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Electrophysiological and biochemical response in rats on intratracheal instillation of manganese

Research Article

Zsuzsanna Máté[1,*], Andrea Szabó[1], Edit Paulik[1], Zsanett Jancsó[2], Edit Hermesz[2], András Papp[1]

[1]*Department of Public Health, Faculty of Medicine, University of Szeged, H-6720 Szeged, Hungary*

[2]*Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged, H-6726 Szeged, Hungary*

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Abstract: Chronic exposure to excess manganese *via* inhalation of metal fumes causes central nervous system damage. For modelling Mn aerosol inhalation, male Wistar rats were intratracheally instilled with $MnCl_2$ solution (0.5 mg/kg b.w. $MnCl_2$; n=12) 5 days a week for 5 weeks. At the end of the treatment, somatosensory cortical evoked potentials, elicited by double-pulse stimulation, were recorded from the animals in urethane anaesthesia. Body weight gain, organ weights, and Mn level in brain, lung and blood samples were also measured. In brain samples, gene expression level of *MnSOD* (Mn superoxide dismutase) was determined. The effect of Mn was mainly seen on the evoked potential amplitudes, and on the second:first ratio of these. Tissue Mn concentration was elevated in brain and lungs, but changed hardly in the blood. Relative weight of heart, thymus, lungs and brain was significantly altered. The level of *MnSOD* transcript in brain tissue decreased. The observed effects showed that Mn had access to the brain and that somatosensory cortical responses evoked by double-pulse stimulation might be suitable biomarkers of Mn intoxication.

Keywords: *Manganese • Intratracheal instillation • Evoked potentials • MnSOD • Rat*

1. Introduction

Although manganese (Mn) is an essential micronutrient (the daily demand being 2-3 mg for adults; [1]) it has been known for a long time that chronic exposure to excess Mn causes dysfunctions in the central nervous system.

Deteriorating effects of occupational Mn overexposure result primarily from inhalation [1]. Miners, metal workers *etc*. are exposed to inorganic Mn aerosols (ore dust, metal fumes). Acute inhalation of Mn-containing fumes can cause adverse health effects, such as metal fume fever, while chronic exposure affects the lungs and the central nervous system. Also for the general population, inhalation may be a significant source of exposure, e.g. from car exhaust (in countries where methylcyclopentadienyl manganese tricarbonyl, an anti-knock petrol additive is used; [2]) but also in the vicinity of industrial emission sources. Cases of alimentary Mn intoxication that caused health damage have also been reported [3,4].

Mn transported by the blood stream can easily cross the blood-brain barrier and reach the brain through receptor-mediated endocytosis (bound to transferrin receptor) or can be carried directly into the cells *via* the divalent metal transporter 1 (DMT-1) or *via* glutamate activated ion channels [5,6]. Direct transport of Mn from the olfactory epithel to the brain along the olfactory nerve fibres has also been described [7,8]. Elimination half life of Mn in the brain is longer than in the whole body [9]. In case of inhalation exposure, Mn deposited in the lungs continues to supply the brain long after the exposure is terminated [10,11]. Mn naturally occurs in all parts of the brain but excess Mn accumulates in certain subcortical regions such as basal ganglia, explaining the nervous system symptoms of Mn intoxication [12].

Altered oxidative status is involved in the mechanism of neurotoxicity of Mn. Mn is a co-factor of the antioxidant

* *E-mail: matezs@puhe.szote.u-szeged.hu*

enzyme Mn superoxide dismutase (MnSOD), localized in mitochondria [13] but at the same time Mn participates in the generation of reactive oxygen species (ROS) and causes oxidative stress [14]. Oxidative stress can be indirectly measured by determination of antioxidant enzymes. MnSOD is the primary antioxidant of the mitochondria, so its synthesis is probably affected by excess Mn [13]. Changes in Mn containing proteins can be seen in many neurodegenerative diseases including manganism (a Mn-induced Parkinson-like syndrome), Alzheimer's disease and amyotrophic lateral sclerosis [15]. Decrease in mRNA of oxidative stress proteins [16] and manganoproteins [13] on experimentally induced oxidative stress is also known.

