.031 | 0.310 ± 0.034 | 0.148 ± 0.027 | 0.915 ± 0.155***# | 3.628 ± 0.291* | 0.208 ± 0.024* | 0.768 ± 0.034*** | 0.0165 ± 0.0021*** |
| ***MC*** | 0.574 ± 0.041*** | 0.319 ± 0.036 | 0.160 ± 0.029 | 1.007 ± 0.148***### | 3.386 ± 0.216 | 0.185 ± 0.035 | 0.725 ± 0.088 | 0.0189 ± 0.0023**# |
| ***FC*** | 0.570 ± 0.040*** | 0.338 ± 0.038* | 0.177 ± 0.011** | 1.107 ± 0.099***### | 3.154 ± 0.168# | 0.206 ± 0.012** | 0.824 ± 0.042***# | 0.0184 ± 0.0016** |
| ***FMC*** | 0.586 ± 0.042*** | 0.331 ± 0.027* | 0.150 ± 0.014 | 1.000 ± 0.056***### | 3.401 ± 0.120 | 0.207 ± 0.031 | 0.749 ± 0.046** | 0.0222 ± 0.0028***### |

Mean±SD, n=8. *, **, *** p<0.05, 0.01, 0.01 vs. *Cont*; #, ### p<0.05, 0.001 vs. the corresponding vehicle controls; °, °°, °°° p<0.05, 0.01, 0.001 vs. corresponding *LD*.

**Table 6** Relative organ weights of rats treated with metal NPs and the corresponding controls (calculation basis: brain weight).

| | ***Relative organ weights (related to brain weight)*** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Groups** | ***Heart*** | ***Thymus*** | ***Lungs*** | ***Liver*** | ***Spleen*** | ***Kidney*** | ***Adrenals*** |
| ***Cont*** | 0.610 ± 0.050 | 0.235 ± 0.028 | 0.712 ± 0.058 | 7.641 ± 1.055 | 0.386 ± 0.028 | 1.592 ± 0.197 | 0.0289 ± 0.0064 |
| ***FMVC*** | 0.509 ± 0.040*** | 0.186 ± 0.052 | 1.030 ± 0.092*** | 6.219 ± 0.665** | 0.381 ± 0.088 | 1.296 ± 0.100** | 0.0303 ± 0.0054 |
| ***FeLD*** | 0.497 ± 0.028*** | 0.184 ± 0.018** | 0.910 ± 0.058***## | 6.337 ± 0.562** | 0.342 ± 0.035* | 1.367 ± 0.126* | 0.0266 ± 0.0042 |
| ***FeHD*** | 0.505 ± 0.043*** | 0.196 ± 0.051 | 1.031 ± 0.065***°°° | 6.412 ± 0.505** | 0.372 ± 0.033 | 1.364 ± 0.103** | 0.0290 ± 0.0051 |
| ***MnLD*** | 0.520 ± 0.044** | 0.201 ± 0.021* | 1.277 ± 0.138***### | 6.251 ± 0.336** | 0.362 ± 0.046 | 1.348 ± 0.089** | 0.0338 ± 0.0050 |
| ***MnHD*** | 0.498 ± 0.027*** | 0.198 ± 0.029* | 1.426 ± 0.138***###°°° | 6.656 ± 0.624 | 0.359 ± 0.036 | 1.277 ± 0.035** | 0.0342 ± 0.0050 |
| ***CrVC*** | 0.510 ± 0.052** | 0.185 ± 0.063 | 0.673 ± 0.047 | 6.199 ± 0.892* | 0.335 ± 0.071 | 1.338 ± 0.142* | 0.0265 ± 0.0047 |
| ***CrLD*** | 0.530 ± 0.055* | 0.213 ± 0.049 | 1.718 ± 0.162***### | 6.004 ± 1.255* | 0.332 ± 0.042* | 1.350 ± 0.126** | 0.0287 ± 0.0031 |
| ***CrHD*** | 0.491 ± 0.021*** | 0.184 ± 0.047* | 2.364 ± 0.204***###°°° | 5.889 ± 0.453*** | 0.331 ± 0.040* | 1.598 ± 0.535 | 0.0275 ± 0.0031 |
| ***VC*** | 0.561 ± 0.022* | 0.295 ± 0.054 | 1.310 ± 0.202*** | 6.566 ± 0.660 | 0.366 ± 0.037 | 1.337 ± 0.055 | 0.0296 ± 0.0036 |
| ***FM*** | 0.537 ± 0.057* | 0.258 ± 0.051** | 1.594 ± 0.292**# | 6.293 ± 0.414* | 0.362 ± 0.046 | 1.334 ± 0.068 | 0.0287 ± 0.0039 |
| ***MC*** | 0.556 ± 0.054 | 0.280 ± 0.055* | 1.752 ± 0.192***### | 5.924 ± 0.520**# | 0.324 ± 0.061 | 1.264 ± 0.138** | 0.0329 ± 0.0026# |
| ***FC*** | 0.593 ± 0.039 | 0.312 ± 0.010 | 1.944 ± 0.143***### | 5.546 ± 0.359***## | 0.362 ± 0.013 | 1.448 ± 0.074*## | 0.0322 ± 0.0008 |
| ***FMC*** | 0.565 ± 0.028 | 0.258 ± 0.033** | 1.715 ± 0.168***## | 5.828 ± 0.478**# | 0.353 ± 0.042 | 1.282 ± 0.102** | 0.0379 ± 0.0044### |

Mean±SD, n=8. *, **, *** p<0.05, 0.01, 0.01 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. the corresponding vehicle controls; °°° p<0.001 vs. corresponding *LD*.

### *3.3.2. Open field motility*

In the OF test, Mn treated rats showed decreased motility: ambulation time and count were decreased significantly in the groups *MC* and *FM*C. Decrease in vertical activity was seen in almost each NP treated groups, except in *FeLD* and *FeHD* (moreover significant increase of rearing count vs. *FMVC*), and in *FM* group (no change vs. *VC*). In accordance to this, immobility and local activity were decreased significantly in these Fe NP treated groups. Mn and Cr increased immobility and local activity, and this change was significant in the combinations *MC*, *FC* and *FMC* (Table 7 and 8). The length of individual events (the time/count value) of rearing also increased in *FeHD* (vs. *Cont*), and that of immobility and local activity increased in *FC* and *FMC* groups, respectively.

