PhD Thesis

FUNCTIONAL NEUROTOXICITY OF ARSENIC AND MANGANESE AS ENVIRONMENTAL AGENTS AND THE POSSIBLE PROTECTIVE ROLE OF NATURAL ANTIOXIDANTS

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The Applicant's Relevant Publications

I. **Sárközi K**, Nagy V, Papp A, Tombácz E, Szabó A (2013): Effects of three natural antioxidants on the general and nervous system toxicity of manganese nanoparticles in rats.
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Abstracts:

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SUMMARY

Oxidative stress apparently plays a major role in the effect of several toxicants, including heavy metals, of environmental or occupational origin. It means inequilibrium between metabolic reactions producing reactive oxygen and nitrogen species (ROS and RNS), and the capacity of enzymatic and non-enzymatic reactions to neutralize these. In itself, oxidative stress is a side effect of oxidative energy production, which can be caused by exposure to a number of environmental xenobiotics, metals and others. Non-neutralized ROS and other radicals damage the nervous system, liver, kidneys etc. Free radicals are more and more held responsible for various chronic non-communicable diseases, and for aging. The central and peripheral nervous system is prone to oxidative damage, due to highly active mitochondrial energy production, to abundance of (unsaturated) structural lipids, and to low antioxidant defence capacity in the brain; and ROS may constitute the final common pathway for several neurotoxicants because oxidative damage to membrane lipids may lead to alterations of the membrane-bound functions crucial to the functioning of neurons. In the present thesis, the relationships of neurotoxicity, oxidative stress and antioxidant protection are investigated in case of two heavy metals with public health importance, arsenic (As) and manganese (Mn).

Arsenic used to have numerous practical applications most of which have been discontinued because of the health risks. Its modern applications e.g. in semiconductors, are apparently safe. Increased presence of As in certain rocks may lead to human exposure by As emissions from mining and smelting non-ferrous metal ores or from burning coal with higher As content, but most importantly via drinking water. The problem of As in drinking water has been quite serious in several regions. According to WHO, As is among the ten most important chemicals of major public health concern. Arsenic occurs in various oxidation states in inorganic or organic compounds. Inorganic arsenite (As^{III}) is more toxic that arsenate (As^{V}), and most of the organic arsenicals are practically nontoxic. Arsenite strongly binds to the –SH group of proteins, inactivating various enzymes including those in the citrate cycle and terminal oxidation, depleting ATP pool and promoting the generation of ROS in the cells. It also increases oxidative stress by depleting reduced glutathione and thioredoxin. The consequences of chronic exposure to inorganic arsenic include nervous system damage. Electrophysiological data on As neurotoxicity are scarce, but in As-exposed workers altered EEG, visual evoked potentials, and peripheral nerve activity were detected, together with signs of oxidative stress.

Manganese (Mn) can have the oxidation states -3 to 7+, indicating its propensity to redox reactions. Mn and its compounds have numerous technical applications (steelmaking, welding, nanotechnology etc). It is an essential trace element, required for normal brain function, and acting as cofactor for several enzymes such as Mn-dependent glutamine synthetase or superoxide dismutase. In high amounts it is toxic, nervous system being an important target. The population can be exposed to food- or waterborne Mn, but neurotoxic manifestations are rare. Occupational Mn exposure (in Mn ore mining and smelting, production of alloys, welding etc.) is due to Mn-containing aerosol, including nanoparticles (NPs, with at least one dimension smaller than 100 nm). NPs are apparently involved in

various chronic non-communicable diseases of humans affecting among others the central nervous system. With their small size, NPs easily penetrate physiological barriers. Their huge and reactive surface induces generation of ROS. Mn disturbs neurotransmission (glutamatergic, cholinergic, dopaminergic, GABAergic), causes mitochondrial dysfunction and cellular energy shortage, and can inhibit voltage-gated Ca-channels in neurons. It induces oxidative stress via oxidation of dopamine and other catecholamines, and by mitochondrial inhibition. Chronic Mn exposure in humans leads to a Parkinson-like syndrome, but disorders with electrophysiological signs after Mn exposure also have been reported.

Cells possess various mechanisms against oxidative stress. These include, beside enzymes (superoxide dysmutase, catalase etc.) small biomolecules called antioxidants. Glutathione, with -SH group in the cysteine moiety, can directly react with ROS, and can reduce oxidized tocopherole and ascorbic acid. Uric acid, final metabolite of purines, is an important antioxidant in the blood plasma. Further endogenous antioxidants are lipoic acid (primarily a cofactor of dehydrogenases) and melatonin. Exogenous antioxidants are foodborne micronutrients, such as the vitamins A, C and E, and various carotenoids and polyphenols. Their role in upkeeping and improving health is a major field of research. The antioxidants used in the present work were ascorbic acid, rutin, curcumin, and green tea polyphenols.

Ascorbic acid is essential (as vitamin C) only for humans and a few other animals. It is able to scavenge ROS and regenerate oxidized glutathione. It is present in the brain due to active uptake process. It is a well-known natural substance, easily accessible for the public, present in various foods and drinks. Rutin is a flavonoid present in, e.g., citrus fruits and rhubarb. Similarly to other flavonoids, rutin acts as free radical scavenger and as metal chelator. Health benefits ascribed to rutin include antioxidative, anti-inflammatory, antiallergic and neuroprotective effects. Curcumin is present in the rhizomes of turmeric. It has multiple pharmacological properties including antioxidant effect, and has been effective in animal models of Alzheimer's dementia and neurotoxicity. Fresh leaves of the tea shrub contain 35–40% flavonoids, and green tea brewed from leaves without fermentation is a rich source of these antioxidants. Tea, a popular drink worldwide, is a major source of total intake of flavonoids, phytochemicals with antioxidant and metal chelating activity which also activate transcription factors and antioxidant enzymes.

Exposure to metals and metalloids remains a source of health damage for the population, and oxidative stress probably plays a significant role in the damages caused by As and Mn, especially in the nervous system. By supporting antioxidant defence with exogenous antioxidants, metal-induced nervous system damages could possibly be diminished. The general aim of this thesis was to observe the neuro-functional damage caused by exposure to arsenic and manganese and the possible protective effect of certain antioxidants of natural origin. The particular questions to be answered on the basis of the expected results were as follows:

- Can the neurotoxic, and other toxic, effects of the used physicochemical form of As and Mn be investigated in identically built-up experiments or together?
- Have the antioxidants included in the experiments any effect on the alterations induced by the two metals?

- What differences, qualitative or quantitative, can be seen between the effects of the antioxidants?
- Which of the antioxidants might be an optimal choice, considering also protection of human health?

These aims have been realized in three experiments, performed on young adult male Wistar rats. They were treated with Mn or As, and the natural antioxidants ascorbic acid (vitamin C), rutin, curcumin, and green tea brew as given in the table below. With physicochemical form and way of application, imitation of real life human exposure was attempted.

	Experiment 1	**Experiment 2**	**Experiment 3**
Duration, agents, application	4 weeks of MnO_2 NPs, 4 mg/kg b.w. intratracheal then 1 week of antioxidants (ascorbic acid, curcumin, rutin or their vehicles), 100 mg/kg b.w. by gavage	6 weeks As 5 and 10 mg/kg b.w. ($NaAsO_2$,) by gavage; and ascorbic acid, 1 g/L, green tea brew, 2.5 g/500 mL, via drinking fluid	6 weeks As 10 mg/kg b.w. ($NaAsO_2$,) by gavage; or MnO_2 NPs, 4 mg/kg b.w. intratracheal and ascorbic acid, 2 g/L, rutin, 1 g/L, green tea brew, 2.5 g/500 mL, via drinking fluid
Investigations	• Body weight • Organ weight • Open field test • Electrophysiology	• Body weight • Food and water consumption • Organ weight • Open field test • Electrophysiology • Biochemical measurements • Tissue metal level	• Body weight • Food and water consumption • Organ weight • Open field test • Electrophysiology • Biochemical measurements • Tissue metal level

The rats' body weight was recorded daily in all experiments. Food and drinking fluid consumption were measured in Experiment 2 and 3. In the open field test, the rats' spontaneous locomotor activity was measured. Electrophysiology meant recording and analysis of spontaneous and stimulus-evoked activity from the primary somatosensory (SS), visual (VIS) and auditory (AUD) cortical surface of the rats, and compound action potentials from the tail nerve, in urethane anesthesia. After the complete behavioral and electrophysiological recording, the rats were overdosed with urethane, were dissected, and organs were weighed. For metal level determination and biochemical measurements, whole brain, liver, kidneys, and 2-3 ml of red blood cells were taken from 3 randomly chosen rats per group. To determine heavy metal concentration, the samples were dried to constant weight, and were digested in 65% HNO_3 at 90°C for 90 min. Measurement was done by inductively coupled plasma mass spectrometry at the Department of Inorganic and Analytical Chemistry, University of Szeged Faculty of Science and Informatics. Biochemical

measurements (indicators of oxidative stress: H_2O_2; $ONOO^-$; TBARS) were done at the Department of Biochemistry and Molecular Biology, University of Szeged Faculty of Science and Informatics. Antioxidant power of the drinking fluids was determined by their ferric reducing capacity.

General toxic effects: Both As and nano-Mn caused reduced body weight gain. This was paralleled partly by reduced food intake in As treatment (Experiment 2 and 3) but almost fully in Mn treatment (Experiment 3). Protective effect of the used antioxidants against the metals' action of body weight was weak or absent, with rutin showing the relatively clearest effect.

In rats instilled with MnO_2 NPs (Experiment 1 and 3) relative weight of the lungs increased massively and this was not influenced by the antioxidants. Else, only the weight of the adrenals reacted on As and Mn exposure, with an inconsistent increase which was weakly influenced by the antioxidants. Elevated As level was measured in the treated rats' red blood cell, cortex, liver and kidney samples (strongest in the latter organ). Antioxidants reduced As deposition but only moderately. In Experiment 3, also Mn was deposited in the tissues but the antioxidants had no effect on that.

Open field behavioral effects: In the OF test, the antioxidants alone had no effect on parameters of motility. As reduced motility slightly in Experiment 2 but more significantly in Experiment 3, with increased local activity and immobility but decreased rearing. The OF effect of Mn was similar (Experiment 1 and 3). Green tea had minimal influence on the OF effect of either As or Mn. In Experiment 3, rutin counteracted the effect of As more clearly than that of Mn while ascorbic acid influenced only the OF effect of Mn. Rutin also reversed the Mn-dependent increased preference of the rats to the corner zones of the OF box in Experiment 1.

Electrophysiological effects: The band spectrum of the electrocorticogram was influenced only by nano-Mn exposure, not by As. The shift to higher frequencies was moderate in Experiment 1 but more pronounced in Experiment 3. The decrease of low-frequency activity was abolished more strongly by green tea than by rutin but not at all by ascorbic acid. There were more marked changes in the parameters of evoked activity. Latency of the cortical evoked potentials was negligibly influenced by the antioxidants alone. Administration of Mn NPs caused significant increase of the latency in each modality, which effect was counteracted by rutin and green tea, less strongly by ascorbic acid, but not at all by curcumin. The effect of As, and the counter effects of the antioxidants on that, was similar. Slowing of the conduction velocity in the tail nerve, seen on treatment with both As and Mn, was counteracted most strongly by rutin, less by green tea and only weakly by ascorbic acid. In Experiment 1, curcumin also had a weak protective effect against decrease of conduction velocity by Mn.

Biochemical effects: The intensity of TBARS reaction level of $ONOO^-$ were altered by exposure to As and Mn, and by antioxidant application, in a way which showed effect and counter effect. The effect of As was most clearly reversed by rutin, and that of Mn, by ascorbic acid. The causal relationship between inner metal doses (tissue As and Mn levels) and the observed neuro-functional and biochemical changes was tested searching for correlations. In Experiment 2 cortical and peripheral electrophysiological changes, and cortical TBARS level, was significantly correlated to cortical As concentration. In Experiment

3, body weight gain was correlated to liver level of As or Mn, while certain electrophysiological and behavioural parameters, and TBARS, to cortical metal levels but also to each other.

In the investigated functional alterations of the treated rats' nervous system, there were some general trends, such as decreased OF motility or slowed electrophysiological responses, indicating possible common mechanisms in the background. Oxidative stress may be one such mechanism, as both As and Mn, as inorganic chemical agents, are know to induce the generation of reactive oxygen species in living tissue, and such effect has been described also in exposed humans. The negative effect of oxidative stress on CNS functionality has also been reported for both metals. The antioxidants tested in this study had some counter effect on the electrophysiological and/or behavioral alterations induced by As and nano-Mn, but to a dissimilar extent. The effect of ascorbic acid was in most cases less than that of rutin or green tea brew, although the measured antioxidant capacity of ascorbic acid solution was higher. Protective actions, independent of direct reduction of oxidized biomolecules, of rutin and green tea flavonoids include first of all chelation of metal ions.

All antioxidants tested are natural, foodborne compounds potentially available for general use in populations exposed to As or Mn, but also to other environmental toxicants, but the real life efficacy of this kind of preventive measures has not yet been verified, so the ideas like "functional drinks in neurodegenerative diseases" still await implementation.

The particular questions of the study can be answered as follows:

- In Experiment 3, the used physicochemical form of As and Mn were investigated in terms of neurotoxicity and general toxicity together in the same experiment. Identical but separate experiments with the two metals were not made, but the answer to the first question is essentially positive.
- The antioxidants included in the experiments had some clearly detectable effects on the alterations induced by the two metals, regarding both nervous system effects (electrophysiological and behavioral) and general toxicity, but:
- There were marked differences between the effects of the antioxidants: 1/ Curcumin had practically no effect. 2/ The protective effect of vitamin C was weaker than that of the applied flavonoids – rutin and green tea constituents. The cause of the difference was apparently not the antioxidant capacity but probably the metal chelating ability of the flavonoids and/or their better local availability in the CNS.
- The examined antioxidants are all easily available natural compounds. As a chemically defined compound, rutin showed better effect than ascorbic acid. Green tea infusion (or an extract or concentrate) is chemically complex but the main constituents have been chemically identified. An "optimal" choice of antioxidant has to consider, beyond verified in vivo protective effect, also technical (sources, processing, formulation) and social (acceptance in the population to be protected) aspects, and requires further studies both within and outside the scope of environmental neurotoxicology.

TABLE OF CONTENTS

ABBREVIATIONS

ANOVA	analysis of variance
ATP	adenosine triphosphate
AUD	auditory
CAP	compound action potentials
CNS	central nervous system
DNA	deoxyribonucleic acid
EcoG	electrocorticogram
EEG	electroencephalogram
EGCG	(–)-epigallocatechin-3-gallate
EPs	evoked potentials
GABA	γ-aminobutyric acid
GLP	good laboratory practice
GSH	glutathione
HEC	hydroxyethyl cellulose
HNO_3	nitric acid
HOO•	hydroperoxyl radical
H_2O_2	hydrogen peroxide
it	intratracheal
$KMnO_4$	potassium permanganate
KOH	potassium hydroxide
MMT	methylcyclopentadienyl manganese tricarbonyl
Mn-SOD	manganese superoxide dismutase
MnO_2	manganese dioxide
MR	magnetic resonance
MS	Microsoft
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NO	nitrogen monoxide
NPs	nanoparticles
$O_2^{\bullet-}$	superoxide anion
$^{\bullet}OH$	hydroxyl radical
OF	open field
PAA	polyacrylic acid
PBS	phosphate buffered saline
po	per os
RBCs	red blood cells
ROS	reactive oxygen species
RNS	reactive nitrogen species
SS	somatosensory
TBARS	thiobarbituric acid-reactive substances
VIS	visual
WHO	World Health Organization

1. INTRODUCTION

1.1. Oxidative stress

1.1.1. Definition and evolutionary aspects

Oxidative stress can be defined as an inequilibrium between metabolic reactions producing so-called reactive oxygen species (ROS) and reactive nitrogen species (RNS) on one side and the capacity of enzymatic and non-enzymatic reactions to neutralize them on the other side (Valko et al., 2007). Oxidative stress and damage is a side effect of oxidative energy production of living organisms, the appearance of which was a fundamental event in the geological and biological evolution of Earth.

Oxygen-emitting photosynthetic activity of primitive cyanobacteria raised the O_2-content of the lower atmosphere to the level we know today ca. 2.4 billion years ago (Buick, 2008). For all organisms which had developed in the previous oxygen-free environment – the ones called today anaerobic – free oxygen was poisonous and induced the development of antioxidative defence mechanisms. It was only a later step of evolution when "purposeful" biological oxidation as the most efficient way of biological energy production appeared. Thanks to the strongly positive standard redox potential of O_2 (vs. H_2O), biological oxidation provides for a high rate of energy production – but at the risk of oxidative damage of biomolecules due to the by-products of the reactions. That is why all living organisms have various antioxidant protective mechanisms which, however, had originally the role to protect ancient anaerobic cells against the new, oxygen-rich environment (Kiss et al., 2007).

1.1.2. Oxidative species: normal cellular metabolism and the effect of xenobiotics

In the absence of exogenous harmful substances, most of the ROS originates from cellular respiration, from the mitochondrial electron transport chain. Complete reduction of an O_2 molecule requires four electrons but these are transported along the electron transport chain (complexes I to IV in the inner mitochondrial membrane) one by one. So, a part of oxygen can remain partially reduced if some electrons "leak" from the transport chain (mostly at complex I) too early and react directly with the O_2 molecule (Valko et al., 2007). The immediate product is the superoxide anion ($O_2^{\bullet -}$), leading (in further reactions with free protons and electrons) to generation of hydroxyl ($^{\bullet}OH$) and hydroperoxyl ($HOO^{\bullet}$) radicals. Reactive nitrogen species (RNS) are also known, the most prominent being nitrogen monoxide ($NO^{\bullet}$) an important signal molecule involved in vascular tone regulation, generated

from L-arginine by NO synthase. Also certain ROS have signal or effector functions, e.g. in the immune response (oxidative burst in macrophages), erythropoetin production, or programmed cell death (Dröge, 2002).