The especial sensitivity of brain stem dopaminergic neurons to oxidative stress, together with dysfunction in the basal ganglia, may explain the partial similarity between manganism and Parkinson's disease [12]. The clinical symptoms of Mn neurotoxicity appear insidiously after years of exposure which renders the recognition of the disorder more difficult. Early detection of Mn intoxication would be crucial because once the symptoms of manganism become apparent the deteriorating effect on the central nervous system is irreversible [17].

The usual biomarkers of Mn exposure are metal levels in blood, urine, faeces and hair. In certain studies [18,19] positive correlation of Mn level in blood and urine with exposure levels was found, but others reported that these are not reliable indicators of Mn exposure [20-23]. Rapid Mn clearance trough biliary excretion from the body and the fact that only a small amount of Mn is excreted into urine [24,25] also suggest that blood and urine Mn levels are not proper indicators of exposure. 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 central nervous system effects of Mn [26-28]. For human cases, Greger [29] suggested the use of neuro-functional tests.

It is also known that excess Mn causes mitochondrial dysfunction by inhibiting mitochondrial complex I and II, and by disruption of the mitochondrial membrane potential [6]. Mitochondrial damage results in disturbance of energy producing metabolism (leading to ATP depletion) and in functional alterations that can be detected by electrophysiological methods. The cortical evoked potentials (EPs) obtained by somatosensory double-pulse stimulation were proven in our previous works sensitive to the action of another mitochondrial toxin, 3-nitropropionic acid in rats [30,31]. Moreover, mitochondrial damage is reflected in changes of *MnSOD* gene expression levels.

Considering the mentioned lack of a specific biomarker for the early detection of Mn intoxication, our aim was to seek for sensitive electrophysiological parameters as potential indicators of Mn neurotoxicity. To do that, rats were subacutely treated by intratracheal instillation of $MnCl_2$. Somatosensory cortical EPs elicited by double-pulse stimulation were used to study functional neurotoxicity, and the mitochondrial effect was detected by the *MnSOD* gene expression level. Body and organ weight, and tissue Mn level, were also measured.

2. Experimental Procedures

2.1 Animals and treatment

Adult male Wistar rats (180±20 g body weight at start) were obtained from the university's breeding centre and were housed in an air conditioned room, maintained at 22°C, with 12-hour light/dark cycle (light on at 06:00) and free access to tap water and standard rodent chow. The animals were divided into three groups of 12 animals each. Those in the *Treated* group received intratracheal instillation of $MnCl_2$ dissolved in distilled water (0.5 mg/kg b.w. $MnCl_2$), the vehicle control (*VC*) group was instilled with distilled water, and further 12 rats constituted the untreated control (*Cont*) group. Intratracheal instillation (volume: 1 ml/kg b.w.) 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 and a pair of non-traumatic forceps. $MnCl_2$ solution (or distilled water) 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. This treatment was performed once a day, 5 days per week for 5 weeks. $MnCl_2$ was from Sigma-Aldrich Hungary, urethane from Reanal, Hungary.

2.2 Electrophysiological investigation

Electrophysiological measurement was done by anaesthetizing the animals with urethane (1000 mg/kg b.w., i.p.) first. The head was fixed in a stereotaxic frame and the left hemisphere was exposed. Wounds were sprayed with 10% Lidocaine and the exposed dura surface was covered with a thin layer of petroleum jelly. After at least 30 min of recovery, the animals were transferred to the recording setup, and a ball-tipped silver electrode was positioned on the somatosensory projection area of the whiskers (barrel field). Somatosensory EPs were obtained by electric stimulation of the corresponding

