SUBACUTE EXPOSURE OF RATS TO CHROMIUM CONTAINING NANOPARTICLES VIA THE AIRWAYS: NEUROLOGICAL AND GENERAL TOXICOLOGICAL EFFECTS

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ABSTRACT: Industrial emission of nanoparticles (NPs) can originate from metal fumes and results in significant inhalational occupational exposure. Respiratory effects of chromium (Cr) compounds, often present in metal fumes, are known but information on their neurological effects is scarce, though Cr is known to cause oxidative stress and reach the brain after inhalation. For investigating the potential nervous system effects of Cr NPs, male Wistar rats were exposed to a nanosuspension of $Cr(OH)_3$ (∅ ca. 50 nm) by intratracheal instillation, once a day, 5 times per week, for 4 weeks. Significant internal Cr exposure was detected in brain, lung and blood samples, together with slowed body weight gain and decreased relative weight of certain internal organs, both effects showing linear correlation with external Cr dose. In the spontaneous cortical electrical activity, Cr caused increase of power in the fast bands (beta1, beta2, gamma) and decrease at lower frequencies (theta and delta). Cortical evoked potentials showed significantly increased latency on the action of Cr NPs. The correlation of Cr levels in tissue samples with general and neurotoxicological parameters pointed to a causal relationship. Further investigation of the functional alterations observed in this study would be important in replacing classical biomarkers or biological exposure indicators with ones more suited to detection of nervous system damage caused by NPs containing Cr.

KEY WORDS: chromium, nanoparticle, toxicity, neurotoxicity, rat

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Abbreviations:

AUD = auditory
b. w. = body weight
CNS = central nervous system
ECoG = electrocorticogram
EP = evoked potential
GLP = good laboratory practice
it = intratracheal
NP = nanoparticle
ROS = reactive oxygen species
SS = somatosensory
VIS = visual

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INTRODUCTION

Chromium (Cr) is a heavy metal with various industrial applications, the most important being production of alloys (stainless steel etc.) and electroplating. The whole life cycle of the metal – from ore mining through smelting and final product manufacturing to waste management and recycling – is concomitant with dermal and inhalational exposure, constituting thus a frequent occupational hygienic problem.

Inhalational exposure results from Cr-containing aerosol (dust, mist, metal fumes) in the workplace atmosphere. While inhalation of soluble Cr compounds (mists or dust) 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, 2008). 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. Dizziness, headache and weakness were also found in the same exposed group, indicating a possible neurotoxic effect. Respiratory symptoms were also described after exposure of workers to chromite ore (containing Cr^{III}), and the number of complaints and clinical signs increased in parallel with the number of respirable chromium (both Cr^{III} and Cr^{VI}) particles (Huvinen et al., 2002). Although ingested Cr^{III} is considered non-toxic, hypoactivity, mydriasis, lacrimation and body weight loss was reported as signs of acute Cr toxicity in rats (Gad et al., 1986). Based on recent findings, Levina and Lay (2008) suggested to pay more attention to the toxic effect of trivalent Cr compounds, among others because certain technologies, using traditionally the more toxic Cr^{VI}, are being transformed for Cr^{III}, a move driven by regulations – such as RoHS (Restriction of Hazardous Substances; European Parliament, 2003) in the European Union – to phase out hazardous industrial chemicals.

Cr_2O_3 is a trivalent Cr oxide compound, often found in industrial emissions. Horie et al. (2011) reported that cytotoxicity of nanoparticulate (60 nm) Cr_2O_3 was higher compared to micrometer-sized particles, and that Cr_2O_3 nanoparticles (NPs) reduced mitochondrial activity, increased intracellular ROS (reactive oxygen species) level, caused oxidative stress and released Cr^{VI} in the medium in vitro. This observation pointed to the role of particle size in the toxicity of heavy metals. In case of airborne particles being inhaled, NPs (diameter below 100 nm) are mostly deposited in the nasopharyngeal and alveolar region (ICRP, 1994). The surface-to-mass ratio of these particles is extremely high, resulting in a huge and reactive overall surface; and inhaled NPs can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs (Oberdörster et al., 2005). A healthy blood-brain barrier 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).