This natural production of ROS and RNS in the cells causes normally no manifest oxidative stress because various antioxidant mechanisms (see 1.1.3.) eliminate the surplus of dangerous molecules, but exposure to a number of environmental substances or xenobiotics can tip over this oxidant-antioxidant balance.

Many of the metals, present in the residential or workplace environment and exposing humans by inhalation, ingestion and other routes, can be involved in oxidative stress. Some of the notable metal xenobiotics, like Mn (for details, see 1.3.2.) but also Fe, Co, Cu and others, can undergo redox cycling and participate in ROS generating processes such as the Fenton reaction. Others, including As (for details, see 1.3.1.) and Cd, inactivate certain compounds that act as antioxidants in the cells (Valko et al., 2005). The resulting imbalance between pro-oxidant and antioxidant processes leads to generation of reactive oxygen, carbon, sulfur and nitrogen radicals. The outcomes at systemic level include neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals (Flora et al., 2008).

The primary consequence of the presence of ROS and RNS in the cells is damage to various biomolecules. Amino acid side chains in proteins can be oxidized, so presence of carbonyl groups in proteins is a good measure of oxidative damage. Peroxidation of lipids – first of all unsaturated fatty acids, important constituents of membrane lipid bilayer – not only destroys the original molecules and impedes the functions bound to their intact state, but generates further reactive radicals, and finally malondialdehyde (Valko et al., 2007). It is a measurable indicator of lipid peroxidation (by means of the thiobarbiturate reaction: Flora, 2011) and also a reactive molecule, itself generating covalent adducts with DNA bases.

It is being realized more and more that in all major chronic non-communicable diseases, such as malignant tumors, cardiovascular diseases, chronic inflammations or diabetes, ROS and RNS are involved in the pathomechanism. The free radical theory of aging states that existence and action of free radicals in the organism, left unneutralized due to inadequate antioxidant capacity, is a leading cause of the gradual decay of all body functions (Harman, 1956).

For the present thesis, oxidative damages to the nervous system are the most important. Central and peripheral parts of the nervous system are both prone to oxidative damage, due to highly active mitochondrial energy production because of the high energy demand, to abundance of (unsaturated) structural lipids, and to low antioxidant defence capacity in the

brain (Guerra-Araiza et al., 2013). Reactive oxygen species may constitute the final common pathway of effect of several neurotoxicants (LeBel and Bondy, 1991) and oxidative damage to membrane lipids in axons and neuronal cell bodies may lead to changes of fluidity and probably to alterations of those membrane-bound functions which are crucial to the functioning of neurons (Coyle and Puttfarcken, 1993).

1.1.3. Protective antioxidant mechanisms of the cells

Under normal conditions, the oxidative species generated in cellular metabolism are neutralized by several biochemical reactions, both enzymatic and non-enzymatic.

Superoxide is converted to H_2O_2 by the superoxide dysmutases; the molecule is then either reduced by glutathione peroxidase to water or decomposed by catalase to water and oxygen (is made harmless in both ways), or can undergo the Fenton reaction with suitable endogenous or exogenous transition metal ions to produce $^{\bullet}OH$ which, being a free radical, is a more reactive ROS than H_2O_2.

The small biomolecules involved in antioxidant defence are collective called "antioxidants".

1.2. Antioxidants

There exist several – not fully compatible – definitions of antioxidants, depending on the actual approach (more chemical or more biological, in vitro or in vivo, etc).

In a purely chemical approach, an antioxidant is a reducing agent that is oxidized instead of another molecule which is to be protected, or reverts that from its oxidized form. In a more biological view, antioxidants can inhibit or prevent the oxidation of oxidizable cell constituents by scavenging free radicals and reducing oxidative stress (Kim and Lee, 2004). The definition given by Dröge (2002) "substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates" is supposed to include both chemical antioxidants and antioxidant enzymes. Frequently a distinction is made between endogenous (enzymes and small molecules synthesized by the organism) and exogenous (foodborne factors like vitamin E, vitamin C for primates, flavonoids etc.) factors acting against oxidative stress, and the term "antioxidant" is used only for the latter. It has to be considered also that, depending on the actual redox conditions, the same compound can act as a reducing (antioxidant) or an oxidizing (pro-oxidant) agent.

1.2.1. Endogenous antioxidant molecules of the human and animal organism

The two most important endogenous small antioxidant molecules are glutathione and uric acid.

Glutathione is a tripeptide: gammaglutamyl-cysteinyl-glycine. It can be synthetized in every cell but the main site of generation is the liver. The active part of the molecule is the –SH group of the cysteine moiety. It can directly react with the $^{\bullet}OH$ radical and singlet oxygen, and can reduce the oxidized forms of tocopherole and ascorbic acid. As an enzyme cofactor, it works with glutathione peroxidase (breaking down H_2O_2 and organic peroxides) and glutathione transferase (linking the tripeptide to xenobiotic molecules to facilitate their elimination) (Valko et al., 2007).

Uric acid is the final metabolite of purines, a product of xanthine oxidoreductase (an enzyme producing also certain ROS). Uric acid is an important antioxidant, especially in the blood plasma (Valko et al., 2007). It has been supposed that uric acid replaced ascorbic acid in those primates (including humans) who lost both the ability to synthesize ascorbic acid and to further metabolize uric acid to allantoin (Proctor, 1970). Consequently, for nearly all other animal and plant species, ascorbic acid (vitamin C) is an endogenous antioxidant.

Further endogenous molecules with antioxidant activity include lipoic acid and melatonin. Lipoic acid is synthetized and used in the cells in covalently protein-bound form and is primarily a cofactor of dehydrogenases. According to some sources, lipoic acid can directly act as antioxidant (Valko et al., 2007) while others state it is more an activator of cellular antioxidant mechanisms (Shay et al., 2008). Melatonin, a serotonin derivative produced in the pineal gland, is, beside its chronobiological function, also a powerful antioxidant (Tan et al., 1993), scavenging ROS directly and interacting with other antioxidants.

1.2.2. Exogenous (dietary) antioxidants

These are micronutrients required to be present in food in sufficient amounts. Some are considered essential, with known recommended daily intake and known health consequences of insufficient or absent supply (Rodler, 2005).

Vitamin A (retinol) molecules have a terpenoid-like structure, and this is the basis of the antioxidant activity of this nutrient, which is independent of its other, more specific effects. Its major sources include liver, eggs and dairy products.

Vitamin C (ascorbic acid) is, as mentioned above, an essential nutrient only for a few species including humans. The best known sources are citrus fruits, green peppers, sea buckthorn etc.

It is one of the antioxidants used in the experiments performed for this thesis that is why it is presented more in detail in 1.4.1.

The word vitamin E refers to a group of lipids (tocopheroles and tocotrienes) chemically related to carotenoids but without conjugated double bonds. The principal antioxidant function vitamin E, found e. g. in vegetable oils and egg yolk, is to scavenge lipid peroxyl radicals, linked via a redox reaction chain to glutathione and ascorbic acid (Wefers and Sies, 1988).

Carotenoids represent a large family of natural lipophylic compounds of tetraterpene structure found in fruits ad vegetables, mostly in those with more intense yellow or red colors. Their antioxidant potency is due to free radical scavenging, and that, to the double bonds in the hydrocarbon backbone. Carotenoids can cooperate with other antioxidants including tocopherol, ascorbic acid, uric acid and some polyphenols, and certain carotenoids are provitamins of vitamin A (Krinsky and Johnson, 2005).

Polyphenols are a group of organic molecules containing one or more aromatic rings with phenolic hydroxyl groups. Most of them are of plant origin, and are produced in the secondary metabolism of the plants as protective agents (against predators and ultraviolet light) and as signal molecules (Lattanzio et al., 2006). They are present, to various degrees, in foods and beverages obtained from fruits and vegetables. The antioxidant property of polyphenols results from their oxidation to quinon structure and reducing thereby the reaction partner. Among them, flavonoids are the largest group. Rutin, another antioxidant used in our experiments (see 1.4.2.), is the glycoside of quercetin which is a flavonoid, more precisely a flavonol (Formica and Regelson, 1995). Various flavonoid-type polyphenols, mainly catechins, are found in the infusion of green tea leaves (that is, the tea drink; Hodgson, 2006; see 1.4.3.). Curcumin (1.4.4.), likewise involved in the experiments performed for this thesis, is just another plant-derived phenolic compound with antioxidant and chelator properties (Kunchandy and Rao, 1990).

1.2.3. Health effects of antioxidants: what is known, supposed and hoped

In the last decade, interest in the potential health benefits of exogenous antioxidants, first of all those of plant origin, has been increasing. Epidemiological studies suggested that long term consumption of diets rich in plant polyphenols can protect against chronic non-communicable diseases such as cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Pandey and Rizvi, 2009). Flavonoids have known anti-inflammatory and antioxidative effects, and they have been described as neuroprotective and

able to reduce damage in CNS diseases, with a number of benefits especially in maintaining cognitive function and memory capacity (Mandel et al., 2005). Ideas have been published to create "functional drinks" rich in antioxidants (vitamin C and E, polyphenols) to prevent or treat neurodegenerative diseases (Zafrilla et al., 2009). However, the beneficial effects observed in cell cultures and experimental animals have been verified in humans, by now, only to a rather limited extent (Albarracin et al., 2012). It is also a question how efficiently the antioxidant content of foods etc. is absorbed and reaches the desired site of action (Pandey and Rizvi, 2009).

1.3. Metals, heavy metals, arsenic and manganese

The use of metals by humans commenced about four thousand years ago and has been increasing since then. Due to that, the level of various metals has been also increasing, and in case of several metals not only in the immediate workplace environment but globally. It is possible that continuous exposure to metals creates a "silent pandemic" in modern societies, being responsible for a decrease in IQ, increased risk of antisocial behavior, neurodevelopmental disorders and brain dysfunctions (Rodríguez-Barranco et al., 2013).
Most of the metals known to be toxic are so-called heavy metals. The meaning of this term has been a matter of debate (Duffus, 2002). Newly, heavy metals are defined on the basis of their place in the periodic table; and so transition metals (d-block elements), post-transition metals and metalloids (found in the p-block) as well the lantanoids and actinoids are regarded as heavy metals (Appenroth, 2010). The place of these elements in the periodic table determines their chemical properties. The biologically most relevant two properties are the existence of more than one oxidation states in many of these metals (promoting their participation in redox reactions) and the tendency for making coordinate bonds (in enzymes working with metal cofactor – but also in the denaturation of proteins by heavy metal ions and enzyme inhibition by false cofactors).
The two heavy metals included in this thesis are arsenic (As) and manganese (Mn).

1.3.1. Arsenic

As a chemical element, arsenic is the 20th most abundant element in the Earth's crust and is ubiquitous in trace amounts. Its increased presence in certain rocks may lead to environmental contamination and human exposure by As emissions from mining and smelting non-ferrous metal ores (Rosado et al., 2007) and from burning coal with higher arsenic content (Bencko et

al., 2009). Arsenic used to have numerous practical applications (pigments, wood preservatives, pesticides, even medicines) most of which have been discontinued by now because of the health risks (ATSDR, 2000). Its modern applications e.g. in semiconductor manufacturing, are apparently safe.

The most important source of human As exposure is drinking water. The presence of As in subsurface waters used for drinking is due to geochemical factors: arsenic had been bound in ferric oxide-hydroxide containing layers in the geological past, at the time of sediment formation, and is being released when the local redox conditions in the bedrock aquifer are changed, typically because of human interference resulting from drilling deep wells and drawing water (Duker et al., 2005). The problem of As in drinking water has been quite serious in regions of Asia (Bengal Basin in India/Bangladesh: up to 3.000 μg/L) and South America (Argentina: up to 10.000 μg/L) (Nordstrom, 2002). In the Carpathian Basin, more exactly in South-East Hungary (Börzsönyi et al., 1992) and the adjacent Romanian and Serbian regions (Lindberg et al., 2006), the problem has become less severe by now, but municipal drinking waters with As concentration above the European Union limit value of 10 μg/L (Council Directive 98/83/EC, 1998) are still found.

Arsenic is defined in some literature sources as a micronutrient (12-25 μg/day may be required for the metabolism of methionine: Nielsen, 1991) while other sources (Guidelines..., 2011) deny the essentiality of this element. In any higher amount than that mentioned above, however, As is poisonous. According to the WHO, As is among the ten most important chemicals of major public health concern (Preventing Disease through Healthy Environment, WHO, 2010).

Arsenic occurs in four oxidation states (-3, 0, +3, +5) and in inorganic or organic forms. Inorganic trivalent arsenite (As^{III}) is more toxic than pentavalent arsenate (As^{V}), and most of the organic arsenicals are practically nontoxic (Ratnaike, 2003). Arsenite strongly binds to the –SH group of proteins, inactivating various enzymes including those in the citrate cycle and terminal oxidation, depleting ATP pool and promoting the generation of ROS in the cells. It also increases oxidative stress by depleting reduced glutathione and thioredoxin, by generating H_2O_2 in connection with oxidation of arsenite to arsenate, and by liberating iron (which then will exert its own ROS-generating effects) from ferritin (Jomova et al., 2011).

Arsenate, if not reduced to arsenite, interferes with phosphate in glucose phosphorylation and ATP synthesis. Methylation of As^{III} prevents these effects and promotes excretion in the kidneys, but increases carcinogenicity (Chouhan and Flora, 2010).

The mostly described consequences of chronic exposure to inorganic arsenic include cardiovascular, hepatic and renal diseases, and skin cancer (Ratnaike, 2003), but nervous system damage is also known. High-As water of a private well caused encephalopathy (with headaches, motor weakness, mental confusion and finally coma) in a family (Armstrong et al., 1984). In schoolchildren exposed to As emitted by a smelter in Mexico, problems of cognitive development were significantly associated with elevated urinary arsenic excretion (Rosado et al., 2007). In Bangladesh, where extreme As levels occur in drinking water (see above) association between children's exposure and motor disorders were found (Parvez et al. 2011) – both reports suggest functional damage of the central nervous system. In children exposed to As by nearby non-ferrous smelters in Europe, altered dopaminergic biomarkers were found (de Burbure et al., 2006).

Electrophysiological data on As neurotoxicity are scarce, but in As-exposed workers of a copper smelter altered EEG, visual evoked potentials, and peripheral nerve activity were detected (Halatek et al. 2009). In the same subjects, signs of oxidative stress were also found and both that and the extent of the electrophysiological alterations were correlated to the internal As burden. Some of the functional neurotoxic effects of As have been modelled in animal experiments (Rodríguez et al., 2001; García-Chávez et al., 2007) including earlier works of the Department (Schulz et al., 2002; Szabó et al., 2006).

1.3.2. Manganese

Manganese (Mn) can have oxidation states from -3 to 7+, indicating its propensity to participate in oxidation-reduction reactions. This, along with the tendency to form complexes, has biological and toxicological relevance (Aschner et al., 2007). Manganese and its compounds have had numerous technical applications, starting with mineral MnO_2 used in glassmaking in the middle ages through using it as alloy component in steelmaking since the 19th century up to its present-day high-tech applications such as contrasting agents for MR imaging (Eschenko et al., 2010) and nanostructures for supercapacitors (Chen et al., 2005).

Mn is an essential trace element for plants, animals and humans. The human body contains about 10 mg Mn, stored mainly in the liver and kidneys, and a daily intake of 2-4 mg is sufficient for adults (ATSDR, 2008). Mn is required for normal brain function in all mammals (Erikson and Aschner, 2003; Keen et al., 2000). It is a cofactor for several enzymes such as Mn-dependent glutamine synthetase (localized in astrocytes and involved in the turnover of glutamate as neurotransmitter) superoxide dismutase (protecting mitochondria against oxidative stress), as well as arginase or pyruvate carboxylase (Erikson and Aschner, 2003;

Normandin and Hazell, 2002). Despite its essentiality, Mn in high amounts will be toxic, and the nervous system is an important target of Mn toxicity (ATSDR, 2008).

The population can be exposed to Mn in several ways when there is an elevated level of Mn in the soil, and therefore in plants, food (cereals) and drinking water. Typical neurotoxic manifestations are rare, occurring only where the Mn content of drinking water is abnormally high (e.g. in Greece, naturally: Kondakis et al., 1989; in the USA, possibly due to pollution: Woolf et al., 2002; or in Japan, due to pollution: Kawamura et al., 1941). A Mn-based anti-knock petrol additive, methylcyclopentadienyl Mn tricarbonyl (MMT), had been developed to replace tetraethyl lead. It was in use in a few countries but has been withdrawn due to the risk of population-level inhalational exposure by the exhaust gases (Davis, 1998).

Occupational Mn exposure is primarily inhalational, and occurs mostly in the metal industry (Mn ore mining and smelting, production of alloys, welding by use of rods with a Mn-containing coating) but also in the manufacturing of zinc-carbon and alkaline dry cells (ATSDR, 2008; Bader et al., 1999). Two Mn-based fungicides (Maneb, Manzoceb) also may cause job-related exposure (Ferraz et al., 1988). Chronic nervous system damage by Mn was first described 150 years ago in workers who, grinding black oxide of Mn (MnO_2, pyrolusite), developed muscular weakness and unsteady gait (Couper, 1837). Today we know that Mn can disturb neurotransmission (glutamatergic, cholinergic, dopaminergic, GABAergic), can cause mitochondrial dysfunction and cellular energy shortage, and can inhibit voltage-gated Ca-channels in neurons (Aschner et al., 2009). Mn induces oxidative stress via the oxidation of dopamine and other catecholamines, as Mn is accumulated in dopamine-rich brain regions, especially in the basal ganglia (Erikson et al., 2004); and by mitochondrial damage (inhibition of complex II: Malecki, 2001; and complex III: Zhang et al., 2003). Also, the activity of Mn-SOD and glutathion peroxidase is decreased if there is a local overdose of Mn (Hamai and Bondy, 2004).