peripheral site (right whisker pad). A double-pulse scheme was used in which the first stimulus (square pulse, *ca*. 4 V, 0.05 ms) was followed by the second one after 300, 240, 180, 120 and 60 ms interstimulus interval (ISI). A complete record, taken once from each rat, involved 20 "runs"; and in one run each ISI was used once in decreasing order. The runs were averaged by the software controlling the whole stimulation, recording and evaluation process (Clampex 8.0, Axon Instruments Inc.). On the averaged EP curves, peak amplitude and peak latency was measured, and the second:first ratio was calculated (each parameter of the second EP was divided by the corresponding parameters of the first EP). 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 was identical to that applied previously with 3-nitropropionic acid [30,31].

The results were tested for significance with one-way ANOVA with post-hoc LSD test. The level of significance was set to $P<0.05$.

2.3 General toxicology

The body weight of the animals was measured regularly during the experiment. After the electrophysiological measurement, the animals were euthanized with an overdose of urethane and were dissected. Blood was collected from the abdominal vein, and the main organs (brain, lungs, thymus, heart, liver, kidneys, adrenal glands and spleen) were removed and weighed. From these data, relative weights were calculated by relating organ weights to 1/100 of body weight.

For Mn level determination, whole brains, lungs and blood (from the *VC* and *Treated* groups only) were quickly frozen and stored at -20°C. For measurement, *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 Mn determination was done by inductively coupled plasma mass spectrometry. Statistical analysis was done by two-sample t-test.

During the whole procedure, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed (based on the ethical licence, permit No. XXI./02039/001/2006).

2.4 Measurement of *MnSOD* gene expression

2.4.1 RNA extraction, reverse transcription and PCR amplification

Brain samples were homogenized in RNA Bee reagent (Tel-Test, Inc.; Friendswood, Texas, USA) and total RNA was prepared according to the procedure suggested by the manufacturer. To detect rat *MnSOD* specific mRNA, an RT-PCR-based strategy was employed. First-strand cDNA was synthesized by using 5 mg total RNA as template. For that the RNA was denatured at 90°C, mixed with 200 pmol of each dNTP (Fermentas), 200 U RevertAid Premium Reverse Transcriptase (Fermentas) and 500 pmol random hexamer primers (Fermentas) in a final volume of 20 ml, and incubated for 10 min at 37°C, followed by 1 h at 42°C. The reaction was stopped by heating at 65°C for 5 min. 1 ml of reverse transcription product was added to 12.5 ml of 2x Dream Taq Green PCR Master Mix (Fermentas). The sequences of primers, used for PCR amplication, were derived from GeneBank entry NM_017051 for MnSOD (forward: 5'GCTGGGGCTGGCTTGGCTTC3' and reverse: 5'CCACCACGGGCCTGACACAC3') and NM_031144 for β-actin (forward: 5'GCAAGAGAGGTATCCTGACC3' and reverse: 5'CCCTCGTAGATGGGCACAGT3').

2.4.2 Measurement of MnSOD mRNA levels

For normalization of the amount of *MnSOD* mRNA, the *β-actin* mRNA level was used as internal standard. Images of ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with GelBase/GelBlot™ Pro Gel Analysis Software (UVP). The relative level of *MnSOD* mRNA is expressed as ratio [*MnSOD*/*b-actin*].

3. Results

3.1 Body weight gain, organ weights, Mn levels

Intratracheal instillation of $MnCl_2$ had no significant effect on the body weight gain in the *Treated* group compared to *Cont* and *VC*; that is, there was no body weight reduction in the treated rats.

However, Mn had a clear effect on the relative weight of certain organs, such as brain, lungs, heart and thymus. The increase of these organs' relative weights was significant by the end of treatment (Figure 1).

Mn concentration both in brain and lungs was highly elevated in *Treated* compared to *VC*, while that of the blood hardly changed (Table 1).