**Table 7** Data of OF motility in rats treated with metal NPs and the corresponding controls.

| **Groups** | ***Cont*** | ***FMVC*** | ***FeLD*** | ***FeHD*** | ***MnLD*** | ***MnHD*** | ***CrVC*** | ***CrLD*** | ***CrHD*** |
|---|---|---|---|---|---|---|---|---|---|
| Ambulation distance (cm) | 2424.62 ± 237.5 | 2128.32 ± 345.09 | 2490.78 ± 300.37 | 2474.47 ± 238.84# | 2260.87 ± 495.62 | 2092.16 ± 489.26 | 2330.38 ± 179.55 | 2292.67 ± 163.53 | 2330.07 ± 144.37 |
| Ambulation time (s) | 285.5 ± 20.78 | 283 ± 23.16 | 284.11 ± 24.76 | 289.2 ± 25.88 | 283.8 ± 43.46 | 287.33 ± 34.82 | 283.2 ± 7.33 | 275.57 ± 14.29 | 276.8 ± 10.28 |
| Ambulation count | 32.4 ± 6.5 | 31.71 ± 5.38 | 32.89 ± 3.62 | 32.2 ± 3.55 | 29.8 ± 4.21 | 31.83 ± 3.71 | 33.20 ± 8.26 | 31.9 ± 4.31 | 32 ± 3.74 |
| *Ambulation speed (cm/s)* | 8.45 ± 1.1 | 7.85 ± 0.63 | 8.66 ± 0.81 | 8.46 ± 0.63 | 7.99 ± 1.02 | 7.53 ± 1.18 | 8.15 ± 0.58 | 8.49 ± 0.54 | 8.38 ± 0.47 |
| *Ambulation time/count (s)* | 9.18 ± 2.54 | 9.42 ± 2.17 | 8.76 ± 1.43 | 9.14 ± 1.79 | 9.81 ± 2.72 | 9.12 ± 1.53 | 9.07 ± 2.38 | 8.93 ± 1.62 | 8.29 ± 0.83 |
| Local activity time (s) | 147 ± 14.8 | 150.25 ± 33.49 | 123.88 ± 13.4* | 123.86 ± 14.84* | 179.13 ± 55.77 | 186 ± 65.11 | 151.33 ± 14.19 | 154.44 ± 32.94 | 155 ± 21.93 |
| Local activity count | 63.75 ± 8.22 | 63.6 ± 8.41 | 51.56 ± 4.42 | 53.5 ± 7.48 | 67 ± 11.11 | 73.25 ± 15.52 | 59.33 ± 2.31 | 64.71 ± 3.3 | 65.2 ± 5.17 |
| *Local activity time/count (s)* | 2.2 ± 0.32 | 2.41 ± 0.21 | 2.43 ± 0.17 | 2.38 ± 0.29 | 2.68 ± 0.42 | 2.5 ± 0.36 | 2.55 ± 0.25 | 2.54 ± 0.34 | 2.36 ± 0.16 |
| Immobility time (s) | 20.5 ± 8.36 | 19.6 ± 5.55 | 16 ± 5.58 | 12.63 ± 3.38## | 23.71 ± 13.82 | 27 ± 2.83 | 19 ± 2.65 | 22.33 ± 7.37 | 23.6 ± 5.55 |
| Immobility count | 14.25 ± 2.22 | 15.33 ± 4.46 | 10.29 ± 2.69**## | 9.13 ± 2.23**## | 18.57 ± 6.63 | 22 ± 2.65**# | 15 ± 3.61 | 16.44 ± 5.83 | 17.40 ± 3.05 |
| *Immobility time/count (s)* | 1.63 ± 0.39 | 1.38 ± 0.17 | 1.45 ± 0.21 | 1.39 ± 0.17 | 1.28 ± 0.45 | 1.32 ± 0.18 | 1.3 ± 0.22 | 1.44 ± 0.07 | 1.34 ± 0.15 |
| Rearing time (s) | 150 ± 33.67 | 144.6 ± 40.4 | 166.13 ± 22.01 | 170.75 ± 27.08 | 130.14 ± 21.34 | 136 ± 1.41 | 148 ± 17.44 | 137.33 ± 27.16 | 134 ± 6.38 |
| Rearing count | 64.67 ± 10.12 | 58.14 ± 6.62 | 66.83 ± 4.67# | 69.33 ± 8.87# | 53.71 ± 15.87 | 47.25 ± 15.06 | 62.5 ± 5.07 | 53.83 ± 5.04# | 53 ± 5.48# |
| *Rearing time/count (s)* | 2.11 ± 0.13 | 2.6 ± 0.49 | 2.44 ± 0.31 | 2.57 ± 0.1*** | 2.09 ± 0.29 | 2.53 ± 0.22 | 2.31 ± 0.12 | 2.57 ± 0.53 | 2.54 ± 0.29 |

Mean±SD, n=8. *, **, *** p<0.05, 0.01, 0.001 vs. *Cont*; #, ## p<0.05, 0.01 vs. the corresponding vehicle controls.

**Table 8** Data of OF motility in rats treated with the combinations of metal NPs and the corresponding controls.

| **Groups** | ***Cont*** | ***VC*** | ***FM*** | ***MC*** | ***FC*** | ***FMC*** |
|---|---|---|---|---|---|---|
| Ambulation distance (cm) | 2424.62 ± 237.5 | 2128.32 ± 345.09 | 2486.45 ± 305.78 | 2144.55 ± 431.8 | 2151.42 ± 194.03 | 1975.24 ± 408.08 |
| Ambulation time (s) | 285.5 ± 20.78 | 283 ± 23.16 | 286 ± 13.3 | 255.71 ± 28.82* | 256.8 ± 28.87 | 255.8 ± 8.67*# |
| Ambulation count | 32.4 ± 6.5 | 31.71 ± 5.38 | 32.9 ± 5.45 | 26.33 ± 3.14* | 27 ± 2.37 | 26.88 ± 2.23*# |
| *Ambulation speed (cm/s)* | 8.45 ± 1.1 | 7.85 ± 0.63 | 8.67 ± 1.29 | 8.01 ± 0.87 | 8.25 ± 0.8 | 8.11 ± 0.55 |
| *Ambulation time/count (s)* | 9.18 ± 2.54 | 9.42 ± 2.17 | 9.15 ± 2.16 | 9.8 ± 1.17 | 9.81 ± 1.05 | 9.34 ± 0.98 |
| Local activity time (s) | 147 ± 14.8 | 150.25 ± 33.49 | 127.5 ± 21.22 | 172.75 ± 20.12 | 182 ± 11.27* | 196.5 ± 29.59*# |
| Local activity count | 63.75 ± 8.22 | 63.6 ± 8.41 | 54.7 ± 10.23 | 69.5 ± 4.85 | 69 ± 7.25 | 76 ± 12.54 |
| *Local activity time/count (s)* | 2.2 ± 0.32 | 2.41 ± 0.21 | 2.37 ± 0.2 | 2.5 ± 0.18 | 2.59 ± 0.21 | 2.69 ± 0.12* |
| Immobility time (s) | 20.5 ± 8.36 | 19.6 ± 5.55 | 14.6 ± 3.91 | 34.83 ± 23*# | 43.33 ± 13.61*# | 26.25 ± 2.63 |
| Immobility count | 14.25 ± 2.22 | 15.33 ± 4.46 | 13.33 ± 6.28 | 21 ± 4.55*# | 18 ± 8.37 | 16.17 ± 2.79 |
| *Immobility time/count (s)* | 1.63 ± 0.39 | 1.38 ± 0.17 | 1.39 ± 0.48 | 1.67 ± 0.29 | 2.23 ± 0.53# | 1.56 ± 0.15 |
| Rearing time (s) | 150 ± 33.67 | 144.6 ± 40.4 | 164.4 ± 21.46 | 130.6 ± 16.79 | 131.25 ± 18.89 | 128.17 ± 21.55 |
| Rearing count | 64.67 ± 10.12 | 58.14 ± 6.62 | 67.33 ± 13.19 | 56.17 ± 5.12 | 61.8 ± 2.78 | 58 ± 10.24 |
| *Rearing time/count (s)* | 2.11 ± 0.13 | 2.6 ± 0.49 | 2.56 ± 0.3 | 2.32 ± 0.24 | 2.13 ± 0.24 | 2.08 ± 0.15 |