Occupational exposure to Cr^{III} and Cr^{VI} has been modelled in rats several times (Suzuki et al., 1984; Derelanko et al., 1999), but experimental results on possible nervous system effects are scarce, in spite of indications that such effects exist (Lieberman, 1941; Tandon et al., 1979). Repetto et al. (1996) reported Cr^{III} toxicity

on a neuroblastoma cell line in vitro, and Diaz-Mayans et al. (1986) found open field hypoactivity in rats exposed orally to Cr^{VI}. Motivated by this lack of data, and by results of comparable works with other toxic heavy metals (Oszlánczi et al., 2010; Horváth et al., 2011; Máté et al., 2011), our aim in the present study was to model human nanoparticulate Cr exposure in rats and to examine the effects on general toxicological and CNS electrophysiological parameters.

MATERIALS AND METHODS

Experimental animals and substances

Young adult male Wistar rats (260–280 g body weight at start) were obtained from the university's breeding centre. The animals were housed, with up to four rats in one cage, under GLP-equivalent conditions (22 ± 1°C, 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.

The rats were exposed to Cr by intratracheal (it.) instillation of a suspension of NPs consisting of $Cr(OH)_3$, the hydrated form of Cr_2O_3. Treatment was performed once a day, 5 times per week, for 4 weeks. This short period was chosen because our previous experience showed that the nanoparticulate form of other metals (e.g. Mn) tends to be more toxic than other (dissolved etc.) forms (Horváth et al., 2012), and it was supposed that in 4 weeks the effects will sufficiently develop.

The NPs (~50 nm diameter) were synthesized at the Department of Physical Chemistry and Materials Science, University of Szeged Faculty of Science and Informatics; their chemical purity was checked by X-ray diffraction, and their particle size by X-ray diffraction and transmission electron microscopy. The NPs were suspended in normal saline by ultrasonication; and were given to the rats in doses given in *Table 1* (high dose, *CrHD*; low dose, *CrLD*). A vehicle control group *(VC)* received saline instillation, and an untreated control *(Cont)* group was also used to see the effects of the treatment procedure properly.

Table 1. Treatment groups and doses

Group code	*Dose and treatment*	*n*
Cont	untreated control	6
VC	1 mL normal saline/kg b.w.; vehicle control	8
CrLD	2 mg $Cr(OH)_3$ NPs/kg b.w.; low dose treated	8
CrHD	4 mg $Cr(OH)_3$ NPs/kg b.w.; high dose treated	8

For intratracheal instillation (1 mL/kg b. w., see *Table 1*) the rats had brief diethyl ether anaesthesia. The unconscious rat was placed on an oblique board (60° to horizontal) hanging with the 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. The NP suspension (or the vehicle) 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.

General toxicological investigations

Body weight of the animals was daily measured during the experiment, to follow up weight gain and to determine the exact daily doses. Weekly body weight gain was calculated, separately for each rat, as the difference of body weight on Friday of the given week and Friday of the pre-treatment week, and these values were averaged group by group. Following electrophysiological recording, the animals were sacrificed by an overdose of urethane, were dissected, and blood was collected from the abdominal vein. 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 to 1/100 of body weight. Blood, brain, lung and liver samples were stored at –22°C.

For metal level determination, three rats were randomly chosen from each group. About 1 g samples of blood, brain and lungs 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 diluted 100-fold, and Cr level was determined by inductively coupled plasma mass spectrometry at the Laboratory of the MOL Hungarian Oil and Gas Company.

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.

Electrophysiological investigation

The rats were prepared for electrophysiological recording, and cortical electrical activity was registered, in urethane anaesthesia (1000 mg/kg b.w. ip.; Mook 2006). For details of preparation, see Papp et al. (2001, 2004).

For recording, 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. Ball-tipped silver recording electrodes were placed on the dura of the exposed left hemisphere, over the primary somatosensory (SS) projection area of the whiskery pad (barrel field), and over the primary visual (VIS) and auditory (AUD) areas. 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.

Spontaneous electrical activity was recorded from these sites simultaneously for 6 min, and the relative spectral power of the frequency bands 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 simpli-

fying, descriptor of the ECoG spectrum in our earlier works (Dési and Nagymajtényi, 1999).