3.2 Electrophysiological effects

Intratracheal instillation of $MnCl_2$ caused mild to significant alterations in the studied parameters of the EPs, mainly in the amplitude values. The peak-to-peak amplitude of the first EP increased in the $MnCl_2$ treated rats and this change was mostly significant (Figure 2). The peak-to-peak amplitude of the second EP showed slight, non-significant decrease in the *Treated* group (*vs*. *Cont* and *VC*). The change in the first EP's peak-to-peak

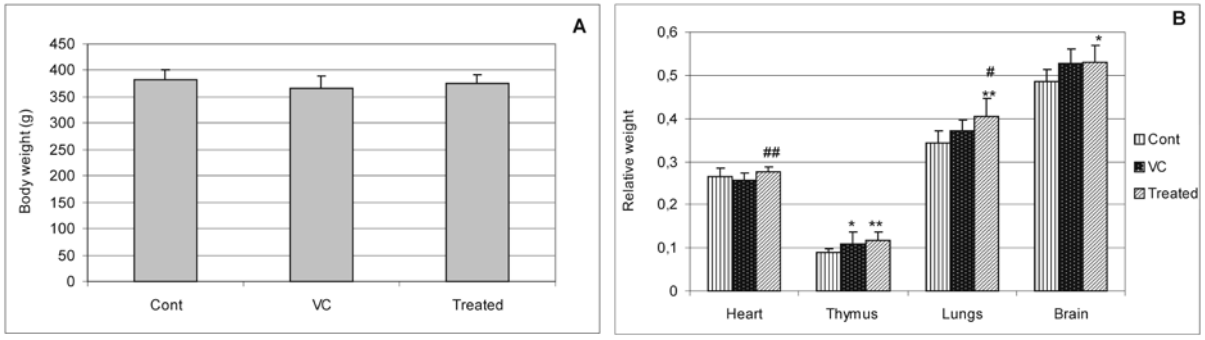

Figure 1. The effect of 5 weeks treatment with $MnCl_2$ or vehicle on the body weight and relative organ weights of the rats. A, body weight at the end of the treatment period; B, relative organ weights. Mean+SD, n=12 for both. *Treated*: intratracheal instillation of 0.5 mg/kg b.w. $MnCl_2$ dissolved in distilled water; *VC*: intratracheal instillation of vehicle (distilled water); *Cont*: untreated control. *, **: P<0.05, 0.01 *vs. Cont*; #, ##: P<0.05, 0.01 *vs. VC*