Mean±SD, n=8. * $p<0.05$ vs. *Cont*; # $p<0.05$ vs. *VC*.

### *3.3.3. Cortical electrical activity*

Generally, in the spectral distribution of the ECoG, the proportion of the fast bands (beta1, beta2, gamma) increased, while that of the slow bands (theta and delta) decreased upon metal NP treatment (Fig. 12). On the SS ECoG, significant decrease of the delta band was caused by Mn treatment, and increase of the gamma band by Mn and Cr (vs. *Cont* and *FMVC*). In the combinations *MC* and *FC*, these changes were more pronounced and partly significant vs. *Cont* and *VC*. The effect of the three-metal combination was less pronounced, and the proportion of bands hardly changed in *FM* group. The observed alterations on the VIS and AUD ECoG (Fig. 12) were similar to those of the SS ECoG, except that Fe NP treatment (and the combination *FM*) decreased gamma band significantly, and that the effect of the metal NP combinations was less pronounced.

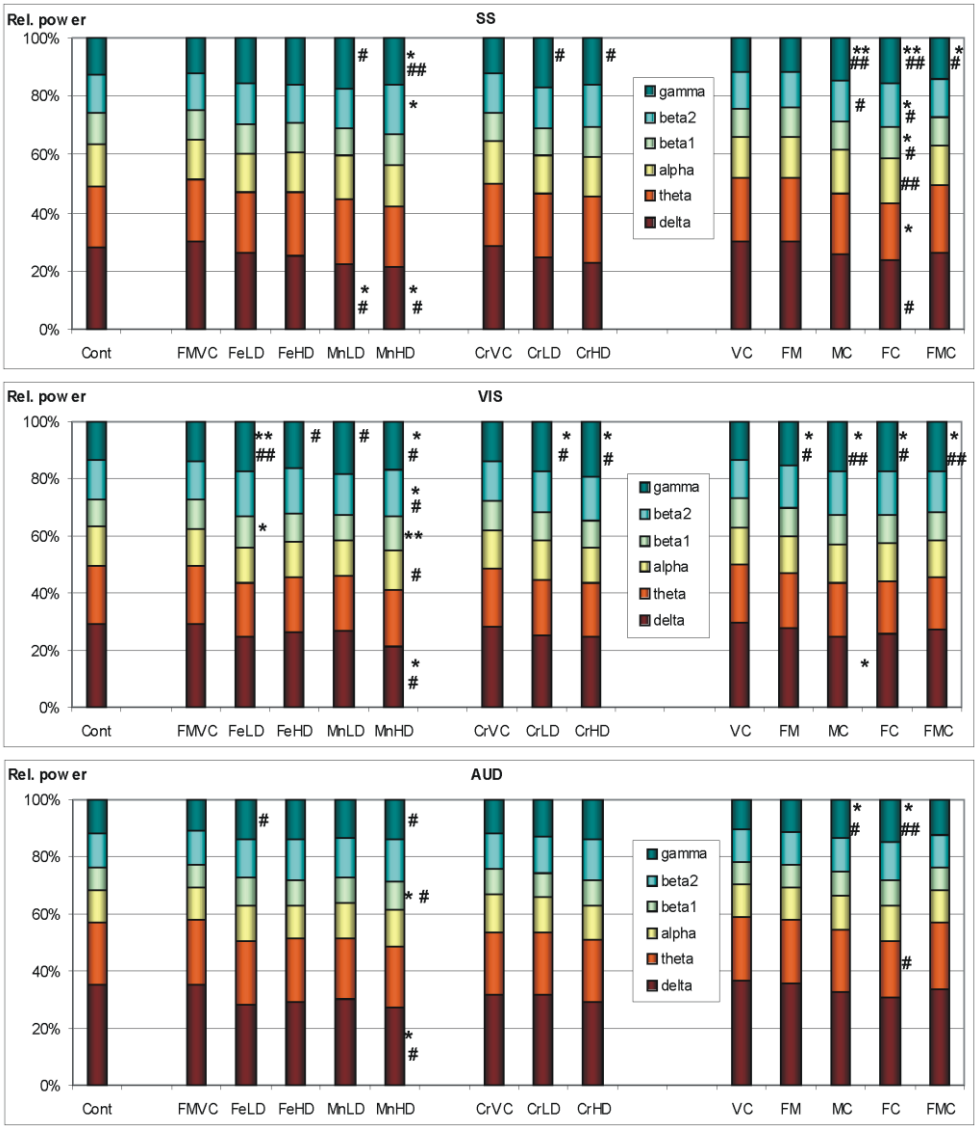


**Figure 12** Experiment III-IV: Band spectrum of the ECoG in rats treated with metal NPs. *, ** $p<0.05$, 0.01 vs. *Cont*; #, ## $p<0.05$, 0.01 vs. the corresponding vehicle controls.

The SS EPs showed significantly increased latency on the action of Mn and Cr NPs, and of *MC* and *FC* combinations. In Fe NP treated groups, however, latency was decreased significantly. The latency increase was seen at all stimulation frequencies, and the steepness of frequency dependence (an indication of the actual state of the sensory pathway, partly similar to the second:frist ratio) was more pronouncedly altered by Mn and MC treatment. The magnitude of latency increase in *MC* and *FC* groups was similar to *CrLD* and *CrHD* groups respectively. Only a slight latency increase was seen in *FMC* group, while in *FM* group, no change was observed compared to *VC*. Fig. 13 shows latency data in relative values, normalized to the latency of the SS EP obtained with 1 Hz stimulation in the corresponding vehicle control groups. Data of the untreated control (*Cont*) are not displayed, in order to avoid crowded graphs and because the difference between *Cont* and the vehicle controls was negligible.

VIS and AUD latency were significantly increased by *MnLD*, *MnHD* and *CrHD* treatment, and the combinations *FC* and partly in *MC*, vs. the corresponding vehicle controls (Fig. 14). Fe caused no noteworthy change alone, or in combination with Mn.