Oxidative stress is involved in the toxicity, including neurotoxicity, of Mn (Aschner, 1997). The ability of Mn-containing welding fumes to induce oxidative stress was proven in vivo (inflammation markers in the bronchoalveolar lavage fluid) and in vitro (depletion of glutathione) by McNeilly et al. (2004). In rats after one month of oral Mn exposure, increased lipid peroxidation and decreased motility was seen (Avila et al., 2008); and the manifestations of oxidative stress in Mn-exposed rats could be reduced by the natural antioxidant, silymarin (Chtourou et al., 2010). Preliminary results of earlier experiments at the Department also indicated that antioxidative substances may reduce the functional alterations in Mn-exposed rats (Nagy et al., 2011).

The typical human neurological disorder caused by Mn, manganism, represents a Parkinson-like syndrome (Normandin et al., 2004) although the two are partly dissimilar. Disorders with electrophysiological signs after Mn exposure include myoclonus in welders (Ono et al., 2002) and epileptic activity in an accidentally exposed child (Hernandez et al., 2003). In young shipyard workers, EEG and visual evoked potential alterations were observed (Halatek et al., 2005). These subjects had blood Mn levels up to 14 μg/L (compared to 5-7 μg/L in reference groups: Bader et al., 1999). EEG and evoked potential disturbances following occupational Mn exposure were also reported by Sinczuk-Walczak et al. (2001) and Sjögren et al. (1996), and were modelled in animals, among others in works done at the Department (Pecze et al., 2004; Vezér et al., 2005).

The mentioned inhalational Mn exposure in workplace settings is due to the presence of aerosol particles containing Mn. It has been discovered relatively recently that nanoparticles (NPs, with at least one dimension smaller than 100 nm, also called ultrafine dust or submicron particles) are of especial concern in terms of health hazard. NPs are supposed to be involved in various chronic non-communicable diseases of humans affecting the central nervous system, the circulatory system and other body parts (Buzea et al., 2007).

Welding fumes are rich in Mn-containing NPs (Antonini, 2003). These not only form an extremely stable aerosol (not removed by sedimentation for days) but have several characteristics in their interactions with living organisms which are not seen with particles in the micrometer range (Oberdörster et al., 2005). Inhaled NPs are either deposited in the nasopharynx or get down to the alveoli (ICRP, 1994). Their entrance into and mobility within the organism is facilitated by their small size, enabling them to easily penetrate physiological barriers like the alveolar, capillary or even blood-brain barrier (Kreyling et al. 2006). After nasal deposition, they can directly reach the CNS by translocation through the olfactory pathway (Elder et al., 2006) and they are less efficiently removed than larger particles by the macrophage clearance mechanisms. Their huge (relative to particle mass) and reactive surface induces generation of ROS, and as a consequence, pro-inflammatory mediators (Stone et al., 2007). The relationship between Mn exposure and neurologic-neuropsychiatric dysfunctions has been established both in massive workplace exposures (Bowler et al., 2007) and in chronic low-level exposure among workers and in the general public (Lucchini et al., 2007, 2009). Motor and olfactory dysfunction, as well as oppositional and hyperactive behavior plus IQ drop, was observed among schoolchildren exposed to high environmental concentration of Mn (Michalke and Fernsnebner, 2014).

1.4. Antioxidants used in this work

1.4.1. Ascorbic acid

Vitamin C (ascorbic acid) is a hexose derivative. It is a vitamin (essential factor) only for humans, other primates, and a few more mammalian species while all other living creatures are able to synthesize it. At physiological pH, ascorbate monoanion is the dominant form and acts as effective antioxidant, that is, electron donor. It shows an auto-oxidation tendency which is strongly pH dependent and is accelerated by catalytic metals (Du et al., 2012). Ascorbic acid is able to scavenge reactive oxygen species such as superoxide radical anion, singlet oxygen and hydroxyl radical (Kim and Lee, 2004). It can improve mitochondrial functions by neutralizing the above mentioned byproducts of terminal oxidation, and can so prevent ROS mediated damage to liver and kidney; and is capable of protecting membrane constituent lipids. Ascorbic acid also improves GSH status by recycling oxidized glutathione (Valko et al., 2007), and it has been reported that ascorbic acid was protective against depletion of GSH and cellular damage in As-exposed rats (Singh and Rana, 2010). Its oxidized forms, ascorbate radical and dehydroascorbate, generated in the reactions described above, are regenerated by several mechanisms which help to maintain the cellular pool of this valuable substance (Du et al., 2012). Intestinal uptake, by Na^+-dependent vitamin C transporters (SVCT) for the intact molecule and by glucose transporters for the fully oxidized form (dehydroascorbate), is limited and apparently depends on the actual need (Du et al., 2012). Uptake into the brain is also an active (SVCT2-dependent) process (Harrison and May, 2009). Depending on the immediate chemical environment, ascorbate can also have pro-oxidant effect – first of all if certain transition metals are present, because it can reduce e.g. Fe^{3+} to Fe^{2+} in a one-electron transition. Fe^{2+} can readily react with O_2, reducing it to superoxide radical, or can react with H_2O_2 to generate Fe^{3+} and oxidizing hydroxyl radical (Fenton reaction). Ascorbate allows recycling of Fe^{3+} back to Fe^{2+}, promoting the rise of highly reactive oxidants (Du et al., 2012).

Vitamin C is a well-known and well-described natural substance, and is easily accessible for the public. It is present in various foods and drinks – typically those of fresh plant (citrus fruits, green pepper etc.) origin – and is commonly recognized as a leading natural nutrient and antioxidant.

1.4.2. Rutin

Rutin (quercetin-3-rutinoside) is a flavonoid present in various in plants. The number one source of natural rutin is buckwheat (*Fagopyrum esculentum*; Kim et al., 2005). Citrus fruits and rhubarb are also rich in rutin (Formica and Regelson, 1995). The relevant part of the rutin molecule in terms of antioxidant effect is quercetin, the aglycone, a flavonol. Similarly to other flavonoids, it acts both as free radical scavenger and as chelator for metals prone to act as pro-oxidants (e.g. iron and copper; Kim and Jang, 2009). Health benefits and pharmacological properties ascribed to rutin include antioxidative, anti-inflammatory, antiallergic, antiviral, anticarcinogenic and anti-hypertensive effects. Rutin is also used in animal feed, cosmetics, as a natural colorant, food preservative, and UV absorbent (Javed et al., 2012). It is also known, however, that bioavailability of rutin – or, more exactly, of quercetin – is limited due to poor intestinal absorption and to degradation by the gut microflora both in humans and in experimental animals (Formica and Regelson, 1995). The neuroprotective effect of rutin has been documented in animal models of brain ischemia (Gomes-Rodrigues et al., 2013) and Alzheimer disease (Javed et al., 2012).

1.4.3. Green tea phenolics

In the weight of fresh leaves of the tea shrub, *Camellia sinensis*, ca. 35–40% are given by flavonoids, first of all catechins (Hodgson, 2006). Green tea is prepared by brewing the harvested but not or minimally fermented leaves. No fermentation means that the leaves' natural phenol oxydases are inactivated by heat treatment, instead of letting them work as in processing of black tea, so that green tea leaves and the drink made from them is rich in flavonoids. Tea is a popular drink worldwide and is a major contributor to total flavonoid intake in many populations. The neuroprotective effect of catechins is based on antioxidant and metal chelating activity; but beyond acting directly as radical scavengers, they also activate transcription factors and antioxidant enzymes. Regarding the prevalence of chronic degenerative central nervous diseases and the popularity of tea, this protective effect has been extensively studied (Weinreb et al., 2004) The major component of green tea, (–)-epigallocatechin-3-gallate (EGCG), is held responsible for the – as yet only partially proven – beneficial effects of green tea such as anticancer, antioxidant and cardiovascular protective function improvement via anti-inflammatory properties (Mandel et al., 2005).

1.4.4. Curcumin

Curcumin (diferuloylmethane) is a yellow substance was isolated from the rhizomes of turmeric (*Curcuma longa*). It has been extensively used in food preparation as spice, preservative and coloring agent in different parts of the world. Curcumin has multiple pharmacological properties such as anti-inflammatory, anti-carcinogenic, anti-mutagenic, anti-ischemic, hypotensive and antioxidant effects, and also has been found effective in animal models of Alzheimer's dementia and chemically induced neurotoxicity (Yadav et al., 2009). Curcumin is a powerful scavenger of $O_2^{\bullet-}$, $^{\bullet}OH$ and NO_2, and has metal binding property (El-Demerdash et al., 2009). Being lipophylic, curcumin easily crosses the blood brain barrier and reduces amyloid deposition in vivo and in vitro (Yadav et al., 2011). Co-administration of curcumin diminished lipid peroxidation in rats subacutely treated with Cd and Pb, and the hippocampal neuronal damage caused by the latter (Daniel et al., 2004).

1.5. Summary and Aims

Exposure to metals and metalloids remains a source of health damage for the population, let the source of exposure be natural or technical, and the exposure situation, job related or general. It follows from the chemical properties of arsenic and manganese, and from the reactions of the cell constituents and biomolecules of the exposed organism, that oxidative stress plays a significant role in the mechanism of metal-induced damages, especially in the nervous system which has limited capacity to counteract an oxidative attack. By supporting antioxidant defence via supplying the exposed organism with exogenous antioxidants, metal-induced nervous system damages could possibly be diminished. Based on that, the general aim of this thesis was to observe, in experiments done in subacutely treated rats, the neuro-functional damage caused by exposure to arsenic and manganese (in doing that, we could rely on previous results of the Department: Szabó et al., 2006; Vezér et al., 2007; Oszlánczi et al., 2010), and the possible protective effect of certain antioxidants of natural origin.

The particular questions to be answered on the basis of the expected results were as follows:

- Can the neurotoxic, and other toxic, effects of the used physicochemical form of As and Mn be investigated in identically built-up experiments or together?
- Have the antioxidants included in the experiments any effect on the alterations induced by the two metals?
- What differences, qualitative or quantitative, can be seen between the effects of the antioxidants?
- Which of the antioxidants might be an optimal choice, considering also protection of human health?

2. MATERIALS AND METHODS

The aims described in 1.5. have been realized in altogether three experiments. The two environmental heavy metals given to the rats were manganese and arsenic; and the natural antioxidants, as putative protecting agents, were ascorbic acid (vitamin C), rutin, curcumin, and green tea infusion (Table 1). Details of the particular experiments (treatment groups, doses, administration, combinations, etc.) will be given later on.

Table 1 Schemes of treatments

Experiments	Experiment 1	Experiment 2	Experiment 3
Duration	4 weeks of treatment with MnO_2 nanoparticles (or vehicle) + 1 week of treatment with antioxidants (ascorbic acid, curcumin, rutin or their vehicles)	6 weeks of simultaneous treatment with $NaAsO_2$ and antioxidants (ascorbic acid, green tea)	6 weeks of simultaneous treatment with $NaAsO_2$ or MnO_2 nanoparticles and antioxidants (ascorbic acid, green tea, rutin)
Investiga-tions	• Body weight • Organ weight • Open field test • Electrophysiology	• Body weight • Food and water consumption • Organ weight • Open field test • Electrophysiology • Biochemical measurements • Tissue metal level	• Body weight • Food and water consumption • Organ weight • Open field test • Electrophysiology • Biochemical measurements • Tissue metal level

2.1. Experimental animals, substances and ways of administration

Young adult, ca. 6 weeks old, male Wistar rats were used for all experiments, obtained from Toxi-Coop (Budapest, Hungary). The animals were housed with three or four rats in one cage (polypropylene, floor 27 x 39 cm, height 19 cm) under GLP equivalent conditions (22±1ºC, 40-60% relative humidity, 12-h light/dark cycle with light on at 06:00), and had free access to standard rodent pellet and drinking fluid (plain tapwater or a treatment solution, see Tables 2, 3 and 4).

For Experiment 1, MnO_2 NPs were produced at the Department of Physical Chemistry and Materials Science, University of Szeged Faculty of Science and Informatics. Synthesis was done in wet reaction in aqueous alkaline medium containing polyacrylic acid (PAA; MW 5000) and ethanol (as reducing agent). $KMnO_4$ solution was dripped into this medium under

stirring, and a sol containing MnO_2 NPs of 25-30 nm diameter was generated. It was administered to rats after adjustment of concentration and pH. Vehicle control animals got the starting medium which was completed with KOH and NaOH, and pH was set to 7.5. The chemical purity of the nanoparticles was checked by X-ray diffraction, and their particle size, by X-ray diffraction and transmission electron microscopy.

For Experiment 3, the MnO_2 NPs were synthesized at the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics. Aqueous $KMnO_4$ solution was mixed with ethylene glycol and sonicated with an ultrasound device. The resulting dark suspension was heated at 200 ºC for 16 hour in a Teflon-lined autoclave oven and then allowed to cool to room temperature naturally. The brownish precipitate formed was filtered and washed with 80 ºC preheated distilled water, and dried at 100 ºC for 1 hour. Chemical purity and particle size (25-30 nm) were checked as above. For administration, the MnO_2 NPs were suspended in 1% hydroxyethyl cellulose (HEC) dissolved in phosphate buffered saline (PBS; pH=7.4). This vehicle slowed the aggregation of the NPs. The suspension was intensively sonicated as it was made, and again before each administration.

The applied dose of MnO_2 NPs was, both in Experiment 1 and 3, 4 mg/kg b.w., instilled intratracheally (it.), and the administration volume was 1 ml/kg b.w. The vehicles of MnO_2 NPs in the two experiments were not the same, but both were toxicologically inert and could be safely applied. PAA is often used in biomedical applications like immunological studies, drug delivery, or enzyme immobilization; it is even a potential candidate as vaccine component (Topuzogullari et al., 2013).

For treatment of the rats with arsenic, crystalline $NaAsO_2$ was purchased from Sigma Aldrich, Hungary. This inorganic form of As was dissolved in distilled water (8.67 or 4.34 mg/mL to obtain 10 or 5 mg/mL As level, respectively) and was administered by gavage (2 mL/kg b.w.).

Vitamin C (ascorbic acid) in solid crystalline form was purchased from the Central Pharmacy of the University of Szeged; while rutin powder (rutin hydrate) and curcumin was bought from Sigma Aldrich, Hungary. The green tea used was a commercially available kind (Chunmee China Green Tea 9366, non-fermented, Fujian Tea Import & Export Company Ltd, China).

For administration to the rats, antioxidants were formulated in different ways in the experiments. In Experiment 1, the antioxidants were given by gavage once a day for 1 week; ascorbic acid was dissolved in distilled water while rutin and curcumin were dissolved in sunflower oil. The dose of each antioxidant was 100 mg/kg, given in 1 ml/kg b.w volume. In Experiments 2 and 3, antioxidants were administered via drinking water for 6 weeks. This

way, a more natural route of administration was attempted, and use of oil as vehicle, requiring a vehicle control group, was avoided. For dissolving vitamin C (to 1 g/L [Exp. 2] and 2g/L [Exp. 3] in drinking water) tapwater was boiled and cooled to room temperature to eliminate dissolved chlorine and oxygen. The pH was set to 7.5 by adding $NaHCO_3$ (in 540 [Exp. 2] or 1080 [Exp. 3] mg/L amount) to diminish breakdown of ascorbic acid (which is strongly pH-dependent with ca. 10 times faster decay at around pH 5 than at physiological pH: Golubitskii et al., 2007). In Experiment 3, for complete dissolution of rutin, the powdered substance was added to the prepared tapwater (1 g/L) under moderate stirring, then $NaHCO_3$ was added until pH≈9.8 where the suspended rutin particles completely dissolved leaving a clear solution. Finally 20% acetic acid (food grade) was added to bring back pH to 7.8 where rutin remained in solution and pH value was not far from neutral. Tea polyphenols, as antioxidants, were used in Experiments 2 and 3, administered via drinking water. Green tea infusion was prepared by brewing 2.5 g tea leaves in 500 ml boiled tapwater. After 10 minutes, the infusion was filtered and was cooled to room temperature.

The ways of administration used in the experiments were oral (by gavage or via drinking water) and intratracheal (it.). For gavage, a slightly bent thin, polished glass tube was attached to a 1 ml syringe. The rats were held firmly by the neck skin under the ears, and the tube was lowered to the stomach as soon as possible without damage of the throat and oesophagus. Due to the low level of discomfort caused to the rats, treatment by gavage needed no anesthesia.

For it. instillation – imitating inhalation of workplace metal fumes, the tyipcal way of NP exposure – rats needed a short anesthesia with diethyl ether, achieved in a glass jar with air-tight lid, saturated with ether vapor. The completely anesthetized rat was suspended on a board tilted to 60º from horizontal, by hanging its upper incisors in a wire loop. Keeping this way the rat in place and its mouth open, the trachea was illuminated transdermally by means of a fibre optic light guide through the animal's neck. The tongue was pulled forward with a pair of non-traumatic forceps, and a custom-made laryngoscope was used to gain access to the glottis. The nanoparticle suspension was instilled into the trachea by means of a 1 ml syringe and 1.2 mm diameter plastic tubing, inserted between the vocal chords. Before taking up the materials, an equal quantity of air was drawn into the syringe and was pushed out after the suspension to assure that the whole amount was emptied from the syringe and tube and delivered into the trachea (Oka et al., 2006). Treatment was performed under an exhaust hood to remove ether vapors.