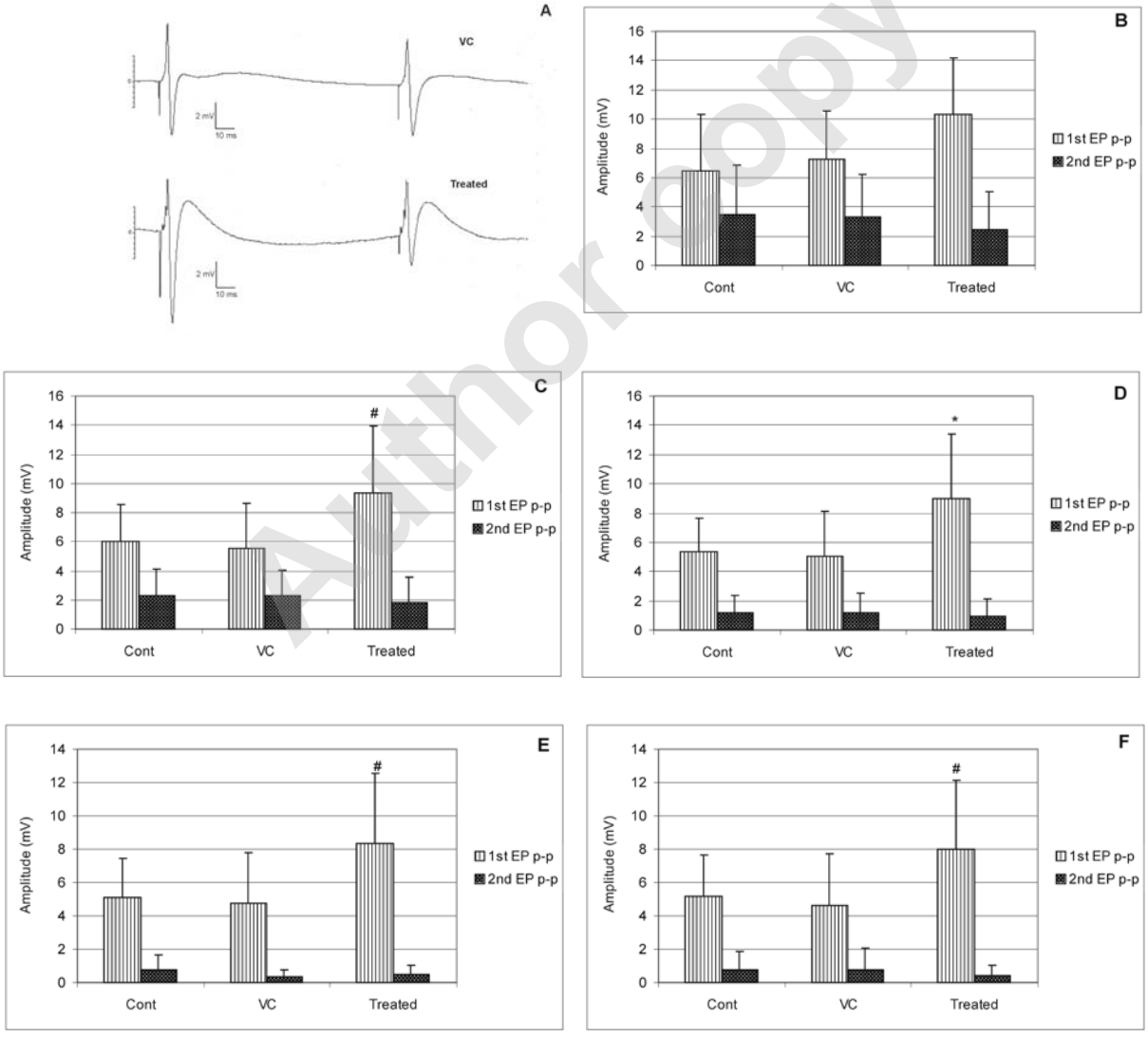

Figure 2. The effect of 5 weeks treatment with $MnCl_2$ or vehicle on the peak-to-peak amplitudes of the first and second EPs. A: Example traces of the EPs of *VC* and *Treated* group, taken with 240 ms ISI. B: 300 ms ISI; C: 240 ms ISI; D: 180 ms ISI; E: 120 ms ISI; F: 60 ms ISI. Mean+SD, n=12; *Treated*: $MnCl_2$ treatment; *VC*: vehicle control; *Cont*: untreated control.*: P<0.05 *vs. Cont*; #: P<0.05 *vs. VC*

Code	Organs		
	Brain	Lungs	Blood
VC	1812.52±176.28	1177.46±160.25	253.76±133.95
Treated	3084.79±472.92***	62022.14±16605.46***	249.10±44.61

Table 1. The effect of 5 weeks treatment with $MnCl_2$ or vehicle on Mn concentration (ppb) in the rats' brain, lungs and blood. Mean±SD, n=6; *Treated*: $MnCl_2$ treatment; *VC*: vehicle control. ***: $P<0.001$ *vs. Cont*