To obtain sensory evoked potentials (EPs), 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. Fifty stimuli of each modality per rat were applied. For VIS and AUD stimulation, 1 Hz frequency was applied, and for SS stimulation 1, 2 and 10 Hz. It was supposed 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 (Papp et al., 2001, 2004). The 50 EPs were averaged and onset latency was measured. The complete recording and evaluation was performed by the software NEUROSYS 1.11 (Experimetria Ltd., Budapest, Hungary).

Data analysis

Data were tested for normality by the Kolmogorov-Smirnov test, and significant differences were detected by means of one-way ANOVA with post-hoc LSD test. Linear regression calculation between tissue metal levels and general and neurotoxicological parameters was done by the "linear fit" function of MS Excel. The level of significance was set to $p<0.05$.

RESULTS

General toxicological parameters

Cr treatment caused dose-dependent significant decrease in body weight gain (vs. *VC* and *Cont*) from the first treatment week on *(Fig. 1)*. Decreased body weight gain was also seen in the vehicle control group *(VC)*, reflecting the effect of the treatment procedure (mainly of repeated diethyl-ether anaesthesia) itself, but the effect in groups *CrLD* and *CrHD* was significantly different from that seen in *VC*, too.

As shown by the data in *Table 2*, it. instillation of Cr NPs caused significant increase in the Cr content of the brain, lungs and also blood (vs. *Cont* and *VC*). The relationship of internal metal load and body weight gain to summed external dose *(Fig. 2)* showed significant linear correlation between summed Cr dose and both body weight gain and Cr levels measured in brain and blood samples. The correlation of metal level in blood and lungs to body weight gain was also significant (data not shown).

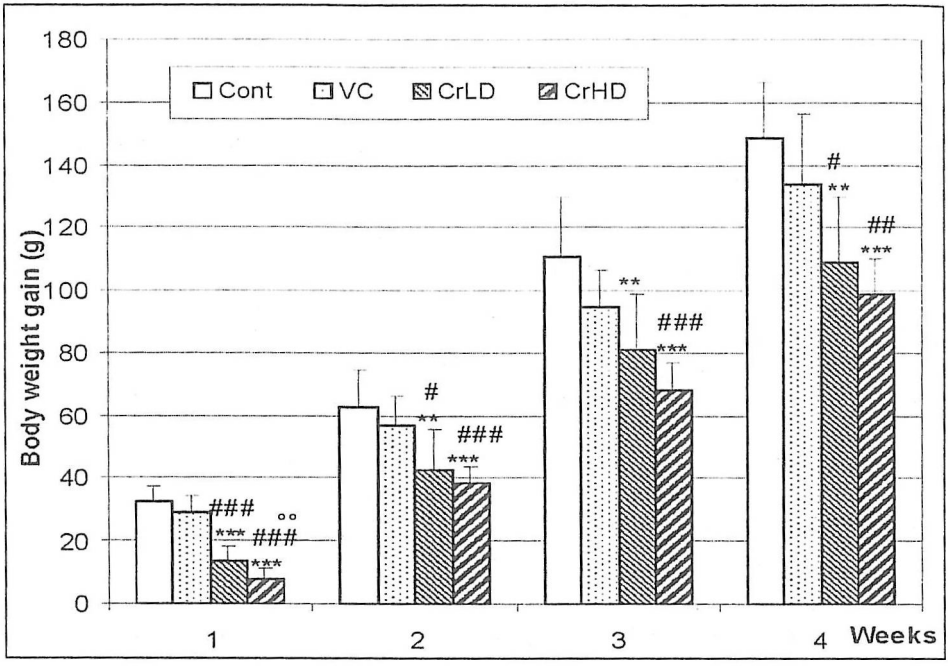

Fig. 1. Body weight gain of the control and treated rats during the 4 weeks of Cr NP exposure. Mean+SD, n=8. For way of calculation, see Methods; for group coding, see insert and Table 1.
, * *p<0.01, 0.001 vs.* Cont; #, ##, ### *p<0.05, 0.01, 0.001 vs.* VC