2.2. Experiments

Experiment 1: Subacute intratracheal nano-manganese treatment followed by 1-week antioxidant administration

During the experiment, 4 mg/kg b.w. MnO_2 was administered by intratracheal instillation. Instillation was done once a day, 5 days a week for 4 weeks. Then, an open field (OF) test was done, and one of three antioxidants – vitamin C (ascorbic acid), curcumin, and rutin – was administered orally by gavage for further 1 week to see if they can influence, or counteract, the effects of MnO_2 NPs (groups *Mn* [without antioxidant]*, MnC, MnK, MnR;* see Table 2). After the 5th week, another OF test was done to see the effect of antioxidants.

Table 2 Description of Experiment 1

Body weight at start		210-230 g	
Duration		4 weeks of treatment with MnO_2 (or vehicle) +	1 week of treatment with antioxidants (or vehicle)
Groups and group codes		**Substance, dose, application**	
		Week 1-4	**Week 5**
Con	Untreated control	-	-
VCon	Vehicle control	PAA, 1 ml/kg b. w., it.	-
OCon	Vehicle control, oil	PAA, 1 ml/kg b. w., it.	Sunflower oil, 1 ml/kg b. w., po. by gavage
VitC	Ascorbic acid	PAA, 1 ml/kg b. w., it.	Ascorbic acid, 100 mg/kg b.w. per os by gavage
Kur	Curcumin	PAA, 1 ml/kg b. w., it.	Curcumin, 100 mg/kg b.w., po. by gavage
Rut	Rutin	PAA, 1 ml/kg b. w., it.	Rutin, 100 mg/kg b.w., po. by gavage
Mn	Manganese nanoparticles	MnO_2, 4 mg/kg b.w. in PAA, it.	-
MnC	Manganese nanoparticles + ascorbic acid	MnO_2, 4 mg/kg b.w. in PAA, it.	Ascorbic acid, 100 mg/kg b.w. po. by gavage
MnK	Manganese nanoparticles + curcumin	MnO_2, 4 mg/kg b.w. in PAA, it.	Curcumin, 100 mg/kg b.w., po. by gavage
MnR	Manganese nanoparticles + rutin	MnO_2, 4 mg/kg b.w. in PAA, it.	Rutin, 100 mg/kg b.w., po. by gavage
Investigations		• Continuous: Body weight measurement • End of 4th and 5th week: open field test • Final: Body weight measurement, electrophysiological recording	

Control group consumed clear tap water (with 30 μg/L manganese content as stated by the municipal waterworks) for 5 weeks (*Con*). For vehicle control groups and parallel antioxidant control groups, see Table 2. Treated groups consisted of 8 animals at start while in the control group there were 6 rats. Body weight change was measured daily during the whole experiment. After the whole administration period, electrophysiological investigations were done and organ weights of the animals were measured.

Experiment 2: Subchronic oral arsenic treatment combined with antioxidants

The scheme of the experiment is seen in Table 3. There were 8 groups of rats with 10 animals each at start and the treatment period lasted 6 weeks. Arsenic was given to the rats by gavage, to model drinking water borne exposure; and a more realistic way of antioxidant treatment, via drinking water, mimicking human uptake from foods and drinks, was used. So in this experiment all the animals were treated simultaneously per os by gavage and via drinking water to see the continuous counterbalancing effect of antioxidants to the effect of As.

Table 3 Description of Experiment 2

Body weight at start		200-220 g	
Duration		6 weeks of simultaneous treatment with $NaAsO_2$ (or vehicle) and antioxidants (or vehicle)	
Group codes and group name		**Substance, dose, application**	
		Po. by gavage	**Via drinking water**
Con	Control	Distilled water	Tapwater
VitC	Ascorbic acid	Distilled water	Ascorbic acid, 1000 mg/L
Tea	Green tea	Distilled water	Green tea, 2.5 g tea leaves brewed in 500 ml water
AsL	Low dose arsenic	$NaAsO_2$, 5 mg/kg b.w.	Tapwater
AsH	High dose arsenic	$NaAsO_2$, 10 mg/kg b.w.	Tapwater
AsLC	Low dose arsenic + ascorbic acid	$NaAsO_2$, 5 mg/kg b.w.	Ascorbic acid, 1000 mg/L
AsHC	High dose arsenic + ascorbic acid	$NaAsO_2$, 10 mg/kg b.w.	Ascorbic acid, 1000 mg/L
AsHT	High dose arsenic + green tea	$NaAsO_2$, 10 mg/kg b.w.	Green tea, 2.5 g tea leaves brewed in 500 ml water
Investigations		• Continuous: Body weight, food and water consumption • Final: Open field test, electrophysiological recording, organ weight and tissue As level measurements, measurement of oxidative stress indicators	

Ascorbic acid solution and green tea brew were given via the drinking fluid (groups *VitC* and *Tea*); these rats were gavaged with distilled water concurrently. Control rats (*Con*) received plain tapwater for drinking (with 7 µg/L arsenic content as stated by the municipal waterworks) and distilled water by gavage. 10 mg/kg b.w. (*AsH*) and 5 mg/kg b.w. (*AsL*) $NaAsO_2$ was given once a day for 5 days a week. In groups *AsLC, AsHC* and *AsHT* arsenic treatment was combined with administration of the antioxidants. The rats received fresh antioxidant solution every two days (measurements showed that antioxidant activity was not lost during this period).

Experiment 3: Subchronic per os arsenic and intratracheal manganese treatment combined with antioxidants

In this experiment per os arsenic (*As*) and intratracheal manganese (*Mn*) treatment were both used, combined with antioxidants (ascorbic acid [*AsC, MnC*], green tea [*AsT, MnT*] and rutin [*AsR, MnR*]) administered via drinking water simultaneously. In this experiment, only those antioxidants were included which were proven effective in the previous experiments (see Results, 3.1. and 3.2.). There were 11 groups of rats with 6 (control groups: *VitC, Tea, Rutin*) or 10 (treated groups) animals each at start. The treatment period lasted for 6 weeks. Open field test was carried out before and after the treatment period. All along the administration period body weight and food/water consumption was measured daily. Electrophysiological investigation was done after the treatment period. Finally organ weights, tissue arsenic levels and oxidative stress indicators were measured. The treatment scheme is given in Table 4.

2.3. General toxicological investigations

Observations during the treatment period: The rats' body weight was registered, as well as their general health state observed, daily in all experiments. Food and water consumption were also measured in Experiment 2 and 3. Body weight data were used to determine the daily dose of directly (gavage or instillation, see above) administered substances for each rat, and to graphically demonstrate the effects on weight gain. Drinking fluid consumption was measured during the experiment to calculate the antioxidant intake. Besides, any signs of toxicity (e.g. rough fur, hunched back, unusual aggressive behavior) were observed and noted. Final observations: After all behavioral and electrophysiological recordings (see below) had been done, the rats were sacrificed by an overdose of urethane, and were dissected. The animals were transcardially perfused with 500 ml PBS to remove blood from the organs.

Table 4 Description of Experiment 3

Body weight at start		200-220 g	
Duration		6 weeks simultaneous treatment with $NaAsO_2$ or MnO_2 and antioxidants	
Group codes	**Substance(s)**	**Dose**	**Application**
VitC	**Ascorbic acid**	2000 mg/L in drinking w.	Via drinking water
Tea	**Green tea**	2.5 g tea in 500 ml drinking w.	Via drinking water
Rutin	**Rutin**	1000 mg/L in drinking w.	Via drinking water
As	**Arsenic**	10 mg/kg b.w. $NaAsO_2$ in distilled w.	Per os by gavage
AsC	**Arsenic + ascorbic acid**	10 mg/kg b.w. $NaAsO_2$ in distilled w. + 2000 mg/L in drinking w.	Per os by gavage + Via drinking water
AsT	**Arsenic + green tea**	10 mg/kg b.w. $NaAsO_2$ in distilled w. + 2.5 g tea in 500 ml drinking w.	Per os by gavage + Via drinking water
AsR	**Arsenic + rutin**	10 mg/kg b.w. $NaAsO_2$ in distilled w. + 1000 mg/L in drinking w.	Per os by gavage + Via drinking water
Mn	**Manganese nanoparticles**	4 mg/kg b.w. in viscous m.	Instilled it.
MnC	**Manganese nanoparticles + ascorbic acid**	4 mg/kg b.w. in viscous m. + 2000 mg/L in drinking w.	Instilled it. + Via drinking water
MnT	**Manganese nanoparticles + green tea**	4 mg/kg b.w. in viscous m. + 2.5 g tea in 500 ml drinking w.	Instilled it. + Via drinking water
MnR	**Manganese nanoparticles + rutin**	4 mg/kg b.w. in viscous m. + 1000 mg/L in drinking w.	Instilled it. + Via drinking water
Investigations		• Continuous: Body weight, food and water consumption • Before and after the treatment period: Open field test • Final: Open field test, electrophysiological recording, organ weight and tissue As level measurements, measurement of oxidative stress indicators	

Organs were removed and weighed, and the relative organ weight (a generally used indicator of toxicity) of the brain, liver, lungs, heart, kidneys, spleen, thymus and adrenal glands was calculated, related to 1/100 of body weight or to the brain weight. Due to the effect of the treatments on body weight, brain-based relative organ weights were mostly the more reliable

(in accordance with Schärer, 1977). During dissection, abnormalities were searched for and noted.

2.4. Chemical and biochemical measurements

For metal level determination and biochemical measurements, tissue samples were taken from only 3 randomly chosen rats per group, due to financial issues. Whole brain, as well as liver, kidneys, and 2-3 ml of red blood cells (RBCs, separated from the freshly drawn heparinised blood by centrifugation) were shock-frozen in liquid nitrogen, and stored at -20°C.

To determine heavy metal concentration, the blood and tissue samples were dried at 80°C to constant weight, and were digested in 65% HNO_3 at 90°C for 90 min (4 mL acid per gram wet tissue), and the digested matter was diluted as needed with distilled water. Measurement was done by inductively coupled plasma mass spectrometry (Pröfrock and Prange, 2012) at the Department of Inorganic and Analytical Chemistry, University of Szeged Faculty of Science and Informatics. In Experiment 2, As level was determined from cerebral cortex, RBCs, liver and kidneys in groups *Con*, *AsH, AsHC* and *AsHT*. In Experiment 3, As and Mn level measurement was done from the same animals taken form control (that is, receiving an antioxidant only) and metal-treated groups.

Biochemical measurements were done at the Department of Biochemistry and Molecular Biology, University of Szeged Faculty of Science and Informatics. The indicators of oxidative stress were determined from cerebral cortex and RBCs, from the same samples as metal level were, in Experiment 2 and 3.

Protein content (for calculation basis) was measured – after haemolysing of the RBCs, homogenizing the brain samples, and diluting them as appropriate – by the method of Lowry et al. (1951). The primary oxidative insult was assessed by measuring the ROS hydrogen peroxide (H_2O_2; method by Villegas and Gilliland, 1998) and RNS peroxynitrite anion ($ONOO^-$; method by Huie and Padmaja, 1993). The level of thiobarbituric acid-reactive substances (TBARS) is regarded as an appropriate indicator of lipid peroxidation caused by toxic metals (Nogueira et al., 2003). The TBARS assay used was published by Serbinova et al. (1992).

The antioxidant power of the drinking fluids was determined by their ferric reducing capacity, using the modified method of Kampfenkel et al. (1995), based on deteection of Fe^{2+} ions by α,α'-dipyridyl. For calibration, a series of L-ascorbic acid solutions was used, and the antioxidant power was expressed in ascorbic acid equivalents.

2.5. Behavioral investigation: open field test

The rats' spontaneous motility was tested in an open field (OF) apparatus (Conducta 1.3 System; Experimetria Ltd., Budapest). This is a standard method to test spontaneous, exploratory motor activity in experimental animals and its changes upon action of exogenous chemicals (Pryor et al. 1983), also included in the OECD guideline for neurotoxicity testing (OECD, 2004). The OF test was usually done on the day following the last treatment (in Experiment 1, also one week before the end of treatment period).

After 20-30 min adaptation in the test room, the animals were put into the centre of the box one by one for a 10 min session. This box was 48x48x40 cm size, equipped with two arrays of infrared beam gates at floor level and at 12 cm height. From the beam interruptions, event counts and summed time of the basic activity forms (ambulation, local activity, rearing, immobility), as well as run length of ambulation, were computed using the following criteria: more than 40 mm shift in the location of interrupted beams at the floor level (i.e., the location of the animal) during a time unit of 1 s was interpreted as horizontal activity; less shift, as local activity; and no shift at all, as immobility. Rearing was recorded if beams at floor level and at the higher level were interrupted simultaneously. This test protocol has been successfully used in the investigation of heavy metal effects on the rats' motor behavior (Vezér et al., 2007). It was also calculated how much of the total session time the rats spent in the corner, side and central zones of the OF box (the whole box area was divided to 9 equal quadratic subfields; the 4 subfields with contact to two adjacent walls were defined as "corner zones", the 4 with one wall contact as "side zones" and the remaining one was the "central zone").

2.6. Electrophysiological investigation

2.6.1. Preparation

The electrophysiological measurement was done on the day following the last open field test. The rats were anesthetized by intraperitoneal injection of 1000 mg/kg b.w. urethane (Maggi and Meli, 1986). The efficiency of the anesthesia was checked by toe pinching; if there was no movement, the animal was ready for surgery.

The head was fixed in a holder frame, the skin on the skull was opened by a mid-sagittal cut, and the muscles and connective tissues adhering to the top face of the skull were removed.

Finally the left hemisphere was exposed by removing the temporal bone along the inner circumference by means of a mini drill. Wounds were sprayed with 10 % lidocaine and the exposed cortex was covered with a thin layer of petroleum jelly. The animals were wrapped in a warm cloth to maintain body temperature. After at least 30 min recovery, the rat was placed into the stereotaxic frame of the electrophysiological apparatus. During the measurement, the animals' body temperature was stabilized by a thermostated (+36.5^{o}C) base plate.

Ball-tipped silver recording electrodes were positioned on the dura over the primary somatosensory (SS) projection area of the whisker pad (barrel field), and over the primary visual (VIS) and auditory (AUD) area. These regions were determined on the base of a somatotopic map (Zilles, 1984). A stainless steel clip was attached to the cut skin edge as an indifferent electrode. SS stimulation was done by a pair of needles inserted into the contralateral whiskery part of the nasal skin, delivering square electric pulses. VIS stimulation was performed by flashes from a high-luminance white LED, positioned to the contralateral eye of the rat. For acoustic stimulation, sound clicks were applied into the ear of the rat through the hollow ear bar of the stereotaxic frame.

To record compound action potential of the tail nerve, a pair of stimulating needle electrodes was inserted at the base of tail (delivering similar electric stimuli as used to stimulate the whiskers), and the compound action potentials were recorded distally by another pair of needles at a distance of 50 mm.

2.6.2. Recording and evaluation

The recording sequence started with six minutes recording of spontaneous activity (electrocorticogram, ECoG) from the three sensory cortical areas. From the ECoG records, the relative spectral power by 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 by the software used. To describe the basal activity of the cortex with a single value, ECoG-index was calculated (Dési and Nagymajtényi, 1999) which is the power ratio of the slow and the fast waves (ECoG index = [delta + theta] / [beta1 + beta2]).

Evoked potentials (EPs) were recorded from the same cortical areas via the same surface electrodes. All stimuli were set and applied as of just supramaximal strength (meaning that, e.g., the stimulus voltage was increased until the evoked response reached maximal amplitude and ca. 5% was added) and well above background. Electrical stimulation of the whiskers (and the base of tail, see below) was done by delivering rectangular electric stimuli (3-4 V, 0.05 ms). The intensity of the visual stimulation was ca. 60 lux, and that of the auditory

stimuli, 40 dB. Trains of 50 stimuli were applied in all three sensory modalities (SS, VIS, AUD) and the EPs were recorded. The frequency of stimulation was 1 Hz in all modalities, plus 2 and 10 Hz for SS stimulation in order to observe frequency-dependent changes.

For compound action potentials (CAP) of the tail nerve, 10 single stimuli were applied first at 1 Hz rate to determine action potential latency, and at higher rates to see the frequency dependence of the latency and amplitude. Then, double stimuli with different inter-stimulus intervals were given, for refractory period calculation (see below).

The complete recording and evaluation was done by the software NEUROSYS 1.11 (Experimetria Ltd, Hungary). The cortical responses and tail nerve action potential were averaged automatically, and latency and duration of the responses was measured manually by means of screen cursors of the software.

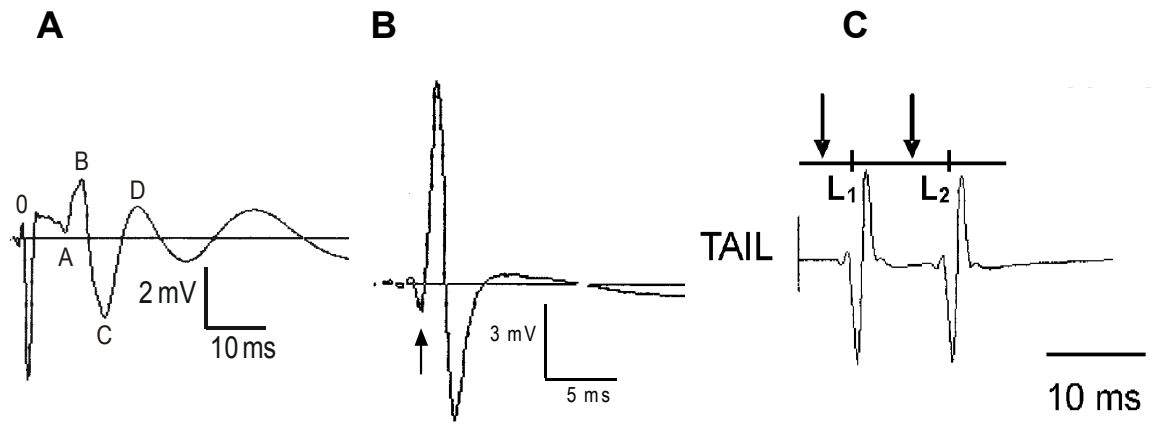

Figure 1. Typical records of evoked electrical responses and explanation of the measurements. **A**, somatosensory evoked potential with the specific measuring points – onset latency was measured between the point marked 0 and A. **B**, compound action potential of the tail nerve – onset latency was measured between the arrow-marked point and the stimulus artefact, and the amplitude, peak-to-peak. **C**, for determining the refractory period, onset latencies of the double action potential were measured between the stimulus onset (arrow) and the points marked L_1 and L_2.