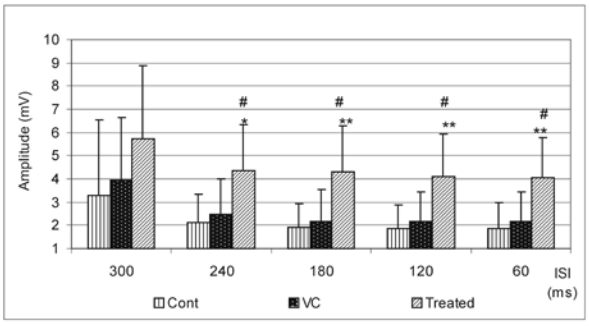

Figure 3. The effect of 5 weeks treatment with $MnCl_2$ or vehicle on the positive peak amplitudes of the first EPs. Mean+SD, n=12; *Treated*: $MnCl_2$ treatment; *VC*: vehicle control; *Cont*: untreated control. *, **: $P<0.05$, 0.01 *Cont*; #: $P<0.05$ *vs. VC*

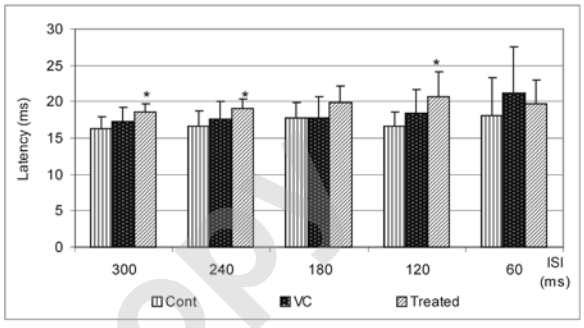

Figure 4. The effect of 5 weeks treatment with $MnCl_2$ or vehicle on the positive peak latencies of the second EPs. Mean+SD, n=12; *Treated*: $MnCl_2$ treatment; *VC*: vehicle control; *Cont*: untreated control. *: $P<0.05$ *vs. Cont*

amplitude was due mostly to the increase of the positive peak, which was also significant compared to *Cont* and *VC* (Figure 3).

The EP latencies generally showed an increasing trend on Mn exposure. This remained below significance except for the positive peak latency of the second EPs (compared to *Cont*) (Figure 4).

The second:first ratio of the peak-to-peak amplitude (a parameter of dynamic interaction in the stimulated sensory system, see Experimental procedures) showed significant decrease in the *Treated* group compared to *Cont* (240 ms ISI) and to *VC* (240 and 180 ms ISIs) (Figure 5). In case of positive and negative peak amplitudes the second:first ratio was lower in all ISIs compared to *Cont* and *VC* groups and showed significance also at 300 and 240 ms ISIs (data not shown).

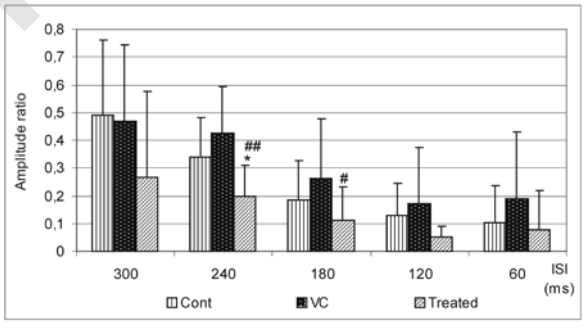

Figure 5. The effect of 5 weeks treatment with $MnCl_2$ or vehicle on the second:first ratio of the peak to peak amplitudes of the EPs. Mean+SD, n=12; *Treated*: $MnCl_2$ treatment; *VC*: vehicle control; *Cont*: untreated control. *: $P<0.05$ *vs. Cont*; #, ##: $P<0.05$, 0.01 *vs. VC*

3.3 *MnSOD* gene expression

MnSOD mRNA level in brain tissue showed a mild decrease after $MnCl_2$ treatment. Compared to *Cont*, no alteration was noticed in *VC* group (Figure 6).

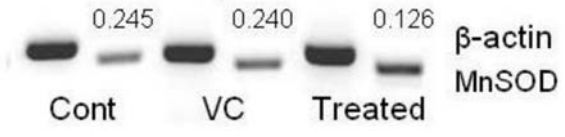

Figure 6. The effect of 5 weeks treatment with $MnCl_2$ or vehicle on the expression of *MnSOD* in the rats' brain, in relative units, compared to *β-actin*. A representative result of the RT-PCR amplification. In parallel with *MnSOD*, *β-actin* was amplified to determine the relative level of *MnSOD*. RNAs for template were prepared from the brain. *Treated*: $MnCl_2$ treatment; *VC*: vehicle control; *Cont*: untreated control.