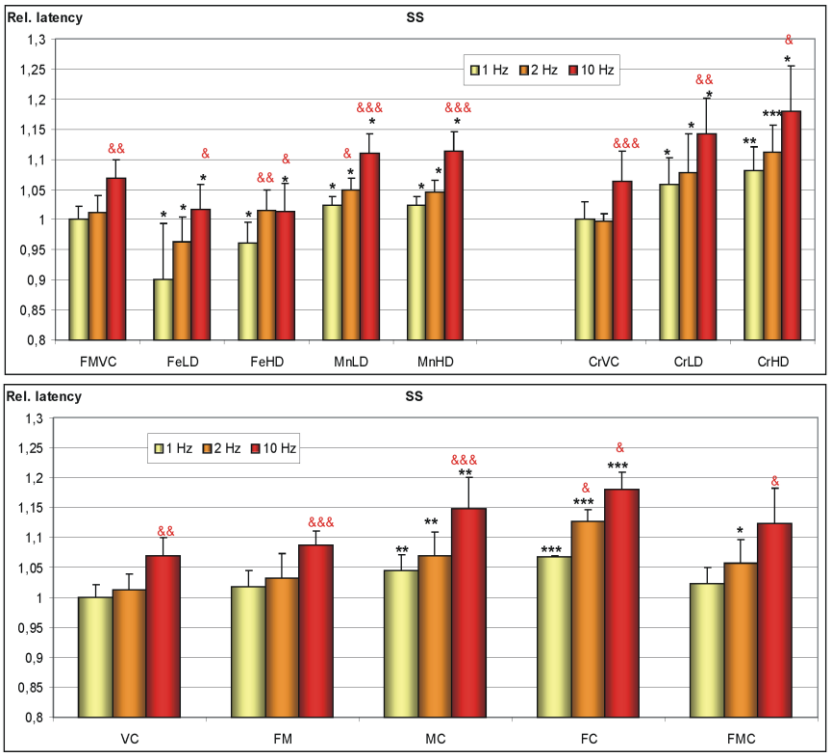


**Figure 13** Experiment III-IV: Latency of the SS EP in rats treated with metal NPs. Relative values, normalized to the latency of the SS EP obtained with 1 Hz stimulation in the corresponding vehicle control groups.
Mean+SD, n=8. *, **, *** $p<0.05$, 0.01, 0.001 vs. the corresponding vehicle control, at identical stimulation frequency (see insert).
&, &&, &&& $p<0.05$, 0.01, 0.001 vs. latency with 1 Hz stimulation within a treatment group.

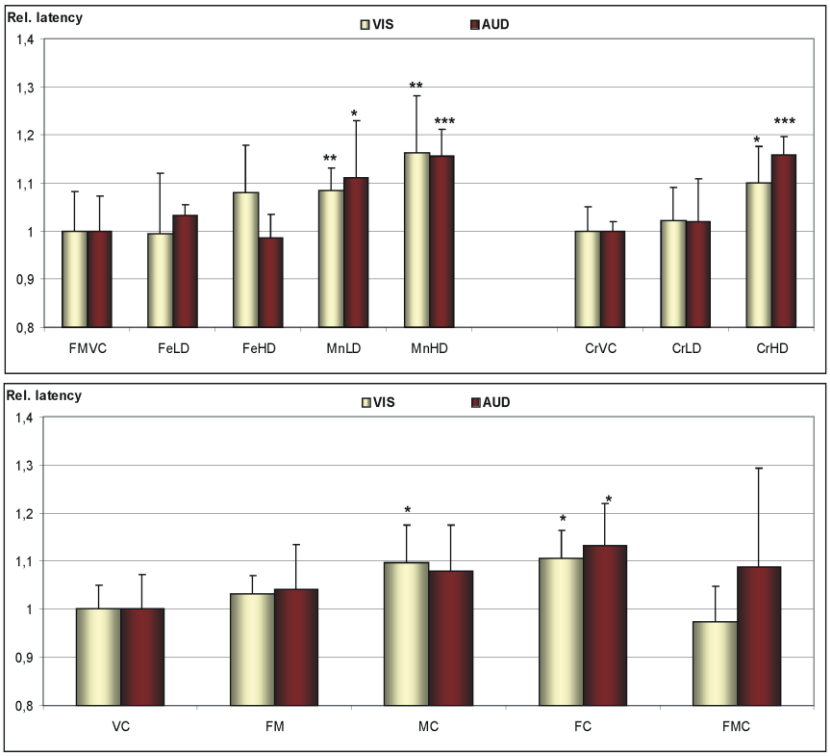


**Figure 14** Experiment III-IV: Latency of the VIS and AUD EPs in rats treated with metal NPs. Relative values, normalized to the latency in the corresponding vehicle control groups.
Mean+SD, n=8. *, **, *** $p<0.05$, 0.01, 0.001 vs. the corresponding vehicle controls.

In case of SS EPs elicited by double pulse stimulation, the positive and negative peak amplitude values of the first EP were increased significantly by Mn (vs. *FMVC*), causing increase of the peak-to-peak amplitude, which was also significant at each ISI. The effect of Cr treatment was opposite to Mn, i.e. the peak-to-peak amplitude of the first EP was decreased significantly compared to *CrVC*. Fe treatment had no noteworthy effect alone, but in combination with Cr (*FC*) caused significant decrease of the positive peak amplitude of the first EP (vs. *VC*); due to the decrease of the negative peak amplitude, however, the peak-to-peak amplitude has not changed. Generally, in case of combined metal NP treatment, no noteworthy change was found in the peak-to-peak amplitudes (data not shown). On the contrary, however, second:first ratio of the peak-to-peak amplitudes showed significant increase in *FC* and *FMC* groups at 120 and 180 ISI compared to *VC* (Fig. 15C). Mn-only treatment caused significant decrease of the second:first ratio of the positive (*MnHD* at 300, 180 and 60 ms ISI), negative (*MnHD* at 300 ms ISI) and peak-to-peak amplitudes (*MnLD* and *MnHD* at 120 and 180 ms ISI) vs. *FMVC* (Fig. 15A). The effect of Cr on the second:first ratio of the amplitude was slight, except for the peak-to-peak amplitude ratio, where significant increase was found at 240, 180 and 120 ms ISI compared to *CrVC* (Fig. 15B).