On the somatosensory EP, onset latency was measured between the stimulus artefact (0 in Fig.1A) and onset of the first peak (*A* in Fig.1A). Duration of the EP was calculated as the difference of the *0-D* and *0-A* times. In case of the visual and auditory EPs, onset latency and duration was measured the same way. The tail nerve CAP had also a biphasic shape. There, onset latency was defined as shown in Fig. 1B. Tail nerve conduction velocity was calculated from the onset latency and the distance of electrodes. From double-pulse records (Fig. 1C), relative and absolute refractory periods were determined, based on the relationship of the

latency of the second and first action potential (ratio of L_2/L_1 in Fig. 1C; Dési and Nagymajtényi, 1999).

2.7. Statistical analysis of the data

The distribution of data was checked for normality by means of the Kolmogorov-Smirnov test. Analysis was done by parametric one-way ANOVA using SPSS 17.0. Post hoc analysis of group differences was performed by Scheffe's test, with probability level at $p<0.05$. Linear correlations between tissue metal levels and neurotoxicological parameters were checked by the "linear fit" function of MS Excel.

The procedures used in the experiments were approved by the Ethical Committee for the Protection of Animals in Research of the University (licenses No. XXI./02039/001/2006 and XXI./151/2013). During the whole procedure, the regulations of the Hungarian Act No. XXVIII of year 1998 on protection and care of animals were strictly followed.

3. RESULTS

3.1. Experiment 1: Manganese and three antioxidants

3.1.1. General toxicity

Intratracheal instillation of MnO_2 NPs reduced body weight gain in the treated groups. Regarding that in week 1 to 4 the groups *Mn*, *MnC*, *MnK* and *MnR* had identical treatment, their body weight data for these weeks were lumped up into a grand average (*Mn gr.*) in Fig. 2. By the 4th week, a considerable weight gain deficit developed under the influence on Mn NPs. In the 5th week, this was counteracted minimally by ascorbic aid, a bit more strongly by rutin, but not at all by curcumin, whereas the same antioxidants alone had no effect on body weight (Fig. 2, top panel). The data of weekly body weight gain (Monday to Monday, bottom panel in Fig. 2) show the same effects more clearly.

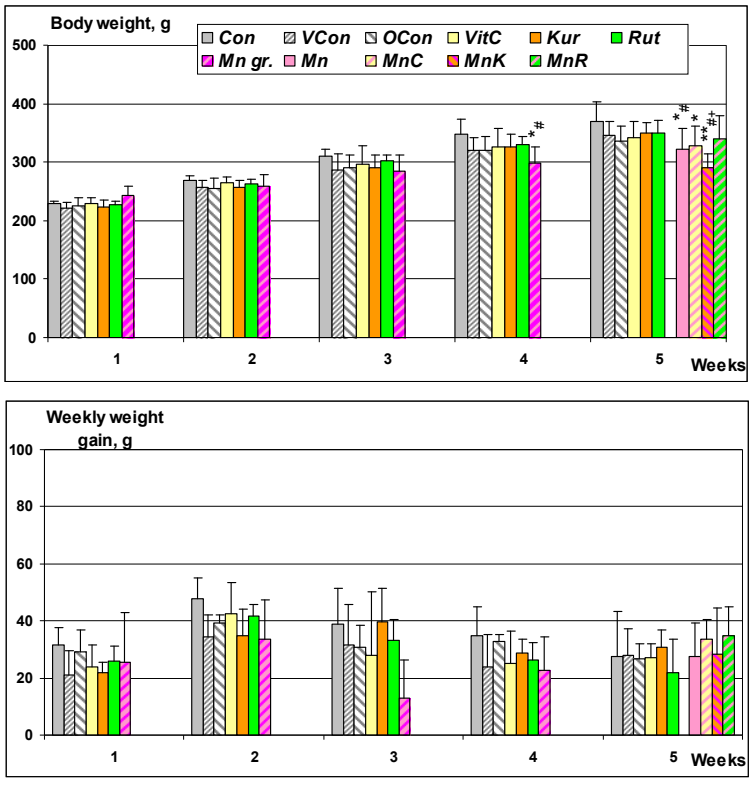

Figure 2 Top: Body weight of the control and treated rats in the course of Experiment 1. Bottom: Weekly body weight gain data of the same rat groups. See insert for group coding. Mean+SD, n=6 (*Con*) or n=8 (all other groups)
*, **: p<0.05, 0.01 s. *Con*; #: p<0.05 vs. *VCon*; +: p<0.05 vs. the same antioxidant alone.

As the final (5th week) body weight was considerably influenced by the treatments, organ weights related to brain weight were chosen for evaluation. Most of the measured organ weights showed no clear trend (Table 5). In all groups instilled with Mn NPs, lung weight increased significantly vs. both *Con* and *VCon*, and vs. the corresponding antioxidant-only groups. There were significant changes also in the relative weight of the adrenals in the combination groups, but there the effect of Mn and the antioxidants could not be clearly separated.

Table 5 Relative organ weights in the control and treated rats in Experiment 1

	Lungs	**Adrenals**
Con	0.8009±0.2421	0.0396±0.0051
VCon	1.0103±0.1620	0.0302±0.0038
OCon	0.9393±0.0869	0.0302±0.0039
VitC	1.0116±0.2048	0.0350±0.0063
Kur	0.9676±0.0662	0.0317±0.0067
Rut	0.9521±0.0839	0.0253±0.0046
Mn	1.5241±0.2393**°°+++	0.0313±0.0044
MnC	1.3187±0.1169***°°##	0.0311±0.0008*°#&
MnK	1.2437±0.0870**°+++###&	0.0392±0.0064***°°°###&&&
MnR	1.4703±0.0973***°°°+++###	0.0402±0.0023**°#&

Mean±SD, n=6 (*Con*) or n=8 (all other groups)
*, **. ***: p<0.05, 0.01, 0.001 vs. *Con*; °, °°, °°°: p<0.05, 0.01, 0.001 vs. *VCon*; +++: p<0.001 vs. *OCon*; #, ##, ###: p<0.05, 0.01, 0.001 vs. the corresponding antioxidant-only group; &, &&&: p<0.05, 0.001 vs. *Mn*.

3.1.2. Effect on open field behavior

The relative share of the four basic activity forms (ambulation, local activity, immobility, rearing) in the total 10 min time of the OF sessions showed that the antioxidants alone – in the 5th week – had no effect on OF motility (Fig. 3). In the Mn NP-treated groups, rearing decreased significantly in the 4th week while both immobility and local activity increased. One week application of the antioxidants (groups *MnC, MnK, MnR*) resulted in nearly complete reversal of Mn effects in case of rutin, but ascorbic acid and curcumin were without effect.

It was also noteworthy that in group *Mn* the changes became more pronounced compared to those in the 4th week even if in the 5th week there was no Mn NP application.

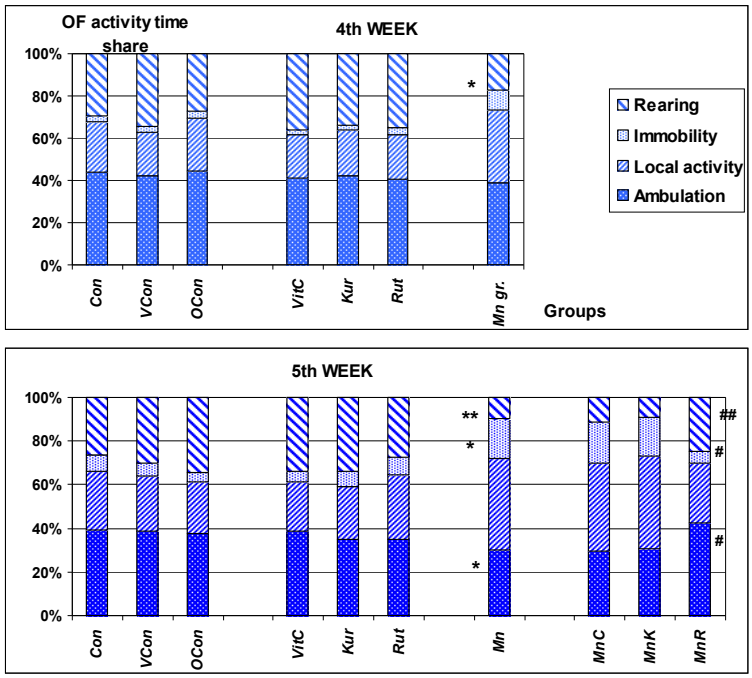

Figure 3 Time share of the OF activity forms in the control and treated rats after the 4th and 5th week of Experiment 1.
*, **: p<0.05, 0.01 vs. *VCon*; #, ##: p<0.05, 0.01 vs. *Mn*.

The changes from the 4th to the 5th week were quantified by calculating the ratio [5th week:4th week] for the OF time data. In all groups without Mn treatment, these ratios indicated the typical age-dependent trend of decreasing motility. In group *Mn*, the decrease of ambulation, and especially rearing, was much more pronounced (significant difference, see Fig. 4). The same calculation in the groups treated with Mn NPs for 4 weeks and an antioxidant for the 5th week verified that rutin abolished the motility-reducing effect of Mn while under ascorbic acid and curcumin the trend towards less and less motion went on (Table 6).

By the zone-based evaluation of OF data (Table 7) it could be shown whether treatment for 4 weeks with Mn and 1 week with an antioxidant influenced the rats' preference to the corners and dispreference to the center of the OF box. The rats' tendency to stay in one of the corner zones and avoid the central zone, an indicator of decreased motivation to explore, was diminished only by rutin.

Table 6 Ratios of the OF time data in the 4th and 5th week.

Groups	Ambulation time	Local activity time	Immobility time	Rearing time
Con	0.8882±0.1809	1.1293±0.2364	2.6698±1.0875	0.8968±0.2782
VitC	0.9349±0.1693	1.1186±0.3768	2.0776±0.8293	0.9361±0.1996
Cur	0.8258±0.1853	1.1124±0.5044	3.1481±1.1404	1.0016±0.3873
Rut	0.8548±0.3249	1.4232±0.1811	2.2580±0.7301	0.7903±0.1533
Mn	0.7742±0.2314	1.2274±0.1819	1.8616±0.2867	0.5709±0.1276
MnC	0.7613±0.3752	1.1746±0.2145	1.9250±1.9185	0.6690±0.7187
MnK	0.7898±0.4195	1.2477±0.3242	1.7990±0.8479	0.5306±0.8570
MnR	1.0896±0.3298	0.8037±0.2976	0.5422±0.2063*#	1.4465±0.4791

Mean±SD, n=6 (*Con*) or n=8 (all other groups).
*: $p<0.05$ vs. *Con*; #: $p<0.005$ vs. *Mn*.

Table 7 Distribution of the presence time of the rats in the various zones of the OF box during the 10 min session.

Groups		Corner zones	Side zones	Central zone
MnC	4th week	0.5223±0.1167	0.3853±0.0799	0.0924±0.1072
	5th week	0.7307±0.1991	0.2375±0.0433*	0.0317±0.0252
MnK	4th week	0.5908±0.1163	0.3349±0.0324	0.0743±0.0453
	5th week	0.6937±0.1527	0.2764±0.0497	0.0299±0.0374*
MnR	4th week	0.4551±0.0559	0.4663±0.0532	0.0786±0.0202
	5th week	0.3889±0.0461	0.5196±0.0533	0.0915±0.0491*

Mean±SD, n=8.
*: $p<0.05$ 5th week vs. 4th week

3.1.3. Electrophysiological effects

In the spontaneous cortical activity (ECoG), the changes caused by Mn NP exposure were similar to those seen in earlier experiments (shift to higher frequencies: Oszlánczi et al., 2010) but were slight and below significance. Hence, any additional effect of the antioxidants could not be identified.

There were, however, marked changes in the parameters of the EPs. Latency of the SS EP (Fig. 4, top panel) was negligibly influenced by the antioxidants alone. Administration of Mn NPs caused significant increase of the latency, which effect was not influenced by curcumin but was to a large extent abolished by both ascorbic acid and rutin. Further, the frequency dependence of the SS EP was more expressed under the effect of Mn and this change was also normalized by the mentioned two antioxidants.

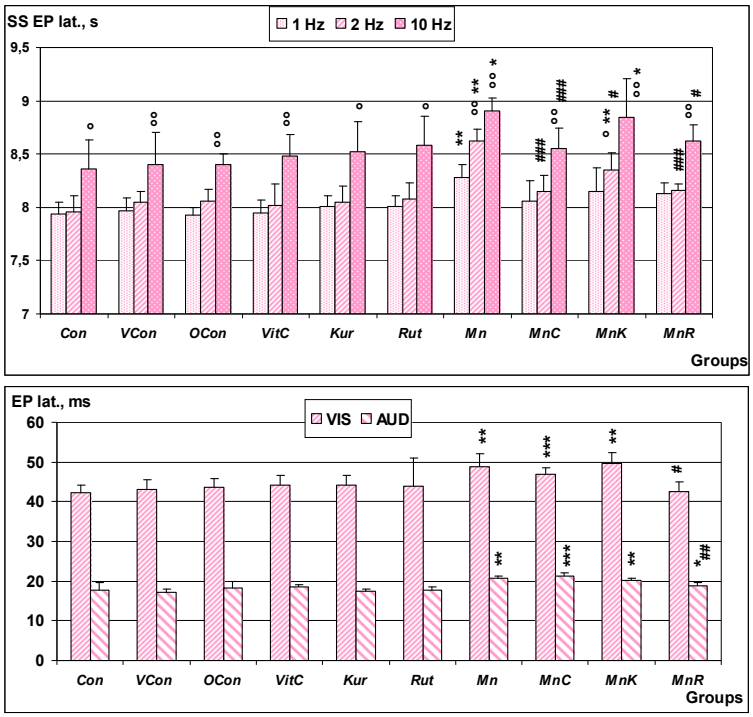

Figure 4 Latency of the somatosensory (top graph) and the visual and auditory (bottom graph) evoked potentials.
Mean+SD, n=6 (*Con*) or n=8 (all other groups)
*, **: p<0.05, 0.01 vs. *Con*; #, ##, ####: 0.05, 0.01, 0.001 vs. *Mn*;
°, °°: p<0.05, 0.01 vs. 1 Hz stimulation in the same group.

The trend of changes in the VIS and AUD responses (Fig. 4, bottom panel) was similar to that seen in the SS EPs, but only rutin had significant effect on the Mn-induced lengthening (totally nullified in the VIS, but only partially in the AUD, EP).

The CAPs of the tail nerve also had increased latency in group *Mn*, indicating decreased nerve conduction velocity. A shown in Fig. 5, conduction velocity and relative refractory period had an approximately antiparallel trend, and the changes caused by Mn treatment were reversed by rutin, partly by curcumin (only the relative refractory period) but not by ascorbic acid.

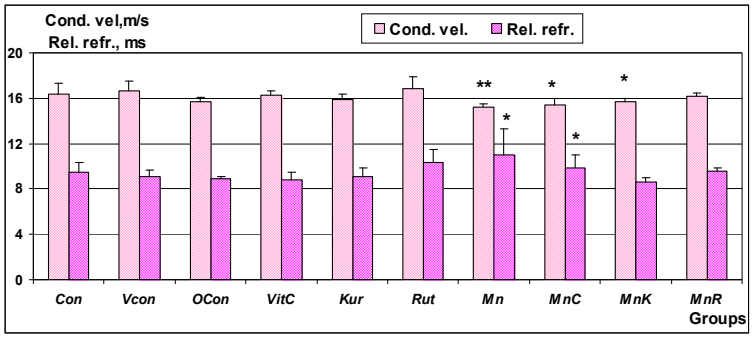

Figure 5 Tail nerve conduction velocity and relative refractory period.
Mean+SD, n=6 (*Con*) or n=8 (all other groups)
*, **: p<0.05, 0.01 vs. Con

Neither tissue metal levels nor parameters of oxidative stress, as a presumed common cause of the observed general toxic and neurotoxic effects, were measured in Experiment 1. A common background could, all the same, be inferred from the correlations of the measured changes. The linear correlation of OF rearing time and SS EP latency (indicating functional neurotoxicity) to body weight gain (indicating general toxicity) was not too robust (Fig. 6) but supported somewhat the existence of such a background.