TABLE 2. Cr levels in blood, brain and lung samples

	Tissue Cr levels (μg/kg)		
Groups	*Blood*	*Brain*	*Lungs*
Cont	142.90 ± 16.06	122.70 ± 15.01	215.45 ± 34.95
VC	121.76 ± 36.32	138.34 ± 12.35	319.28 ± 113.53
CrLD	4809.74 ± 972.68** ##	213.82 ± 55.27	6891780.11 ± 792670.54***###
CrHD	12714.67 ± 2278.13***### oo	4514.75 ± 1995.67*# o	9708166.15 ± 1923132.60***###

Mean±SD, n=3
*, **, *** p<0.05, 0.01, 0.01 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. *VC*;
°,°° p<0.05, 0.01 vs. *CrLD*

Due to the significant effect of Cr NP treatment on body weight, relative organ weights calculated on the basis of brain weight were chosen for evaluation. Relative weight of the heart, thymus, liver, spleen and kidney decreased significantly (vs. *Cont*), while relative lung weights showed dose related significant increase (vs. *Cont* and *VC*; *Table 3*).

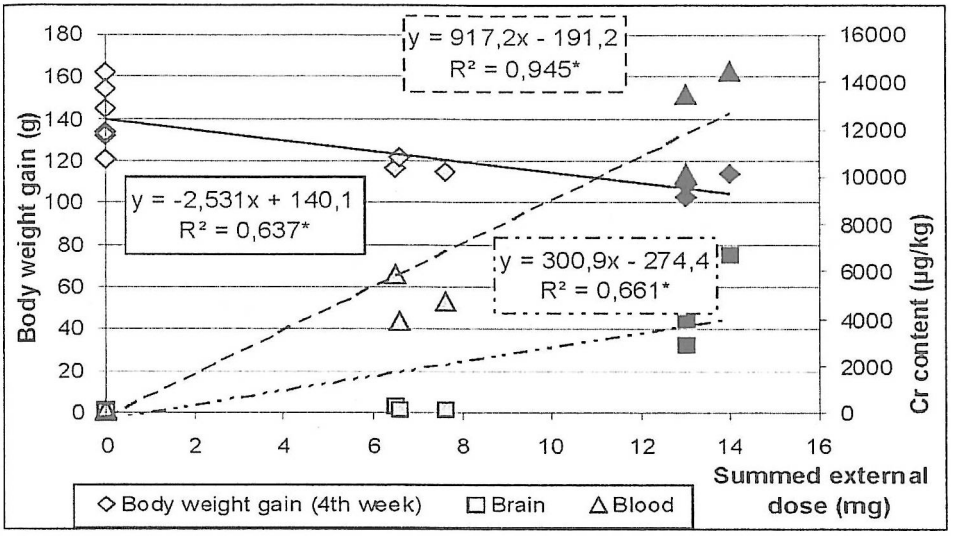

Fig. 2. Correlation of body weight gain (left ordinate), and of blood and brain Cr levels (right ordinate) to summed external Cr dose (abscissa). Each point represents a data pair (see insert at the bottom for kinds of data) from an individual rat chosen for metal level measurement (see Methods)
*Light symbols, Cont; light grey symbols, VC; dark grey symbols, CrLD; black symbols, CrHD. Inserts with outlines corresponding to the fitted lines: equation and R^2; *: $p<0.05$ (F-test for the linear fit)*

TABLE 3. Relative organ weights of rats (calculation basis: brain weight)

	Groups			
	Cont	*VC*	*CrLD*	*CrHD*
Heart	0.610 ± 0.050	0.510 ± 0.052**	0.530 ± 0.055*	0.491 ± 0.021***
Thymus	0.235 ± 0.028	0.185 ± 0.063	0.213 ± 0.049	0.184 ± 0.047*
Lungs	0.712 ± 0.058	0.673 ± 0.047	1.718 ± 0.162***###	2.364 ± 0.204***###ooo
Liver	7.641 ± 1.055	6.199 ± 0.892*	6.004 ± 1.255*	5.889 ± 0.453***
Spleen	0.386 ± 0.028	0.335 ± 0.071	0.332 ± 0.042*	0.331 ± 0.040*
Kidney	1.592 ± 0.197	1.338 ± 0.142*	1.350 ± 0.126**	1.598 ± 0.535
Adrenals	0.0289 ± 0.0064	0.0265 ± 0.0047	0.0287 ± 0.0031	0.0275 ± 0.0031