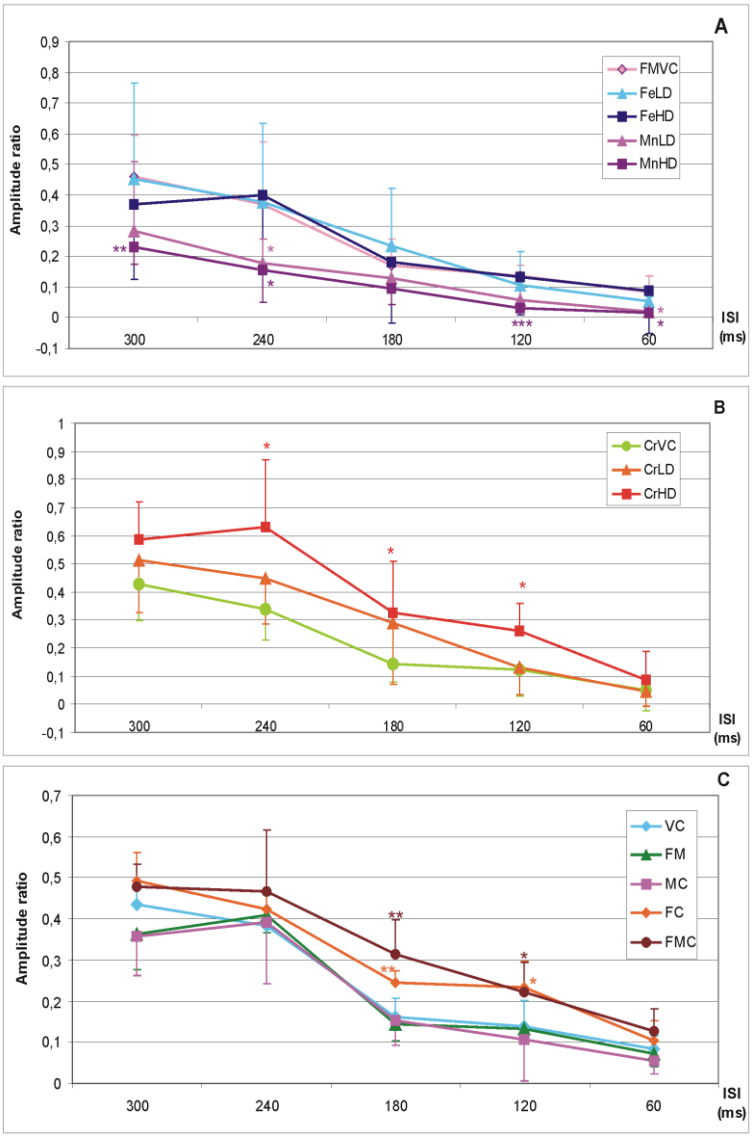


**Figure 15** Experiment III-IV: Second:first ratio of the peak-to-peak amplitudes of the first and second EPs at different ISIs.
Mean+/-SD, n=8. *, **, *** p<0.05, 0.01, 0.001 vs. the corresponding vehicle controls.

### *3.3.4. Tissue metal levels*

Tissue metal levels are given in Table 9. It. instillation of Mn and Cr NPs caused significant increase in the Mn or Cr content respectively of the brain, lungs, and also blood (vs. *Cont* and the corresponding vehicle controls). No effect of Fe was seen on tissue Fe levels, except for the lungs, where (due to the way of administration) Fe content increased significantly (vs. *Cont* and *FMVC*). In case of combined treatment, Mn levels were similar to

those measured in *MnLD* (in which the same dose, 2 mg/kg b. w. was applied), but Cr levels were influenced by the other metals applied. For example, although Cr content of the lungs after combined metal NP treatment was as high as in *CrLD*, Cr levels measured in the brain and blood was less in *MC*, *FC* and *FMC* groups (still significantly increased to *Cont* and *VC* in the blood, but not in the brain).

The correlation of internal metal load and certain toxicological parameters is shown by the plots in Fig. 16 and 17. Significant correlation was found between brain Mn levels and SS and VIS ECoG index and SS and VIS latency in Mn-only treated groups (Fig. 16A) and also in combination with Cr in *MC* group (Fig. 16B). The second:first ratio of the peak-to-peak amplitude showed significant correlation to brain Mn content at 240 and 180 ms ISI (Fig. 17). The correlation of body weight gain with blood, brain and lung Mn levels was also significant, and so was the correlation with blood and lung Cr content (data not shown).

**Table 9** Metal levels in blood, brain and lungs of rats treated with metal NPs and their combinations and of the corresponding controls.

| Tissue metal levels (µg/kg) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Blood | | | Brain | | | Lungs | | |
| Groups | Fe | Mn | Cr | Fe | Mn | Cr | Fe | Mn | Cr |
| ***Cont*** | 2298240.75 ± 20150.90 | 278.84 ± 79.56 | 142.90 ± 16.06 | 85243.51 ± 3719.38 | 2010.17 ± 354.19 | 122.70 ± 15.01 | 386146.30 ± 27269.51 | 1256.71 ± 417.31 | 215.45 ± 34.95 |
| ***FMVC*** | 2307059.97 ± 58563.53 | 234.64 ± 39.21 | - | 99076.46 ± 8099.00 | 1686.92 ± 91.21 | - | 407461.09 ± 32214.79 | 1374.90 ± 90.98 | - |
| ***FeLD*** | 2335767.20 ± 151597.71 | - | - | 90939.48 ± 4145.61 | - | - | 19664708.85 ± 1213088.83***### | - | - |
| ***FeHD*** | 2095880.12 ± 482384.32 | - | - | 104527.91 ± 14339.96 | - | - | 35252158.04 ± 4104496.59***###°° | - | - |
| ***MnLD*** | - | 533.44 ± 99.94*## | - | - | 5548.28 ± 518.42***### | - | - | 11739.03 ± 458.71 ***### | - |
| ***MnHD*** | - | 715.21 ± 176.01*## | - | - | 8159.31 ± 673.97***###°° | - | - | 11747.39 ± 7547.83 | - |
| ***CrVC*** | - | - | 121.76 ± 36.32 | - | - | 138.34 ± 12.35 | - | - | 319.28 ± 113.53 |
| ***CrLD*** | - | - | 4809.74 ± 972.68**## | - | - | 1112.89 ± 1526.02 | - | - | 6891780.11 ± 792670.54***### |
| ***CrHD*** | - | - | 12714.67 ± 2278.13***###°° | - | - | 5335.12 ± 1981.74*# | - | - | 9708166.15 ± 1923132.60***### |
| ***VC*** | 2249378.68 ± 396766.73 | 309.41 ± 97.19 | 138.71 ± 10.67 | 80222.65 ± 9683.69 | 1897.94 ± 289.96 | 146.84 ± 42.40 | 347220.77 ± 61025.06 | 1550.57 ± 279.68 | 179.54 ± 33.43 |
| ***FM*** | 2369477.75 ± 58384.64 | 530.46 ± 79.46*# | - | 82831.18 ± 10200.72 | 5495.31 ± 512.58***### | - | 9933993.95 ± 3392692.31**## | 6947.20 ± 2134.28*# | - |
| ***MC*** | - | 432.76 ± 37.21* | 639.85 ± 107.24**## | - | 6183.42 ± 495.33***### | 277.04 ± 180.34 | - | 8696.07 ± 1321.46 ***### | 7862358.03 ± 960490.48***### |
| ***FC*** | 2374525.02 ± 207877.33 | - | 520.79 ± 99.00**## | 90167.49 ± 1728.98 | - | 194.59 ± 92.21 | 8561935.37 ± 1442308.71***### | - | 6141161.46 ± 1252683.66**## |
| ***FMC*** | 2324487.14 ± 52554.01 | 436.16 ± 22.51* | 520.85 ± 165.54*# | 80890.50 ± 5209.78 | 6351.18 ± 671.70***### | 200.26 ± 94.02 | 7693587.62 ± 934597.04***### | 10162.93 ± 2215.36**## | 5162283.66 ± 489893.10***### |

Mean±SD, n=3. *, **, *** p<0.05, 0.01, 0.01 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. the corresponding vehicle controls; °° p<0.01 vs. corresponding LD.