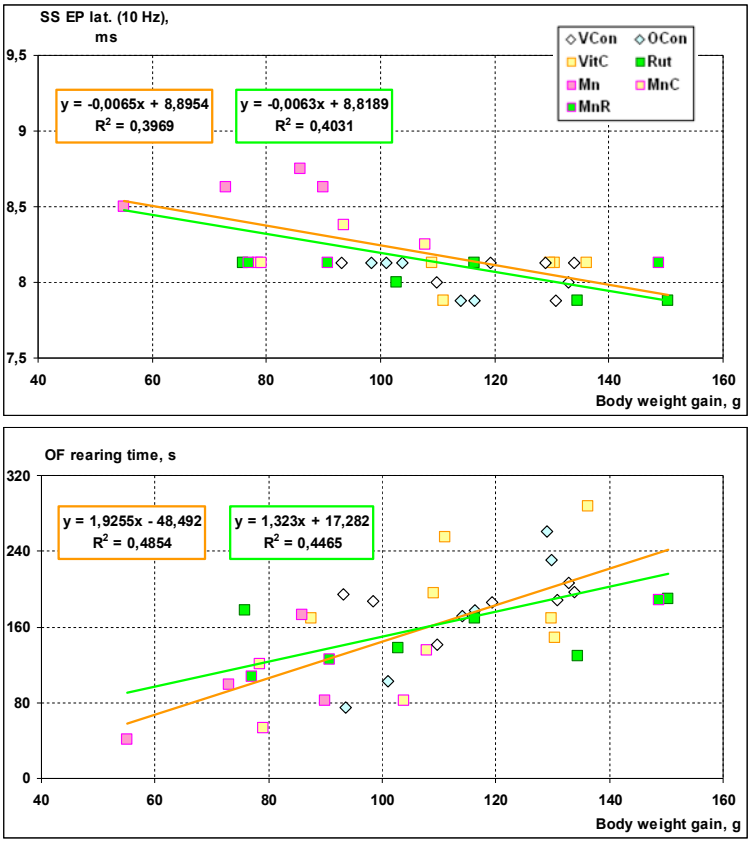

Figure 6 Correlation diagrams between the individual rats' body weight gain with somatosensory evoked potential latency (top) and open field rearing time (bottom).
Yellow trend line: groups *VCon*, *VitC*, *Mn* and *MnC*; green trend line: groups *VCon*, *OCon*, *Rut*, *Mn* and *MnR*. Outline of the equation boxes is the same color as the corresponding trend line.

3.2. Experiment 2: Arsenic with ascorbic acid and green tea

3.2.1. General toxicity

Orally applied inorganic arsenic caused diminished body weight gain. Top panel in Fig. 7 shows that the mean body weight in groups *AsL* and *AsH* was, from the 2nd week on, clearly less than in any of the groups without As exposure (*Con*, *VitC*, *Tea*). The difference became more and more pronounced, and in the groups with high As dose significant, towards the end of the treatment period; this is demonstrated also by the average weekly body weigh gains in the bottom panel in Fig. 7. This graph also shows food intake data, and a comparison of the two sets of values indicated that the differences in body weight gain were only partly due to differences in food intake.

The relative organ weight data showed no clear effect of As or the antioxidants (not shown).

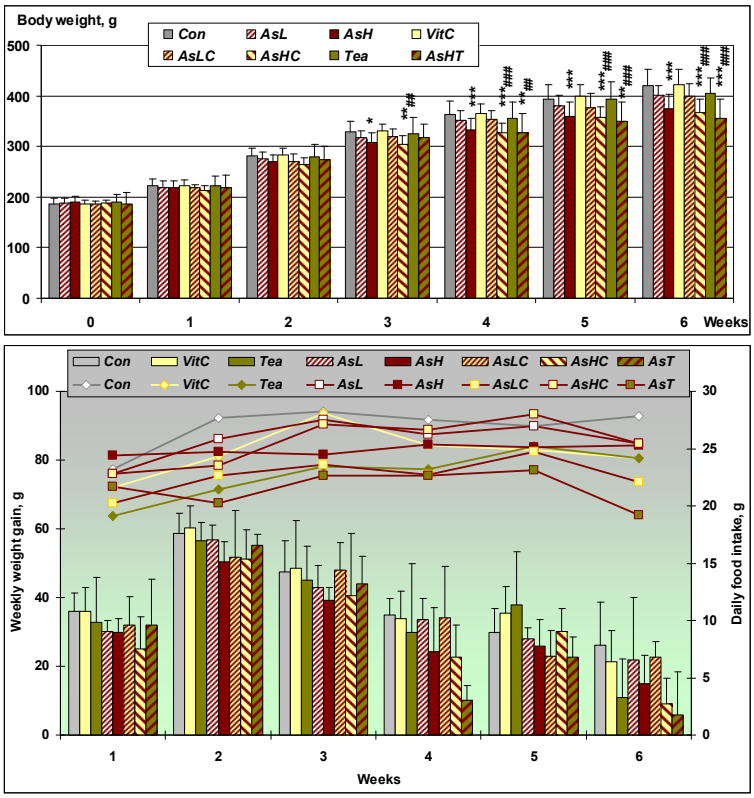

Figure 7 Top: Body weight of the control and treated rats in the course of Experiment 2. Bottom: Data of weekly body weight gain and food intake in the same rat groups. See insert for group coding.
Mean+SD, n=10.
*, **, ***: p<0.05, 0.01, 0.001 vs. *Con*
#, ##, ###: p<0.05, 0.01, 0.001 vs. the corresponding antioxidant-only group.

3.2.2. Effects on open field behavior

The OF results of Experiment 2 were inconclusive. All observed changes were slight and well below significance. Their direction, however, was similar to that observed both in the later Experiment 3, and in an earlier behavioral experiment with As exposure (Sárközi et al., 2012). Treatment with As increased the time spent by the rats in the OF activity forms indicating reduced motility (local activity and immobility) and ascorbic acid and green tea partially reversed this change.

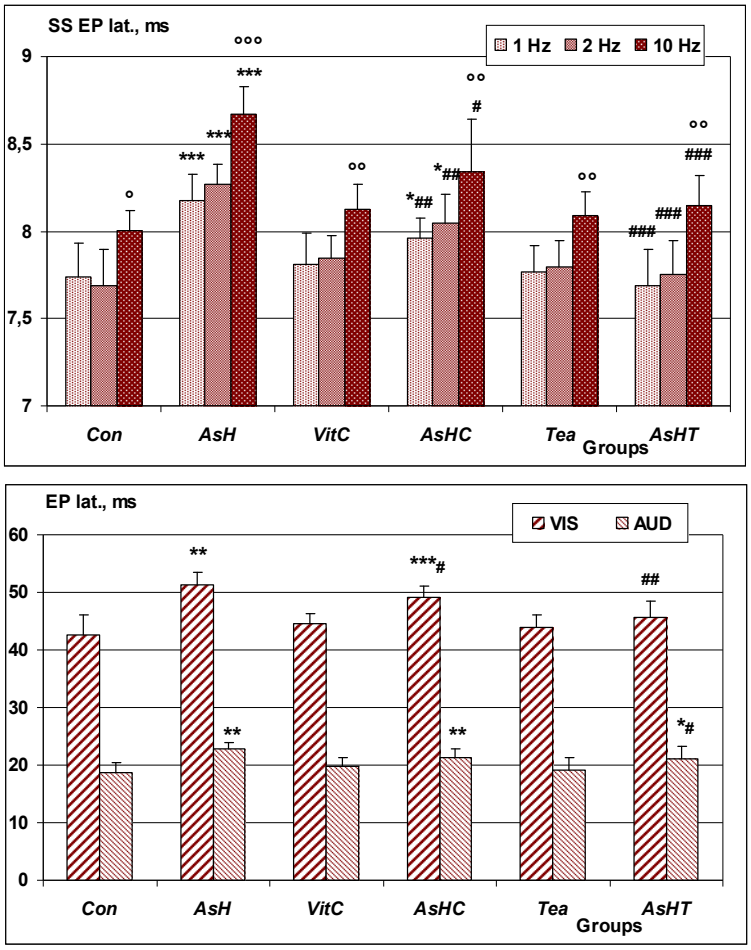

Figure 8 Latency of the somatosensory (top graph) and the visual and auditory (bottom graph) evoked potentials.
Mean+SD, n=10.
*, **, ***: p<0.05, 0.01, 0.001 vs. *Con*; #, ##, ###: p<0.05, 0.01, 0.001 vs. *AsH*
°, °°, °°°: p<0.05, 0.01, 0.001 vs. 1 Hz stimulation in the same group.

3.2.3. Electrophysiological effects

The band power spectrum of ECoG was not influenced by oral application of arsenic – in contrast to what was observed with manganese.

The numerical parameters of cortical evoked activity were, however, markedly changed. Fig. 8 shows the significant lengthening of EP latency of effect of the higher dose of As in all three modalities (SS, VIS, AUD) investigated. In group *AsL*, on the contrary, the change was weak, similarly to the body weight effect shown in Fig. 7, and was hence not further analyzed. Ascorbic acid and green tea infusion had, on their own, no effect on latency, but significantly reduced the lengthening caused by As whereby the ameliorating effect of green tea appeared to be higher than that of ascorbic acid. The frequency-dependent latency increase of the SS EP was significant only at 10 Hz vs. 1 Hz but this also was increased by As and again decreased by the antioxidants.

The CAPs recorded from the tail nerve indicated decreased conduction velocity in group *As* vs. *Con* (Fig. 9). Similarly to the cortical EPs, the effect was counteracted by both antioxidants, more efficiently by green tea than by ascorbic acid. The frequency dependence of the conduction velocity was, unlike with the cortical EPs, not much altered by As and/or the antioxidants.

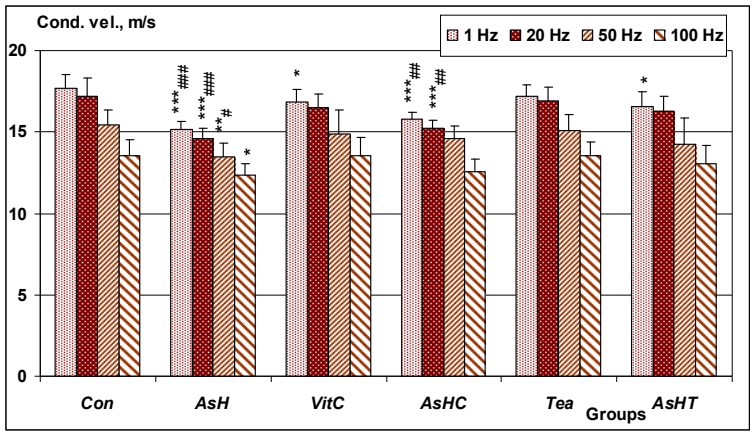

Figure 9 Tail nerve conduction velocity in the rat groups of Experiment 2.
Mean+SD, n=10.
*, **, ***: p<0.05, 0.01, 0.001 vs. *Con*; #, ##, ###: p<0.05, 0.01, 0.001 vs. *VitC*.

3.2.4. Tissue arsenic levels and biochemical indicators of oxidative stress

Oral application of inorganic As by gavage for 6 weeks corresponded to the total As doses given in Table 8 (both Table 8 and Table 9 include those groups only for which the complete set of As level determination and biochemical measurement has been made). The amounts of As shown resulted in greatly elevated tissue As levels; 4- to 6-fold the control level in RBCs and in cortex and liver samples, but ca. 40-fold in the kidneys, the most important site of

deposition of this element. The only noteworthy effect of the antioxidants on the tissue As levels was a marked, but not significant, reduction in the liver.

Table 8 Summed As amounts received by the rats and As levels in the tissue samples.

		Groups			
		Con	*As*	*AsHC*	*AsHT*
Summed external As dose, mg/rat		---	89.0±6.2	88.2±5.4	97.3±19.2
Tissue As (mg/kg dry weight)	RBCs	194.83± 11.04	1325.06± 44.22***	1338.41± 72.56***	1299.22± 268.11**
	Cortex	3.92±3.20	29.14± 11.88*	24.22± 4.81*	26.01± 5.99*
	Liver	4.94±3.02	19.84±13.03	23.10±7.59*	12.38±7.29
	Kidneys	10.09±7.64	396.61± 98.45*	467.49± 38.24**	432.97± 32.43**

Mean±SD, n=10 (external As dose) or n=3 (tissue As levels).
*, **, ***: $p<0.05$, 0.01, 0.001 vs. *Con.*

To achieve comparability, the summed amount of antioxidants given to the rats was calculated in activity – ascorbic acid equivalents – instead of milligrams of the corresponding substance. The summed volume of fluid consumed by the rats, and the mean of the antioxidant activity measured in the ascorbic acid solution and the green tea infusion in fresh state and after using them in the watering bottles for 48 hours, were used for the calculation, and the results are given in Table 9 (for the not included groups *VitC* and *Tea*, the value was 1044.21±174.04 and 461.5±49.6 mg/rat, respectively). According to these data, rats in group *AsC* consumed more antioxidant activity than those in *AsT* but the effects of As were apparently more strongly reduced in the latter group.

Table 9 also shows the measured oxidative stress indicators. The increase caused by As in lipid peroxidation was more significant than that in H_2O_2 and ONO_2^- (oxidative agents induced by As and contributing to lipid peroxidation). Significant reduction of any of these parameters from the As-induced elevated level was achieved only by green tea, not by ascorbic acid, which was in parallel to the two agents' effects seen on evoked cortical and peripheral nervous activity.

Table 9 Summed antioxidant doses and oxidative stress indicators.

		Groups			
		Con	*As*	*AsHC*	*AsHT*
Summed dose of antioxidant, in ascorbic acid equivalents, mg/rat		---	---	998.52±166.42	444.30±63.47
H_2O_2 (µmol/mg protein)	RBCs	1.780±0.890	2.057±0.558	1.564±0.591	1.287±0.241#
	Cortex	0.595±0.027	0.656±0.052	0.543±0.065	0.665±0.060
ONO_2^- (nmol/mg protein)	RBCs	1.645±0.083	2.122±0.287*	2.434±0.565*	1.756±0.354
	Cortex	1.937±0.136	2.231±0.225	2.231±0.202	2.141±0.088
TBARS (nmol/mg protein)	RBCs	0.219±0.035	0.298±0.063*	0.318±0.047*	0.230±0.010
	Cortex	0.315±0.017	0.350±0.006*	0.360±0.050	0.300±0.015#

Mean±SD, n=10 (summed antioxidant dose) or n=3 (oxidative stress indicators).
*: $p<0.05$ vs. *Con;* #: $p<0.05$ vs. *As*

3.2.5. Correlations of various kinds of data in Experiment 2

The causal relationships of As, ascorbic acid and green tea infusion to the investigated neuro-functional and biochemical alterations, suggested by the data presented so far, was further tested by means of plotting data pairs of rats in correlation diagrams. The R^2 values (determination coefficients) belonging to the fitted lines in the correlation plots shown in Fig. 10 were rather low, but this was, at least partly, due to the low number of data pairs the reason for which was given in Materials and Methods.

However, even these weak correlations were found significant by the F-test included in the "linear fit" function of MS Excel used for these correlation plots, so that the above mentioned relationships were confirmed indeed.

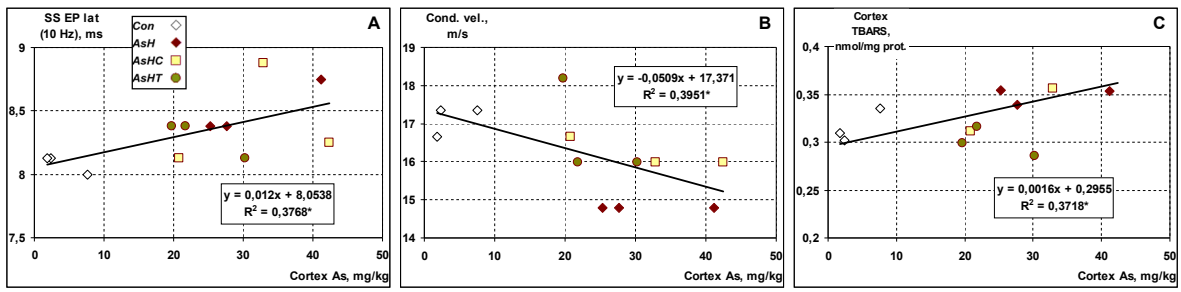

Figure 10 Correlation diagrams of data on electrophysiological (A, evoked potential latency; B, nerve conduction velocity) and biochemical (C, lipid peroxidation in the cortex) effects of As exposure and cortical As levels.
*: $p<0.05$ for the correlation.

3.3. Experiment 3: Involving both metals and the three antioxidants

3.3.1. General toxicity

The effect of orally applied As on body weight developed gradually, similarly to what was seen in Experiment 2. Significant weight deficit appeared by the end of the 3rd week (Fig. 11, top panel). Each applied antioxidant apparently diminished the body weight deficit, with rutin showing the best effect. The data of weekly body weight gain (Fig. 11, bottom panel) were more scattered but indicated the body weight effect of As application earlier, while the line graphs in the same panel showed that the body weight effect may have resulted partly from reduced food intake (just as in Experiment 2) because from the 4th week on the values for groups receiving or not receiving As were clearly separated.

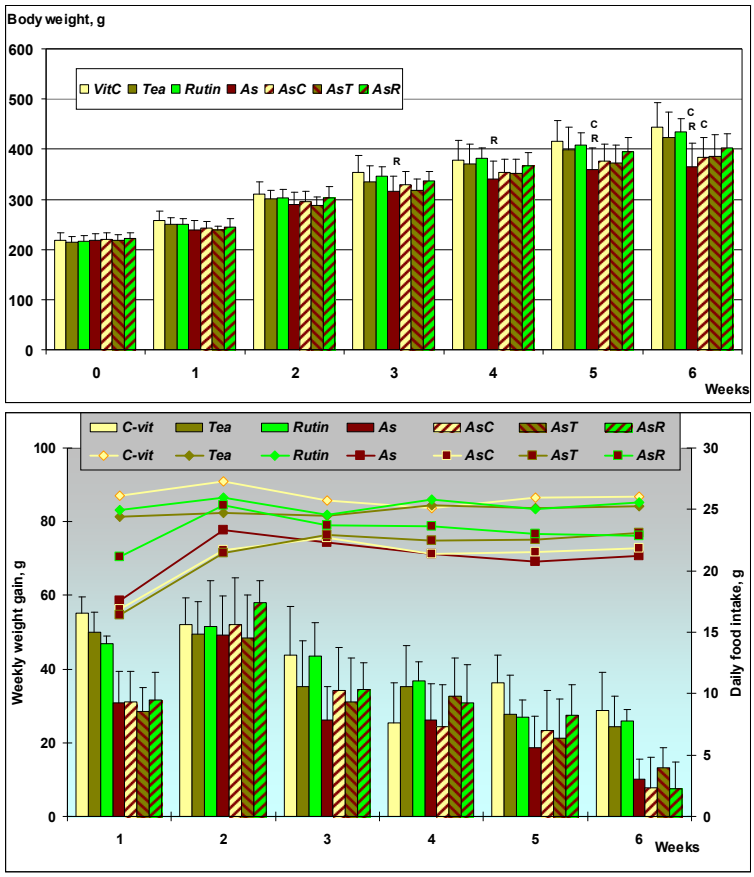

Figure 11 Top: Body weight of the control and As-treated rats in the course of Experiment 3. Bottom: Data of weekly body weight gain (bars) and food intake (line) in the same rat groups. See insert for group coding. Mean+SD, n=9.
C, R: significant difference ($p<0.05$) of the marked bar's value vs. group *VitC* or *Rut,* respectively.