Mean±SD, n=8
*, **, *** $p<0.05$, 0.01, 0.01 vs. *Cont*; ### $p<0.001$ vs. *VC*; ooo $p<0.001$ vs. *CrLD*

The general condition of the lungs after instillation of Cr NPs was emphysematic, and dark spots of metal deposition were seen on dissection. No other organ-specific effect of the metal NP treatment was found (decreased relative organ weights in *VC* vs. *Cont* reflected the effect of the anaesthetic procedure also here).

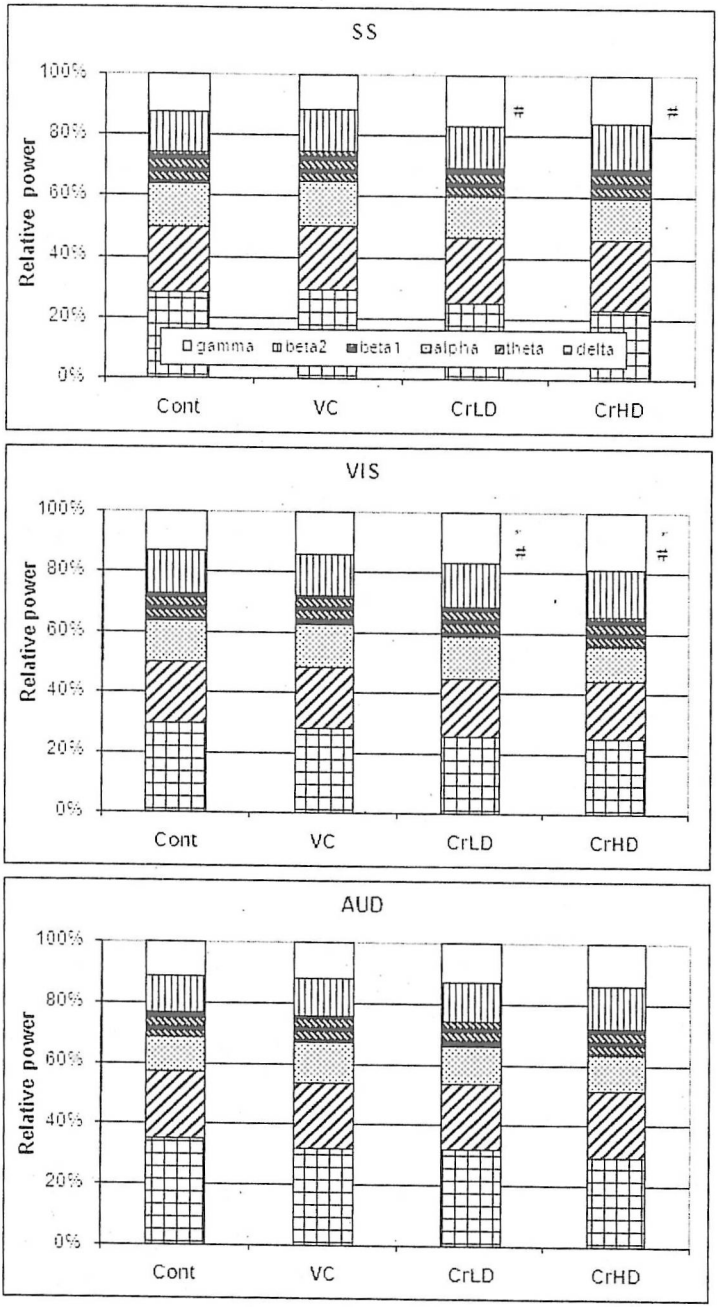

*Fig. 3. Power spectrum of the electrocorticogram from the three cortical areas after 4 weeks of exposure to Cr NPs. Abscissa, groups; ordinate, relative power of ECoG bands (see insert in the graph for SS); * $p<0.05$ vs.* Cont; # $p<0.05$ *vs.* VC

Cortical electrical activity

In the band spectrum of the ECoG, the proportion of the fast bands (beta1, beta2, gamma) increased, while that of the slow bands (theta and delta) decreased upon Cr NP treatment *(Fig. 3)*. In the SS ECoG, significant increase of the gamma band was observed (vs. *VC*) while delta band showed a mild, non-significant, decrease. The alterations in the VIS and AUD ECoG were similar. The correlation of brain Cr level with the ECoG index (see Methods) was significant for the VIS and AUD records *(Fig. 4)*: the relationship for the SS ECoG index was of similar direction but less clear (not shown).