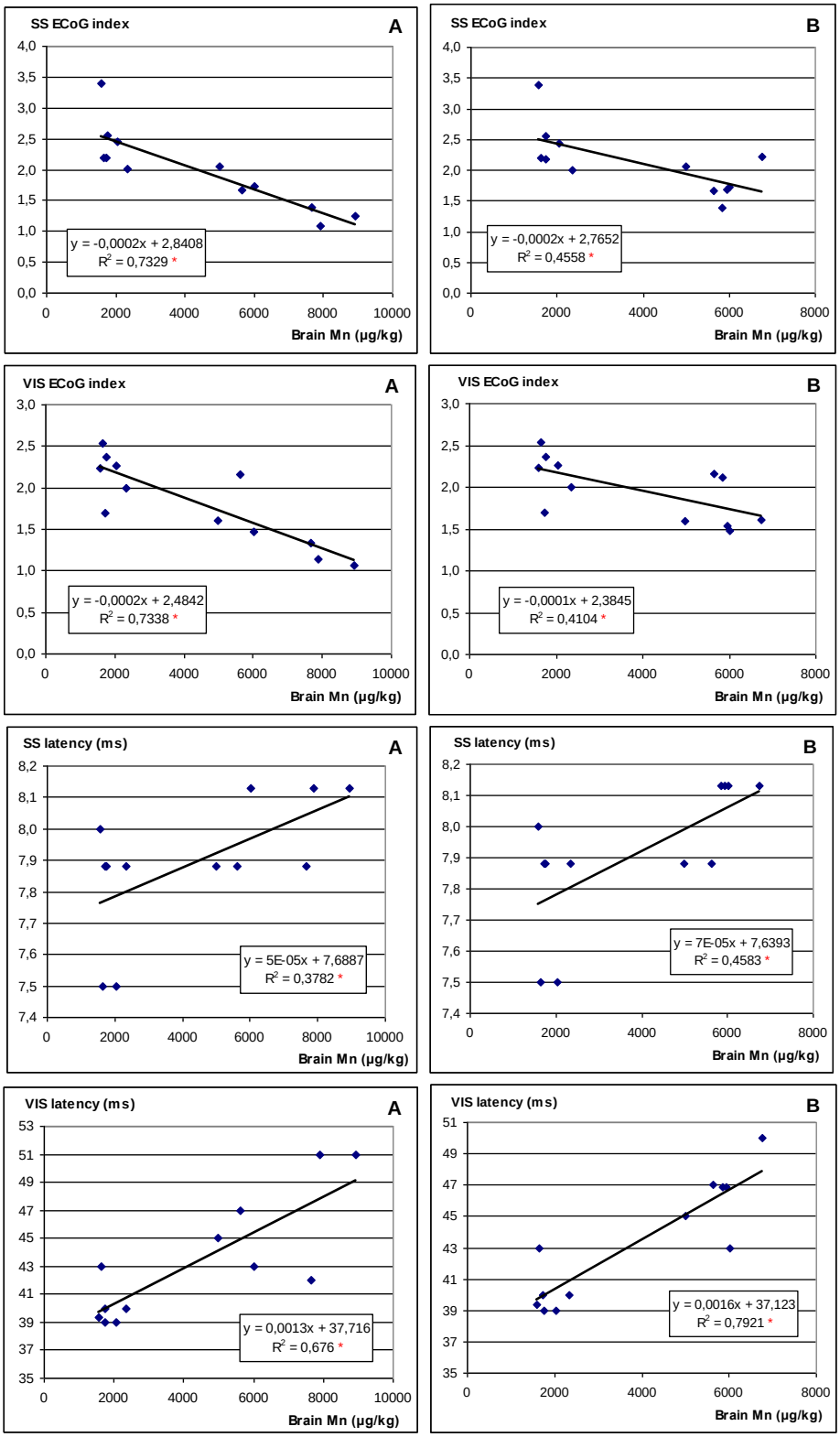


**Figure 16** Experiment III-IV: Correlation of the brain Mn level to certain measured neuro-functional parameters. A: *Cont*, *FMVC*, *MnLD*, *MnHD*; B: *Cont*, *FMVC*, *MnLD*, *MC*.
* $p<0.05$ for the linear fit.

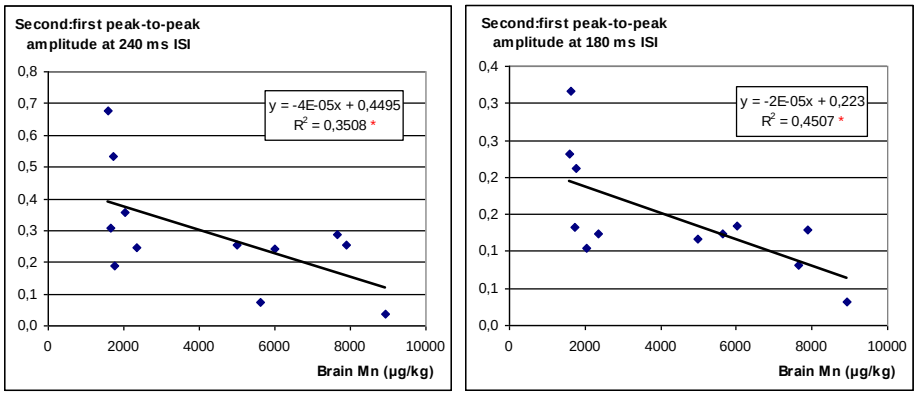


**Figure 17** Experiment III-IV: Correlation of the brain Mn level to the second:first ratio of the peak-to-peak amplitudes.
* $p<0.05$ for the linear fit.