The body weight effect of intratracheally applied nano-Mn was massively present from early on in the course of exposure (Fig. 12, top panel). The cause could be partly the application procedure itself (see Methods 2.1.) similarly to the data of group *VCon* in Experiment 1 – first of all the drop of food intake from the 1st to 2nd week (Fig 12, bottom panel) could have resulted from that. Later on, food intake was partly normalized (but remained clearly below that of Mn-free groups) the body weight deficit, however, did not vanish and was not influenced by co-administration of any of the antioxidants.

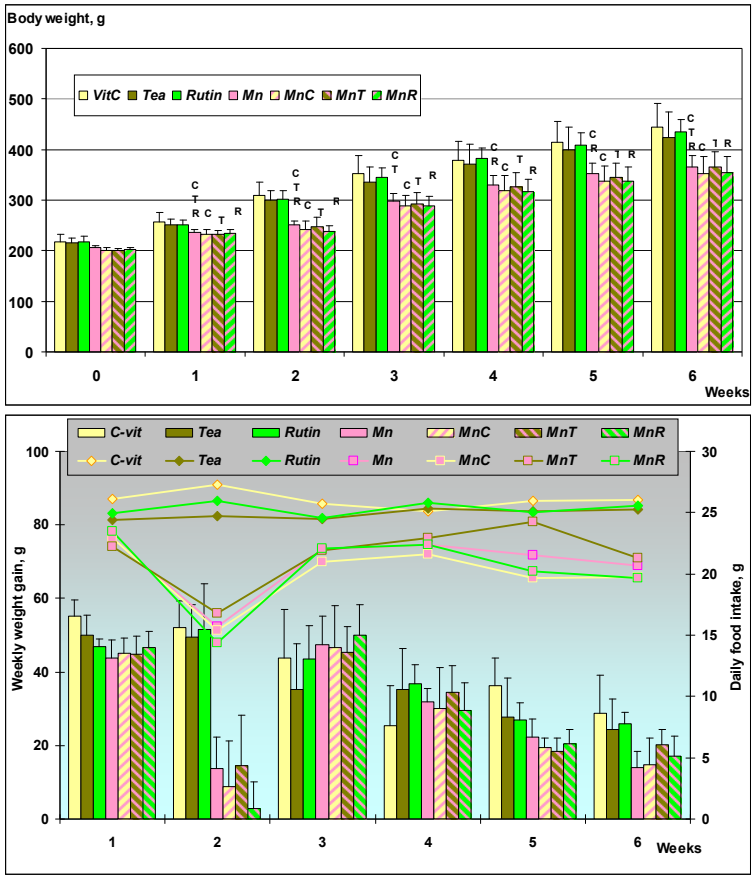

Figure 12 Top: Body weight of the control and Mn-treated rats in the course of Experiment 3. Bottom: Data of weekly body weight gain (bars) and food intake (line) in the same rat groups. See insert for group coding.
Mean+SD, n=9.
C, T, R: significant difference ($p<0.05$) of the marked bar's value vs. group *VitC*, *Tea* or *Rut*, respectively.

Of all organ weights, that of the adrenals (relative weight to brain weight) was increased by both metals. To facilitate the detection of significant changes, a "Control grand average" (*Con gr.*) was obtained from the organ weight data of the groups *VitC, Tea* and *Rut* (which received only an antioxidant). This calculation, similar to *Mn gr.* in Experiment 1, was possible

because it was seen in the outcomes of Experiment 1 and 2 that none of the antioxidants, given alone, had any noteworthy effect on body and organ weights. For As, the increase vs *Con gr.* was significant and so was the counter effect of rutin (*Con gr.*: 0.0310±0.0068, *As*: 0.0408±0.0031, *AsR*: 0.0295±0.0037, $p<0.05$ for both). Similar changes were found also with Mn (*Con gr.*: 0.0310±0.0068, *Mn*: 0.0362±0.0028, *MnR*: 0.0334±0.0027, $p<0.05$ for both). The Mn-caused decrease of relative weight of the liver (*Con gr.*: 7.206±1.403, *Mn*: 5.927±1.051, $p<0.05$) was only slightly reversed by any of the antioxidants.

3.3.2. Effects on open field behavior

Both metals had some significant effects on open field behavior. The effect of As (Fig. 13, top panel) was seen mostly as increased local activity and immobility, and decreased rearing. These changes were much more clear-cut than in Experiment 2, and were in their majority significant. Mn-treatment (Fig. 13, bottom panel) caused decreased ambulation while local activity and immobility increased. Most of these effects were also significant, but were partly at variance with Experiment 1 where rearing was more affected than ambulation.

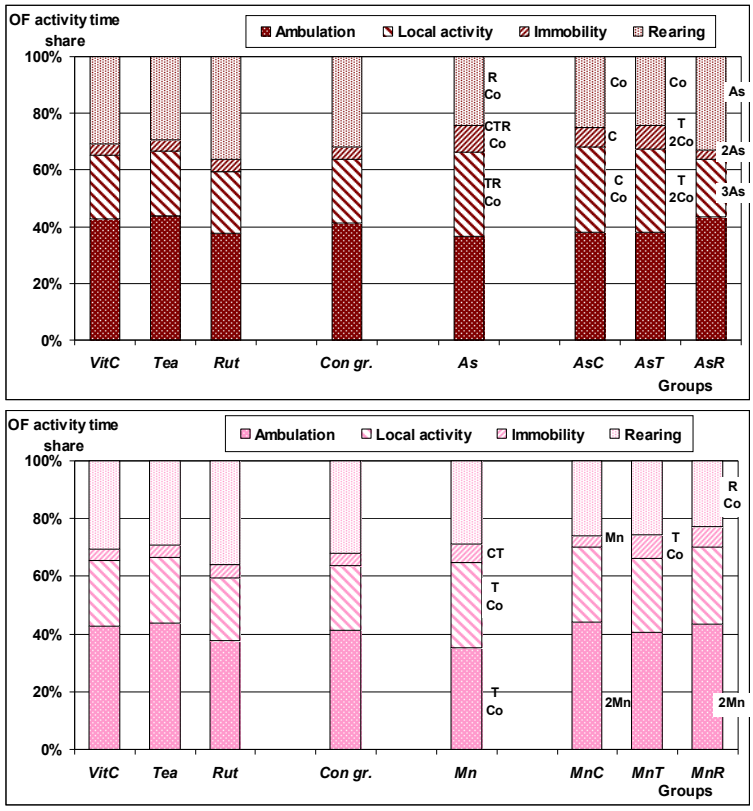

Figure 13 Time share of the OF activity forms in the rat groups of Experiment 3 treated with antioxidants and As (top) and antioxidants and Mn (bottom).
Co, C, T, R, Mn: significant difference ($p<0.05$) vs. group *Con gr.*, *VitC*, *Tea*, *Rut* or *Mn, respectively*. 2Mn: $p<0.01$.

Green tea had no major influence on the OF effect of either As or Mn. In case of As, only rutin had a significant counter effect, while in case of Mn ascorbic acid reversed the OF time values approximately to those of group *VitC*, and rutin increased ambulation but did not significantly change the other activity forms (*MnR* vs. *Mn*).

To see the changes caused by the various treatments more clearly, the ratio of identical OF parameters before and at the end of treatment (that is, in the 6^{th} and 0^{th} week) was calculated. In group *As*, the $6^{th}/0^{th}$ week ratio of ambulation distance was significantly lower (i.e., indicated more marked decrease) than in *Con gr.* or *Tea* (*As*: 0.6008±0.1111, *Tea*: 0.7733±0.0855, *Con gr.*: 0.7473±0.1207, $p<0.05$ for both); and the overall speed of ambulance – summed distance covered divided by summed ambulation time – behaved similarly (*As*: 0.8172±0.1133, *VitC*: 0.9126±0.0587, *Con gr.*: 0.9291±0.1762, $p<0.05$ for both). In the combinations, ascorbic acid partly reversed the effect of As and Mn (values in *AsC* and *MnC* were between those of the corresponding metal-only groups and *VitC*, not differing significantly from either.

3.3.3. Electrophysiological effects

The ECoG band spectrum was not altered by As, in agreement with the findings in Experiment 2. Mn administration caused a moderate shift in the ECoG to higher frequencies.

Fig. 14 (top panel) shows the spectrum of ECoG in various treatment groups from the SS area. Here and also in the two other recorded areas (VIS and AUD) decrease of slow activity in group *Mn* was observed which was abolished more strongly by green tea than by rutin but not at all by ascorbic acid. The ECoG index (Fig. 14 bottom panel) changed accordingly.

The latency of the cortical EPs was strongly lengthened by both metals. The SS EP in arsenic-treated rats (group *As* in Fig. 15, top panel) had significantly increased latency at every stimulation frequency, vs. each antioxidant-only group (*VitC*, *Tea*, *Rut*), and the frequency-dependent extra lengthening was also more pronounced.

On the VIS and AUD EP (Fig. 15, bottom panel) As-induced latency lengthening was almost completely reduced by rutin and green tea but only partly by ascorbic acid. The effects seen on the duration of the EPs were similar but less clear-cut and were therefore not further analyzed.

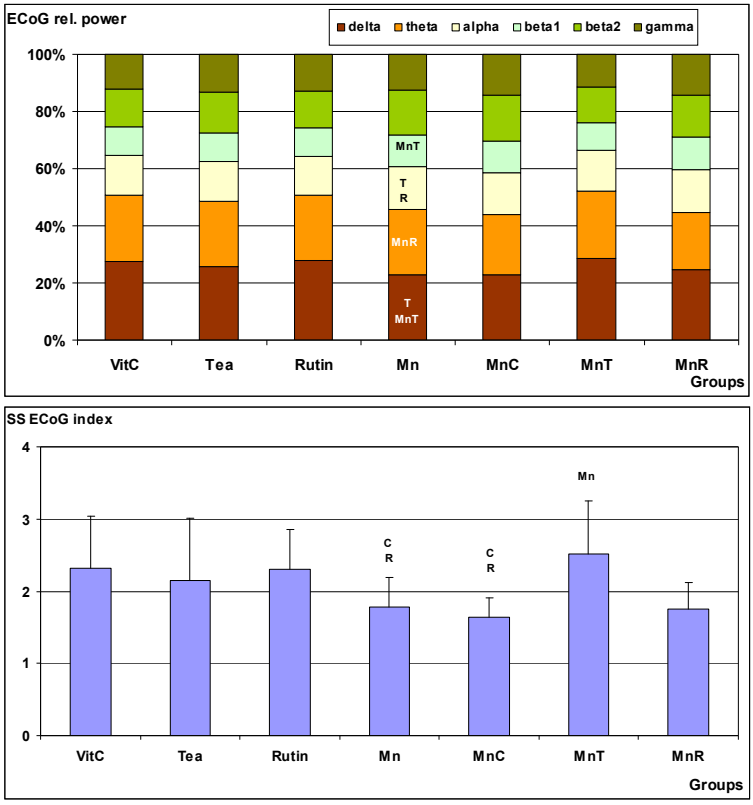

Figure 14 Top: Band spectrum of somatosensory cortical activity (ECoG) in the groups treated with antioxidants and Mn. Bottom: ECoG index of the same groups. Significance marking as in Fig. 12 and 13.

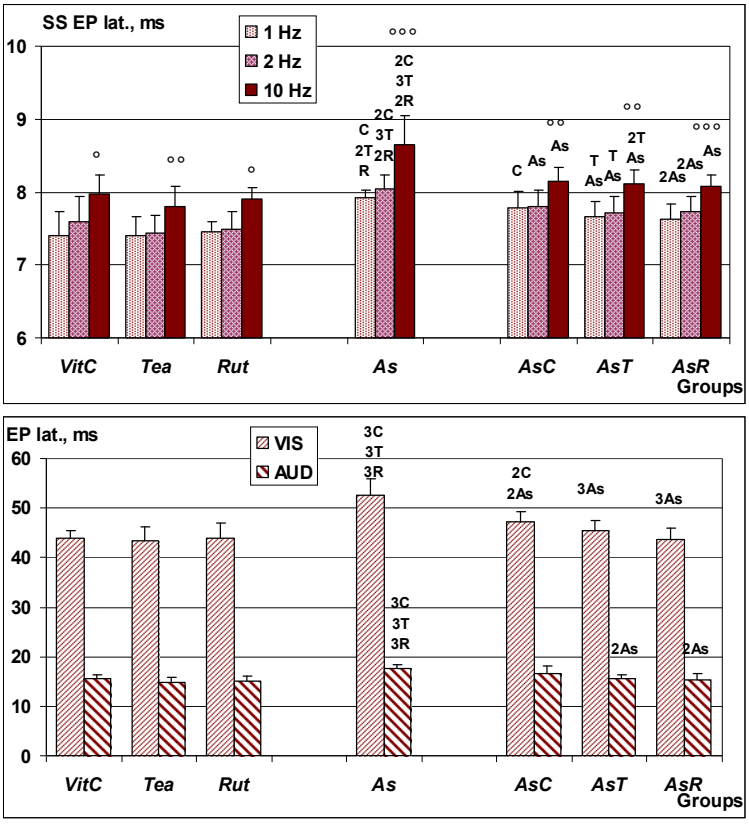

Figure 15 Latency of the somatosensory (top graph) and the visual and auditory (bottom graph) evoked potentials in rat groups treated with As and the antioxidants. Mean+SD, n=9. Significant inter-group differences marked as in Fig. 12 and 13; 2 and 3 marks mean $p<0.01$ and $p<0.001$. For the SS EPs, significant frequency-dependent differences marked as in Fig. 4.

Each antioxidant could significantly diminish the As-caused increase of SS EP latency but the effect of rutin was the most profound: the latency difference in *AsR* vs. *Rut* was not significant while by green tea and ascorbic acid latency was brought to a level between that in group *As* and the corresponding antioxidant-only group (i.e, the effect of As was only partially abolished).

The effect of Mn on the latency of SS EP was nearly as strong as that of As, but the frequency-dependent lengthening did not become more significant (Fig. 16, top panel). The Mn-induced latency increase was significantly reduced by green tea and rutin while ascorbic acid had only a partial effect (shown by the lack of significance both vs. *As* and vs. *VitC*).

The EP latency in the two other modalities (Fig. 16, bottom panel) was more strongly lengthened by Mn than by As. The counter effect of ascorbic acid was moderate (significant only on the AUD EP), that of rutin was more pronounced, and of green tea, nearly complete (VIS EP) or ca. as strong as with rutin (AUD EP).

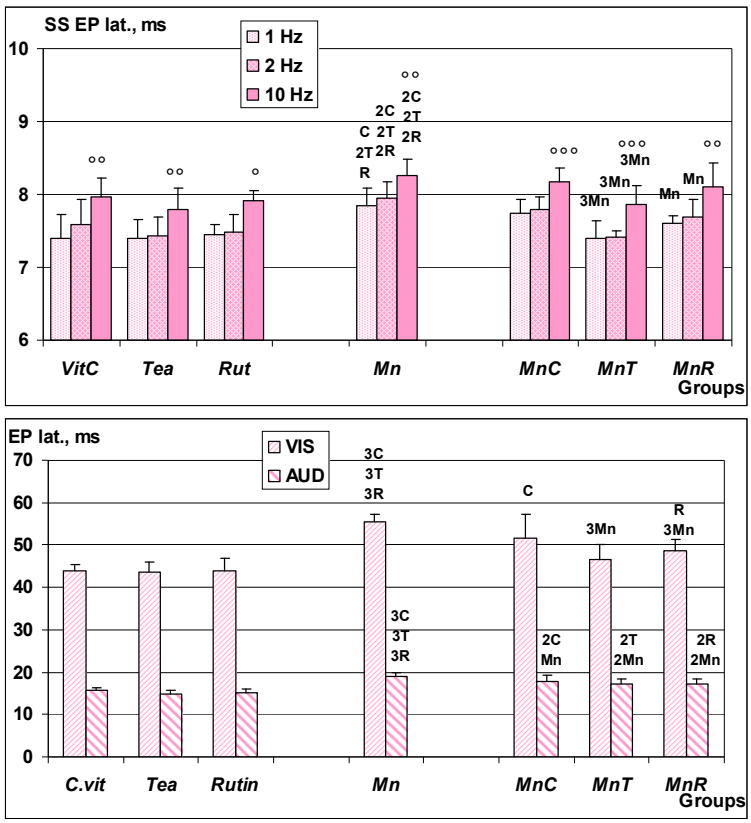

Figure 16 Latency of the somatosensory (top graph) and the visual and auditory (bottom graph) evoked potentials in rat groups treated with Mn and the antioxidants.
Mean+SD, n=9.
Significance marking as in Fig. 15.

Conduction velocity of the tail nerve was significantly reduced both by As and by Mn (Fig. 17) but the effect of As was somewhat stronger. Rutin normalized this significantly in case of both metals, and green tea in case of Mn, while the effect of ascorbic acid was weak. All the same, the velocity values in all groups treated with a metal and an antioxidant were significantly lower than in the corresponding antioxidant-only group which meant that even the best counter effect of any antioxidant was only partial. The relative refractory period was substantially increased by As and this effect was significantly reduced by the antioxidants. In case of Mn, both the metal effect and the counter effects were less clear.