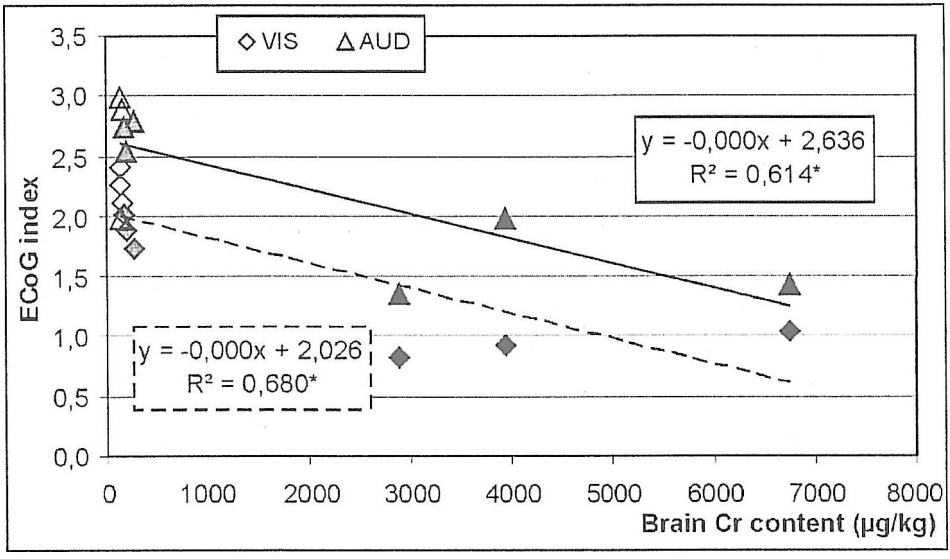

Fig. 4. Correlation of VIS and AUD ECoG index (ordinate) to brain Cr levels (abscissa). The meaning of data points and the way of display is as in Fig. 2

The SS EPs *(Fig. 5A)* showed significantly increased latency on the action of Cr NPs. The latency increase was seen at all stimulation frequencies, and the extent of frequency dependence (an indication of the actual state of the sensory pathway) was also altered by Cr. VIS and AUD latency values significantly increased by *CrHD* treatment vs. *VC (Fig. 5B)*. *Fig. 5* shows latency data in relative values, normalized to the latency in the vehicle control *(VC)* group. Data of the untreated control *(Cont)* are not displayed because the difference between *Cont* and *VC* was negligible. Significant correlation was found between brain Cr level and EP latency *(Fig. 6)*.