# 4. Discussion

One of the most conspicuous outcome of the results was that more or less similar internal Mn doses, metal levels in tissue (first of all brain) samples, were achieved with highly dissimilar external doses – administered daily or total amounts – in experiments I, II and III. The brain Mn levels given in Table 3 were caused by more than 1000 times higher amount of the same Mn compound ($MnCl_2$) when delivered po. than when it. This is partly explained by the bodily need dependent, regulated character of intestinal absorption (Davis et al., 1993) opposed to the non-functional absorption from the alveoli. In an earlier study (Oszlánczi et al., 2010a) it was shown that dissolved $MnCl_2$ was better absorbed from the olfactory mucosa of rats than suspension of $MnO_2$ NPs, and had stronger effect on ECoG and cortical EPs. In the present work, it has been shown that dissolved Mn was efficiently absorbed also from the lungs. 0.5 mg/ml of $MnCl_2 \cdot 4H_2O$ was clearly a hyposmotic solution, driven from the lumen of bronchioli and alveoli towards the blood by the osmotic potential difference. Inhalation of water-dissolved Mn may seem unrealistic but has been raised as a health hazard from high-Mn drinking water sprayed in showers (Elsner and Spangler, 2006).

Timing may also be of importance. In Horváth et al. (2012) $MnCl_2$ was applied by daily gavage for 6 weeks. A mean summed applied dose of ca. 180 mg Mn/rat caused in that study similar alteration of the SS EP as here in Experiment I (summed dose ca. 1600 mg/rat), and the resulting brain Mn levels were also similar (from data in Horváth et al.: 3140±621 µg/kg; Exp. II: 2532±257 µg/kg). Dumping the rat's organism with Mn by the amount forced into the stomach via gavage might not be counteracted by the mechanisms which may more efficiently limit intestinal absorption, and/or increase biliary excretion, in case of continuous uptake with the drinking water.

The relationship of daily weight gain and summed Mn dose (data displayed in Fig. 11) likewise supported the decisive role of physicochemical form and way of administration. In po. application by gavage (*MnL6*: ca. 15 mg/kg b. w. $MnCl_2$ po. for 6 weeks in Horváth et al., 2012), summed external dose was much higher but weight gain was not as much reduced as in partly (*MnL3+3*: 15 mg/kg b. w. $MnCl_2$ po. for 3 weeks, then 2.63 mg/kg b. w. $MnO_2$ NPs it. for 3 weeks) or fully (*MnLD* and *MnHD* in the present study) it. application – even if the data from two distinct studies are not fully comparable. Weight gain data reflected also some interaction between the metals used. The growth curves (Fig. 10), and the data of daily (Fig. 11) and overall (Table 4) weight gain indicated an apparently protective effect of Fe, at least

for *FM* and *FMC* vs. *MnLD*. With Cr, no such effect on weight gain was seen. A possible explanation for this may be that Mn and Fe NPs were administered in one suspension, whereas Cr was given separately and with 4-6 hours delay (for technical reasons explained in Methods, 2.2.4.). A similar interaction was observed also on the parameters of cortical electrical activity (Fig. 12, 13 and 14).

On the tissue metal levels, a dissimilar interaction of the metals was seen. Blood and brain Cr levels were much lower in the combination groups than in *CrLD*, but the levels of Mn were hardly influenced by co-exposure to Fe or Cr. This discrepancy supports the statement by Roels at al. (1992) and Greger (1998) that blood Mn levels are of limited use and neuro-functional biomarkers of Mn effect should be developed instead. All three metal in this study are transported by Tf into cells and through the BBB (Takeda, 2003; Thompson et al., 2001; Vincent, 2001) so an interference would be expected, at least if the binding capacity of Tf were reached in the combination groups. However, except for Cr in blood and brain (Table 9; in accordance with Offenbacher et al., 1997) no interference was seen, and the hardly changing Fe levels in spite of Fe NPs administration also suggested that no (or at least no lengthy) saturation of Tf occurred (temporary saturation, or down-regulation of Tf synthesis, due to Fe overload cannot be excluded: Thompson et al., 2001). In vitro it was described (Quarles et al., 2011) that Cr binding to Tf was influenced only by extreme levels of Fe which were probably not present in the present study as indicated by the blood and brain levels in Table 9.

The measured metal levels could be caused by the presence of NPs themselves in the blood and brain of the treated rats, because NPs easily reach other organs by transcytosis across respiratory epithelium (Oberdörster et al., 2005). From the blood, NPs are able to enter the brain through the BBB (Kreyling et al., 2006). Alternatively, the metals may be dissolved in the low pH (<4.5) of phagolysosomes after being phagocyted by pulmonary macrophages (Lundborg et al., 1985), and reach targets organs in free form. In exposing rats to actual welding aerosol, the role of the dissolved fraction (containing Mn, Cr, Fe and other metals) was predominant in causing oxidative stress and airways inflammation (McNeilly et al., 2005).

Oxidative stress was obviously a key factor in the effects of the metals. Even reduced body weight gain, observed in the treated animals, might be due to the metabolic disturbance caused by the presence of free radicals (Merry, 2002). In the brain, generation of reactive oxygen species was detected in Mn exposure (Zhang et al., 2009), resulting in membrane lipid peroxidation (Avila et al., 2008). Accumulation of Fe in the basal ganglia and induction of

oxidative stress might be involved in the pathogenesis of Parkinsonism (a potential pathomechanism common with Mn; Sayre et al., 1999). Oxidative brain damage was also reported after oral exposure of rats to $Cr^{6+}$ (Soudani et al., 2012). Damaged lipids lead to changes of fluidity and other membrane properties which, in turn, disturb all membrane- and receptor-bound phenomena, such as synaptic transmission, and these are likely to be reflected e.g. in various forms of cortical electrical activity. This could be a possible way of synergistic neurotoxicity of the three metals.

Of the two kinds of cortical activity recorded, EPs reflect alterations in synaptic transmission more directly. Increased latency in the Mn and Cr treated rats might be, at least partly, due to decreased synaptic efficiency. $Mn^{2+}$ ions block some of the presynaptic Ca channels, but also penetrate them (Nelson, 1986) and cause intracellular Ca mobilization (Kita et al., 1981) so that synchronous release of the transmitter will be less, but spontaneous release more, likely. In case of Cr, similar effect (in the adrenal medulla: Liu and Lin, 1997) was described. $Fe^{2+}$ ions also pass through neuronal Ca channels (Gaasch et al., 2007). The ion channel effects of the metals could be present along the whole sensory pathways, acting also on axonal conduction. The main excitatory transmitter in the CNS is glutamate. After being released into the synaptic cleft and having activated the receptors, glutamate is taken up and converted to glutamine by the astrocytes. $Mn^{2+}$ decreases both the uptake (Erikson and Aschner, 2003) and the transformation to glutamine (Normandin and Hazell, 2001). First of all the interactions of metals – the effect of Mn, but not of Cr, being neutralized by Fe – suggested, however, that other factors were involved, such as the above-mentioned oxidative stress (detected in an antecedent study for Mn by Szabó et al., 2011; and for Fe in a related work by Szalay et al., 2011), or energetic shortage due to disturbed mitochondrial function..