4. Discussion

The observed changes in the electrophysiological parameters showed that the applied animal model

is suitable for the follow up of Mn intoxication *via* the airways. The observed functional alterations, and the significantly elevated levels of Mn (*Treated vs. VC*) measured in brain and lung (but not blood) samples, indicated that intratracheally instilled Mn reached the central nervous system, and also confirmed that the observed electrophysiological alterations were due to Mn accumulation in the brain.

Mn accumulates specifically within mitochondria, consequently it impairs (beside other functions of mitochondria, especially Ca homeostasis) energy metabolism, and causes oxidative stress [14,32]. As MnSOD is sensitive to oxidative stress, measurement of *MnSOD* gene expression level in brain samples can adequately support the toxic effect of Mn. Here we detected that *MnSOD* synthesis was, though without significance, inhibited by subacute Mn exposure. Morello *et al*. [13] also found that chronic oral Mn overload down-regulated the expression of manganoproteins in certain parts of the rat brain.

From previous experiments of the department [30,31] it was concluded that electrophysiological investigation, and especially somatosensory double-pulse stimulation, is a suitable method to detect functional changes caused by mitochondrial dysfunction in rats. Similarly to our results, Pecze *et al*. [33], and Takács *et al.* [34] also found that excess Mn caused noteworthy changes in the parameters, mainly in the amplitude, of the somatosensory EP. In those two studies, significant increase in amplitude and a non-significant change in the latency of the EP were found after acute parenteral Mn administration. Following subchronic oral Mn treatment, lengthening of the latency was detected by Vezér *et al*. [35], as it was found in the present experiment.

In the present study, diminished second:first ratio of the peak-to-peak amplitudes was observed, resulting from the above mentioned significant increase of the peak-to-peak amplitude of the first EP and a weaker, non-significant decrease that of the second one. In case of Mn, such an effect is explained (beside the mentioned mitochondrial effect) by the action of Mn on glutamatergic transmission [36]. Having reached the brain, Mn inhibits the uptake and the breakdown of glutamate in astrocytes [37]. Higher transmitter level in the synaptic cleft and the more powerful excitation will then result in increased somatosensory evoked activity. This is seen first of all on the first, positive peak of the EP which, according to the accepted mechanism of cortical EPs, directly reflects the transient polarization of the pyramidal cells upon an EPSP nearer the soma, and so the enhanced synaptic transmission. A more powerful EPSP causes also stronger after-effects *i.e.* a longer refractory period, which explains the amplitude decrease of the second EP in the pair. The mild, non-significant decrease might be the final effect of the direct (more glutamate in the synaptic cleft) and indirect (longer relative refractory period of the synapse) action of Mn.

The standard way to determine and follow Mn load in humans has been to measure Mn level from blood [38], but this is, as mentioned in the Introduction, far from the optimal. There is no way to directly measure Mn in the human brain but fine functional alterations, revealed by electrophysiological measurements, may indicate the magnitude of Mn exposure, and even more its consequences. And, as far as the mechanistic explanation outlined above is correct, the suggested method is all the more adequate as the glutamatergic effect of Mn is also involved in the human CNS damage caused by it.

Electrophysiological tests are not or minimally invasive, and somatosensory double-pulse stimulation has been used in human patients for a long time [39]. The second:first calculation has the advantage that it uses only data from the individual in question (let it be human or animal) and does not rely, or depend, on an eternal basis of comparison. As the amplitude of the somatosensory EP proved to be a reliable parameter of Mn intoxication in rats [33,34], our final conclusion and suggestion is that the electrophysiological test described here could be used to detect and/or confirm human Mn intoxication.

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