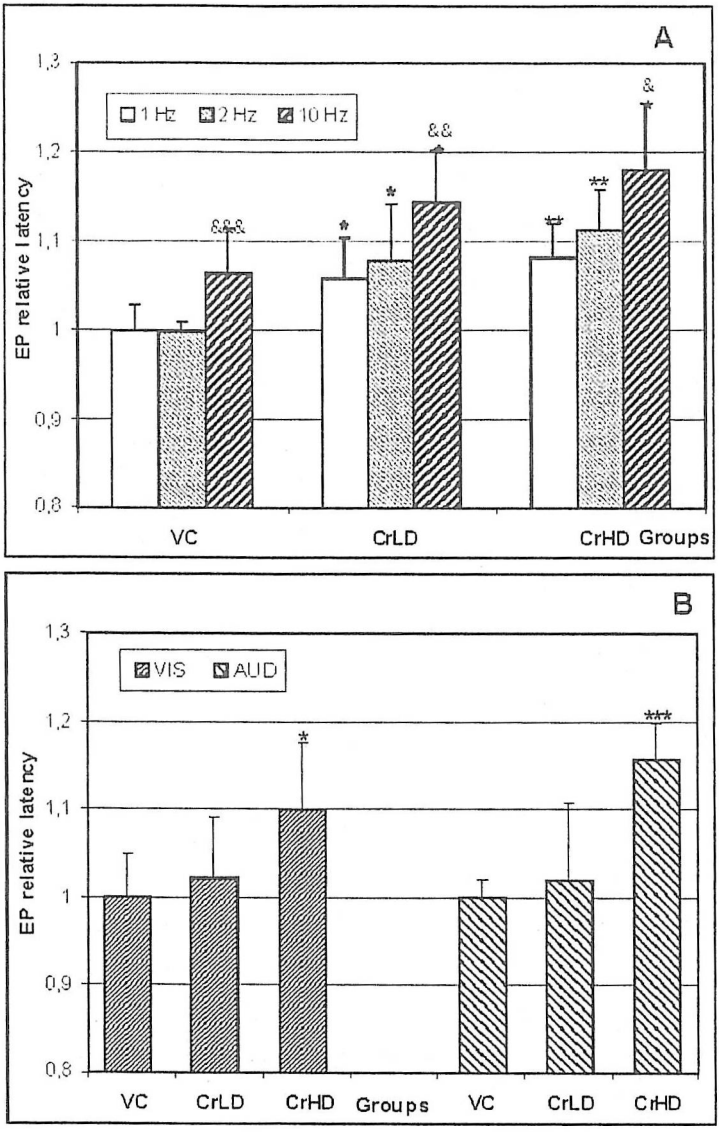

*Fig. 5. Latency of the somatosensory (A), and visual and auditory (B) cortical evoked potentials. Relative values, normalized to the latency in group VC (for the somatosensory EPs, to the latency obtained with 1 Hz stimulation, see insert in A). Mean+SD, n=8. *, **, ***: p<0.05, 0.01, 0.001 vs. VC (for the SS EPs, at identical stimulation frequency).*
&, &&, &&&: for SS EPs, p<0.05, 0.01, 0.001 vs. 1 Hz stimulation within the same group

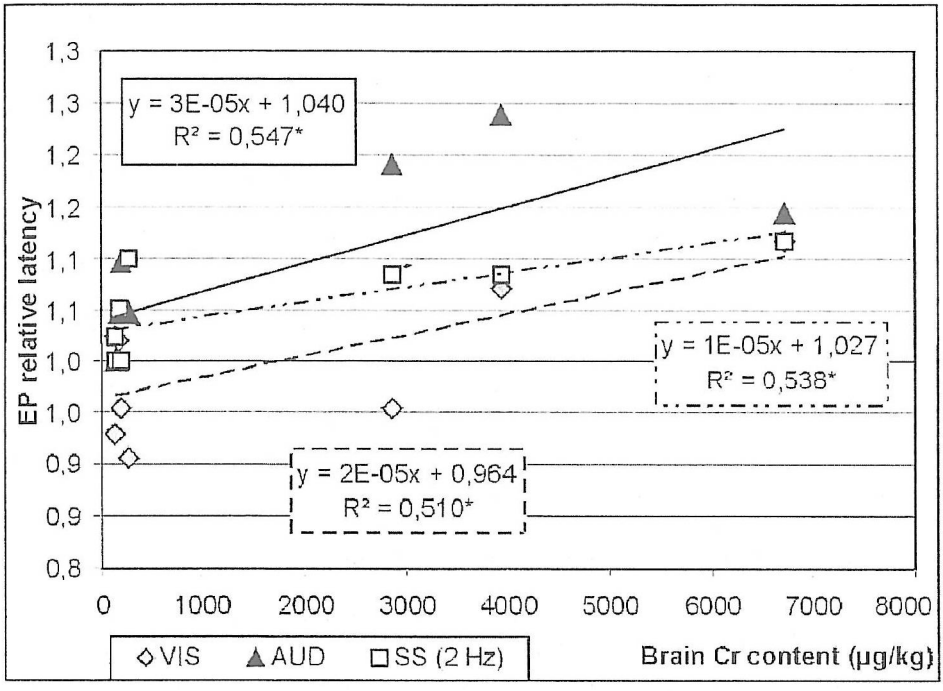

Fig. 6. Correlation of the latency of the EPs to brain Cr levels.
The latency values represented by the points are relative values, shown also in Fig. 5, *(for SS EP, those obtained with 2 Hz stimulation frequency). Else, the meaning of data points and the way of display is as in* Fig. 2