Mn is a well-documented mitochondrial toxin, acting on complex II (Malecki, 2001) and complex III (Zhang et al., 2003). Cr in the mitochondria oxidizes NADH and inhibits alpha-ketoglutarate dehyrdogenase (Cohen et al., 1993). In mitochondrial Mn-SOD, surplus Fe tends to replace Mn, decreasing enzyme activity (Bacon et al., 1993). Fenton reaction, a redox cycle characteristic for all three metals studied (Valko et al., 2005) can generate dangerous hydroxyl radicals within the mitochondria.

Energetic shortage could have slowed ECoG activity, like in cases of human mitochondrial encephalopathy (Smith and Harding, 1993). Glutamatergic overactivity (due to decreased astrocytic uptake and metabolism) could have the opposite effect via increased activation of the ascending reticular formation by collaterals of the sensory pathways. The

resulting net change will finally depend on the relative strength of the two opposite influences.

The background idea in applying double-pulse (Experiment I and II) or varied frequency (Experiment III and IV) stimulation to the somatosensory system was that this could indicate the interaction between closely sequenced excitation processes which in turn would be dependent on the effects of the neurotoxicants being tested. Fig. 13 shows that the extra latency lengthening on higher frequency stimulation (2 or 10 Hz vs. 1 Hz) was more in the Mn and Cr treated rats than in the controls. This could reflect energetic shortage, impaired synaptic transmission, or both, and has been suggested as an indicator of neuro-functional damage (Papp et al., 2001).

Double pulse stimulation itself is a well known method in electrophysiology, but has been applied predominantly in vitro. Centonze et al. (2001) used double pulse stimulation on in vitro (more exactly, ex vivo) brain slices from rats exposed to Mn in the drinking water (20g/l $MnCl_2$). Striatal excitatory postsynaptic potentials were recorded and second:first ratio calculated. With double pulse stimulation, the second stimulus may cause increased transmitter release due to the remaining Ca ions in the presynaptic terminals – but Mn can interfere with stimulus-dependent transmitter release as described above. Glial removal of the released glutamate (Hazell and Norenberg, 1997) can also be affected by Mn, so a change in the second:first ratio was expected, but not found, in the mentioned work.

Under in vivo conditions, double pulse stimulation was used by Liepert et al. (2001) on patients with mitochondrial encephalomyopathy, and strongly decreased intracortical inhibition was found. The effects seen by Szabó et al. (2005) in rats treated with 3-nitropropionic acid were similar to that but the double pulse method in the present study brought less clear results. 3-nitropropionic acid is a mitochondrial toxin by its principal effect while in case of Mn a number of mechanisms, outlined above, can contribute to the final result.

In interpreting the changes in open field motor behaviour, the dopaminergic system must be considered first of all. Motivation, determining spontaneous open field locomotor activity, is regulated by mesolimbic/mesocortical dopaminergic structures (Alexander et al., 1990). Dopaminergic neurons are especially vulnerable to oxidative stress due to the auto-oxidizing tendency of dopamine and to the presence of monoamine oxidase producing hydrogen peroxide (Alexi et al., 2000). In presence of $H_2O_2$ and Mn, Fe or Cr, the Fenton reaction can take place and produce hydroxyl radicals (Valko et al., 2005). Of the activity forms measured, vertical motility is an especially sensitive indicator of striatal dopaminergic

activity (Sedelis et al., 2001). Diminished rearing was observed in the rats exposed to Mn and/or Cr but not Fe, possibly because the brain Fe level did not increase. Increased immobility fits well in the picture of general hypomotility, a phenomenon possibly analogous to what has been described in welders suffering from Parkinson-like syndrome, in which both Mn (Bowler et al., 2006) and Cr (Sayre et al., 1999) can have a causative role.

Based on the results described and evaluated above, it can be stated that the attempt to model welding fume exposure in rats was successful. In a world where the large-scale use of various metals is daily reality and results in occupational exposure and environmental pollution the health effects in general, and in particular the effects on sensitive systems like the nervous system, are of primary concern. Nanotechnological application of metals is another new source of exposure to metal-containing particles, a new feature to the old problem. Especially in neurotoxicity, the study of functional alterations is important because classical biomarkers – such as levels of toxic metals in available human biological samples (blood or urine) – do not indicate well the damage to central or peripheral nervous system. This problem has been repeatedly raised in the literature (e.g. Manzo et al., 1996; Myers et al., 2003). Animal model experiments can contribute to the development of neuro-functional biomarkers which may be better suited for this purpose.

The questions formulated in the particular point of aims (1.6.) can now be answered as follows.

- The general and nervous system effects of Mn were approximately identical to those found in previous experiments. The effects of Cr were, under identical conditions, partly similar to those of Mn. This similarity could be due to shared mechanisms of action. Data on neurotoxic effects of Cr are scarce in the literature so the findings described in this thesis may be novel. The effects of Fe were partly minimal, partly opposite to those of Mn.
- The interactions of the three metal studied were dissimilar on various parameters. The effect of Mn on body weight gain, and on electrophysiological and some OF parameters, were counteracted by Fe, but Cr was apparently not involved in such interaction. In the tissue metal levels, Fe acted on Cr but not on Mn. All that indicated that measured internal dose is not the sole determinant of the functional alterations and so, not an ideal biomarker of effect.

- Even when Mn was applied alone, the physicochemical form and site of application greatly influenced the resulting internal dose which may be of importance in case of various forms of human exposure.
- Electrophysiological tests, including double pulse stimulation and second:first ratio calculation, may well be suitable for detection and follow-up of functional damages in the nervous system but the particular form applied in the present work turned out not to be optimal.

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# 7. Appendix

**I.** Horváth E, *Máté Zs*, Takács Sz, Pusztai P, Sápi A, Kónya Z, Nagymajtényi L, Papp A (2012) General and electrophysiological toxic effects of manganese in rats following subacute administration in dissolved and nanoparticle form. The Scientific World J, Article ID 520632. IF: 1.524

**II.** *Máté Zs*, Szabó A, Paulik E, Jancsó Zs, Hermesz E, Papp A (2011) Electrophysiological and biochemical response in rats on intratracheal instillation of manganese. Centr Eur J Biol 6, 925–932. IF: 0.685

**III.** *Máté Zs*, Szabó A, Paulik E, Papp A (2011) Experimental neurophysiological alterations caused by combined nano-manganese exposure. Fiziologia (Timisoara) 21, 17–20.

**IV.** *Máté Zs*, Szabó A, Oszlánczi G, Papp A (2011) Per os Mn-expozíció modellezése és funkcionális idegrendszeri hatásának vizsgálata patkányban. Egészségtudomány 55, 71–81.

**V.** *Máté Zs*, Szabó A, Papp A (2008) Looking for parameters of early and delayed functional neurophysiological alterations caused by manganese. In: Galbács Z. (Ed.): 15th Symposium on Analytical and Environmental Problems, Szeged, 102–105.