DISCUSSION

In the treated rats' brain (and also blood and lung) samples, increased Cr levels were measured after it. instillation of Cr NPs, indicating that the applied external load was sufficient and the treatment was effective. The correlation of blood and brain Cr levels, and body weight gain, to summed external Cr dose, showing that the (possibly causal) relationship between the applied treatment and the general toxicological effect, was dose related and significant *(Fig. 2)*.

In the interaction of Cr with the organism, absorption, transport and metabolism are all strongly dependent on the valence state. Cr^{VI} is able to enter into the cells to a greater extent than Cr^{III} (Klasing et al., 2005); in NP form, however, Cr^{III} was transported more efficiently in an in vitro experiment, apparently depending on particle size, surface charge and hydrophobicity (Zha et al., 2008). The measured internal metal levels could be due to the presence of NPs themselves in the blood and brain of the treated rats, or Cr ions may have been dissolved from the NPs in the acidic environment of phagolysosomes, after NPs being phagocyted by pulmonary

macrophages (Lundborg et al., 1985). Both were likely, because NPs easily cross barriers, including the blood-brain barrier (Kreyling et al., 2006); but in rats exposed to welding fumes containing Cr, the role of the dissolved metal fraction was found predominant in causing oxidative stress and airway inflammation (McNeilly et al., 2005).

Oxidative stress, whether caused by Cr^{III} as a chemical agent (Horie et al., 2011) or by surface interactions on the Cr NPs (Oberdörster et al., 2005), was obviously a key factor in the observed toxic effects. Cr in the body is primarily transported by the iron-transport protein transferrin (Vincent, 2011). The massive elevation of blood Cr level in the treated groups might lead to displacement of Fe from transferrin bond, and the increased free Fe level might contribute to the oxidative stress and CNS damage (Thompson et al., 2001).

Reduction in body weight gain observed in the treated animals might be due to the presence of free radicals and the metabolic disturbance caused by them (Merry, 2002). Lipid peroxidation (described also in Cr-treated rats: Becerra-Torres et al., 2009) might lead to changes of fluidity and other membrane properties in the brain which, in turn, disturb all membrane- and receptor-bound functions (Coyle and Puttfarcken, 1993) including synaptic transmission and all phenomena depending on that e.g. cortical electrical phenomena.

Of the two kinds of cortical activity recorded, EPs reflect alterations in synaptic transmission more directly. Beyond the membrane damage mentioned above, increased EP latency in the Cr treated rats might be, at least partly, due to decreased synaptic efficacy. Cr^{3+} ions partly block, but partly pass through, voltage-dependent Ca channels at presynaptic endings, leading to decreased stimulus-dependent but increased spontaneous transmitter release (Liu and Lin, 1997). The extra latency lengthening on higher frequency SS stimulation (2 or 10 Hz vs. 1 Hz) was more pronounced in the Cr treated rats than in the controls *(Fig. 5A)*. Beside the disturbed synaptic transmission mentioned, this could also be the result of energetic shortage resulting from the mitochondrial effect of Cr (oxidation of NADH and inhibition of alpha-ketoglutarate dehydrogenase: Cohen et al., 1993).

Inhibition of acetylcholinesterase in the rats' brain, as it was found by Soudani et al. (2012) after exposing the animals to $K_2Cr_2O_7$, could explain the shift of the ECoG band spectrum to higher frequencies in group *CrHD* in the present work, via increased ascending cholinergic activation of the cortex (Metherate et al., 1992).

In our days, with the ubiquitous presence of metals as pollutants and technological materials, the health effects of metals in general, and in particular the effects on sensitive systems like the nervous system, are of primary concern. Investigation of functional alterations may be of especial importance in the aspect of neurotoxicity 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 (Manzo et al., 1996). Animal model experiments can contribute to the development of neuro-functional biomarkers which may be better suited for this purpose.

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