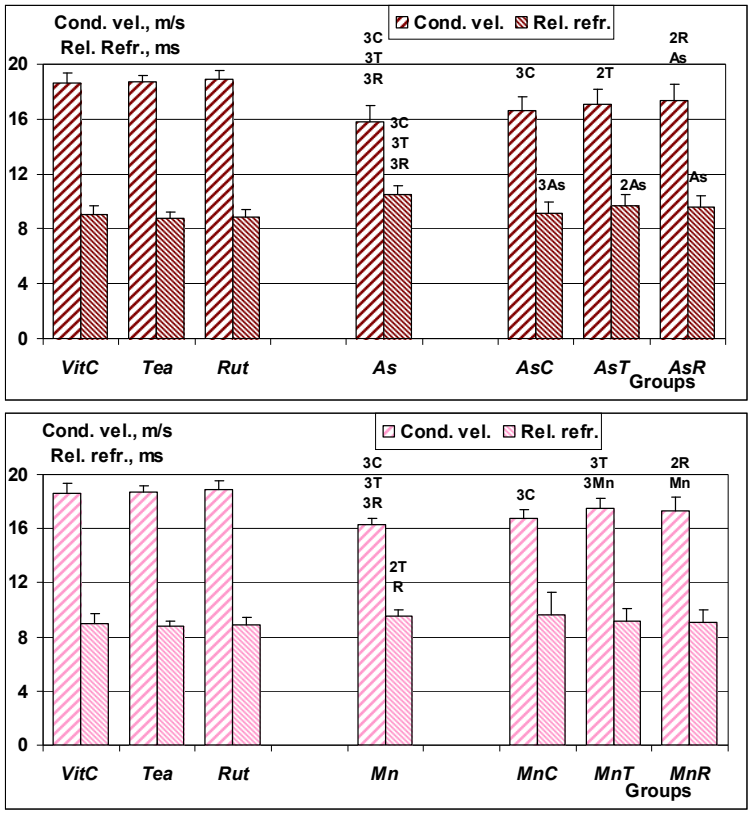

Figure 17 Tail nerve conduction velocity and relative refractory period in the groups treated with As and the antioxidants (top) and Mn and the antioxidans (bottom)
Mean+Sd, n=9.
Significance marking as in Fig. 15.

3.3.4. Tissue metal levels and biochemical indicators of oxidative stress

Both orally applied water soluble As and it. applied nanoparticulate Mn caused massive internal load in the treated rats' organs (Table 10). As level increase was highest in the kidneys. The table also shows the calculated total amount of As or Mn (i.e., the external load) the rats received during the 6 weeks of exposure. The antioxidants had some lowering effect on internal As doses but no such effect (or even an opposite effect) was seen with Mn.

Table 10 Summed metal doses and tissue metal levels in Experiment 3.

		Treatment groups				
Arsenic treatment		*Con gr.*	*As*	*AsC*	*AsT*	*AsR*
Summed external As dose, mg/rat		---	92.08±16.77	95.84±17.45	94.11±17.33	99.04±18.60
Tissue As (mg/kg dry weight)	RBCs	14.420± 1.3432	1565.12± 53.42***	1488.16± 15.87***	1429.49±15.72***	1385.85±99.52***$^{\#}$
	Cortex	1.0539±0.699	71.767±17.716*	43.825±6.966**$^{\#}$	50.725±21.526*$^{\#}$	29.561±9.149***$^{\#\#\#}$
	Liver	1.4389±1.179	27.992±5.868**	22.299±16.880*	12.588±2.887**$^{\#}$	9.493±3.093*$^{\#\#\#}$
	Kidney	6.2076±3.6143	388.945±62.458	416.927±38.298	405.372±114.206***	390.643±54.041***
Manganese treatment		*Con gr.*	*Mn*	*MnC*	*MnT*	*MnR*
Summed external Mn dose, mg/rat		---	36.12±6.61	34.95±6.54	35.51±6.76	35.00±6.37
Tissue Mn (mg/kg dry weight)	RBCs	0.8007±0.1045	1.3653±0.2089	1.7114±0.6293	1.5316±0.3979	1.3295±0.1201
	Cortex	20.935±6.962	46.403±2.386**	44.614±6.962**	54.040±2.565***$^{\#\#}$	51.802±3.004**$^{\#}$
	Liver	7.934±0.514	10.266±0.505*	9.268±0.592*	10.672±1.884	10.227±1.377*

Mean+SD, n=3
*, **, ***: p<0.05, 0.01, 0.001 vs. *Con gr.*
$^{\#}$, $^{\#\#}$, $^{\#\#\#}$: p<0.05, 0.01, 0.001 any combination group vs. the corresponding metal-only group (*As* or *Mn*)

Table 11 Summed antioxidant doses and biochemical indicators of oxidative stress.

		Treatment groups						
Arsenic treatment		*VitC*	*Tea*	*Rut*	*As*	*AsC*	*AsT*	*AsR*
Summed antioxidant dose, mg vitamin C eq./rat		1756.13±319.46	997.14±376.93	1005.40±256.40	---	1165.29±476.48	769.20±311.25	986.49±387.60
H_2O_2 (µmol/mg protein)	RBCs	1.7330±0.3781	1.9313±0.2241	1.7789±0.4501	1.5928±0.2862	1.4404±0.0610	1.7375±0.3468	1.7697±0.2658
	Cortex	0.5755±0.0696	0.5941±0.05783	0.5891±0.0620	0.5549±0.0732	0.6002±0.0702	0.5495±0.0437	0.6541±0.0525
ONO_2^- (nmol/mg protein)	RBCs	2.467±1.290	1.250±0.2241	1.406±0.2756	2.5438±0.7714	3.8058±0.4214	2.6513±0.8727	2.1744±0.3560
	Cortex	2.0428±1.5625	1.9472±0.3425	1.9406±0.2984*	2.4442±0.1253	2.1575±0.2587	2.6188±0.2165	2.0746±0.3123$^{\#}$
TBARS (nmol/mg protein)	RBCs	0.2346±0.0423	0.2635±0.0356	0.2182±0.0501*	0.2809±0.0728	0.2895±0.0173	0.2144±0.0229$^{\#}$	0.2148±0.0573$^{\#}$
	Cortex	0.4021±0.0396	0.3380±0.0413	0.2994±0.0348	0.3437±0.0374	0.3612±0.0410	0.3367±0.0296	0.2853±0.0323$^{\#}$
Manganese treatment		*VitC*	*Tea*	*Rut*	*Mn*	*MnC*	*MnT*	*MnR*
Summed antioxidant dose, mg vitamin C eq./rat		1756.13±319.46	997.14±376.93	1005.40±256.40	---	1025.27±329.49	721.11±207.36	741.43±179.64
H_2O_2 (µmol/mg protein)	RBCs	1.7330±0.3781	1.9313±0.2241	1.7789±0.4501	1.6611±0.2100	1.7375±0.1118	1.8052±0.0909	2.7302±0.0710
	Cortex	0.5755±0.0696	0.5941±0.0452	0.5891±0.0621	0.5722±0.0272	0.5660±0.0365	0.6560±0.0569	0.6031±0.0638
ONO_2^- (nmol/mg protein)	RBCs	2.467±1.290	1.250±0.2241**	1.406±0.2756*	3.8685±0.7618	2.8864±0.7918	4.1058±0.5801	2.6591±0.3180
	Cortex	2.0428±1.5625	1.9472±0.1724*	1.9406±0.2314	2.4603±0.35311	2.0324±0.2541	2.7406±0.3049	2.6822±0.1765
TBARS (nmol/mg protein)	RBCs	0.2346±0.0423*	0.2635±0.0356	0.2182±0.0501*	0.3166±0.0457	0.2941±0.0190	0.2760±0.0185	0.3384±0.0670
	Cortex	0.4021±0.0396	0.3380±0.0427	0.2994±0.0312	0.3023±0.0281	0.2375±0.0195$^{\#}$	0.2757±0.0274	0.2924±0.03021

Mean+SD, n=3

*, **: $p<0.05$, 0.01 any antioxidant-only group vs. the corresponding metal-only group (*As* or *Mn*)

$^{\#}$,: $p<0.05$ any combination group vs. the corresponding metal-only group (*As* or *Mn*)

The summed antioxidant doses and the measurements results on biochemical indicators of oxidative stress are given in Table 11.

The intensity of thiobarbiturate reaction (indicating lipid peroxidation) and the level of peroxinitrite were altered by exposure to the two metals and by antioxidant application in a way which showed effect and counter effect. The data also showed that the effect of As was most clearly reversed by rutin, and that of Mn, by ascorbic acid. All the same, the data were rather scattered with wide error ranges (standard deviations), precluding any firm conclusion.

3.3.5. Correlations

Correlation plots made of data on functional alterations and chemical or biochemical measurements in Experiment 3 were, however, more informative (which indicated also that the unfavorable error ranges mentioned above resulted more from inter-individual variation of the rats and less from measurement errors).

Body weight gain during the exposure period (an indicator of general toxicity manifested among others in metabolic disturbances) was most strongly related to As load of the liver (the central metabolic organ; Fig. 18). Parameters related to functional neurotoxicity, like OF local activity time or SS EP latency, but also the parameter for lipid peroxidation (TBARS), had stronger correlation to the As load of the CNS, and the correlation of the measured biochemical and neuro-functional damage (lipid peroxidation and OF local activity) was not less strong either. The location of the points in the plots, determined by the data pairs, showed, in agreement with Table 10, that the antioxidants, first of all rutin, reduced both internal As load and its functional consequences.

Similarly to As exposure, body weight gain during the exposure period was most strongly related to Mn load of the liver. The OF and electrophysiological indicators of neuro-functional damage included in Fig 19 were better correlated to Mn load of the brain (more exactly, the cortex). Correlation between the functional and biochemical effect of the toxic metal could be demonstrated also for Mn. The distribution of the data pair points suggested further that mainly green tea (in case of EP latency and OF ambulation distance) but also rutin (in case of ambulation distance) could diminish the extent of functional damage without reducing brain Mn level.

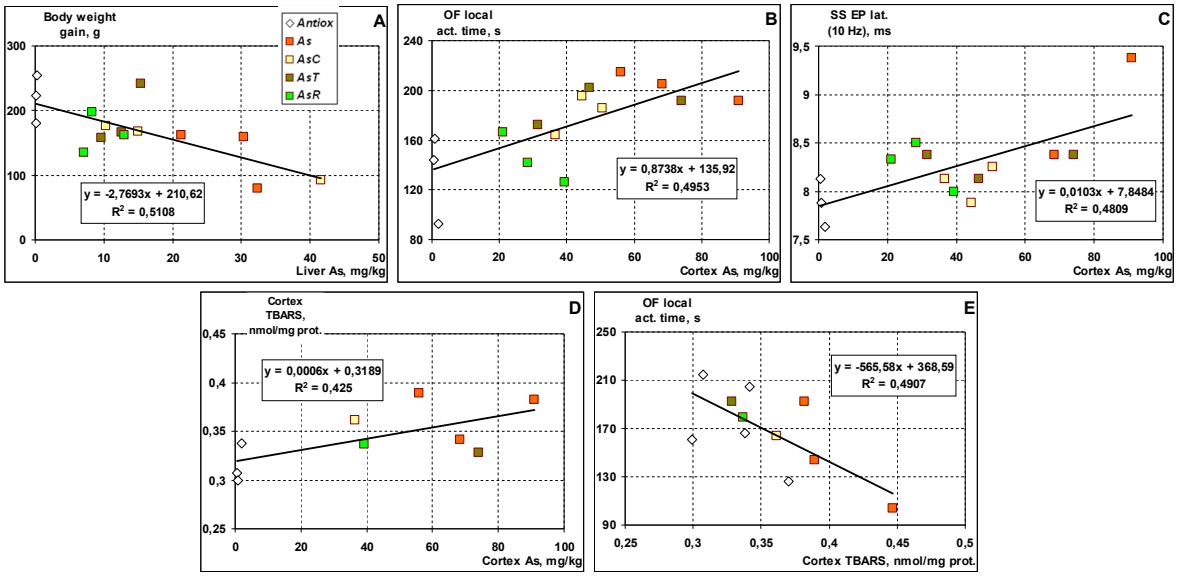

Figure 18 Correlation plots of the data on internal As exposure and its consequences. **A**, body weight gain and liver As load; **B**, **C**, **D**, neuro-functional and biochemical alterations and cortex As load; **E**, neuro-functional and biochemical effects. In the group marking (insert in A), *Antiox* denotes data from any of the antioxidant-only groups.

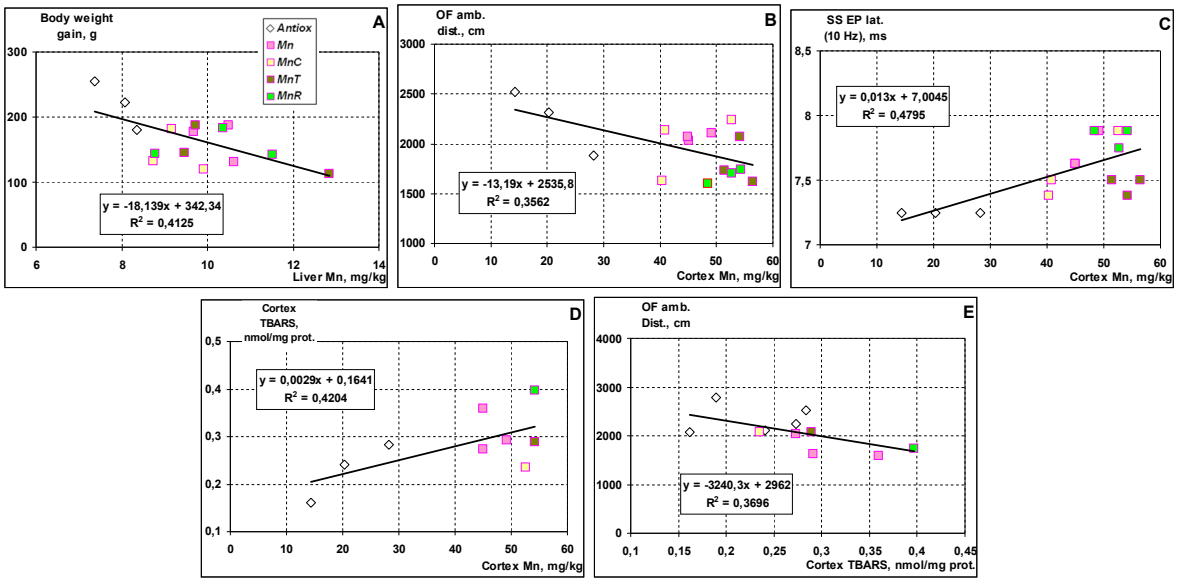

Figure 19 Correlation plots of the data on internal Mn exposure and its consequences. The same display as in Fig. 19.

4. DISCUSSION

The data in Results, and the background literature, permit the statement that the applied way of modelling human exposure to arsenic and manganese was realistic and efficient. In case of As, the main way of human exposure is oral, via drinking water (Nordstrom, 2002). Direct modelling of that is possible and has been used in several reports but dosing by gavage was deemed safer for the staff and the actual amounts applied to the rats were more accurate. The most important measure of internal load – As level in the cerebral cortex – was in the same order of magnitude with those in comparable experiments (10 mg/kg b.w As by gavage for 4 weeks by gavage, ca. 10 mg/kg As in the brain: Rodríguez et al., 2001; or 1.2 mg/kg As in the brain after only 2 mg/kg b.w. for 4 weeks: Yadav et al., 2012). Direct comparison with human data is difficult because the mostly used biological exposure index is urinary (and not blood) As level. In Lahore (Pakistan) humans who drank water with ca. 2.4 mg/l As had ca. 1.3 μg/l As in blood (Bibi et al., 2015). For a 70 kg human drinking 2 L water a day, these data would mean ca. 0.07 mg/kg b.w. dose; our rats in Experiment 2 and 3 received 100x higher daily doses but developed 1000x higher blood levels.

With manganese, most intoxications affecting the nervous system are seen in occupational exposure to Mn-containing metal fumes (Antonini, 2003; ATSDR, 2008). This was successfully modelled by instillation of suspended NPs in previous studies of the Department (e.g., Oszlánczi et al., 2010; Takács et al., 2012). In heavily exposed workers, ca. 14 μg/L blood Mn was found (Bader et al., 1999; Halatek et al., 2005) vs. 5–7 μg/L in reference groups (Bader et al., 1999). In our present work, and in the previous ones cited above, blood Mn levels were much higher but this was equally true for rats being or not being administered Mn, so that the difference in magnitude cannot be ascribed solely to the doses. Moreover, the difference between blood Mn of exposed and non-exposed rats was ca. twofold, similar to that of exposed and non-exposed humans reported in Bader et al. (1999). By assuming daily ventilation volume of 0.5 m^3/kg b.w. for the rats (based on physiological data in Strohl et al., 1997) the it. dose of 4 mg/kg MnO_2 NPs corresponds to ca. 24 mg/m^3 for 8 hours per day. In a survey published by Korczynski (2000), the maximal Mn level in workplace air polluted by welding fumes was around 5 mg/m^3.

Reduced body weight gain in the treated rats indicated that the internal As and Mn load took effect. Rutin was the only antioxidant with a noteworthy effect on the weight gain reduction caused by Mn (Experiment 1) and by As (Experiment 2) – however, in Experiment 3 (albeit

with dissimilar timing and administration of the antioxidants) this was not verified. Reduced food consumption as a cause was more pronounced in Mn-treated rats in Experiment 3 than in As-treated ones in Experiment 2 and 3, but any noteworthy correlation between food consumption and weight gain was seen only in a few cases – most notably in the 2nd week of Experiment 3 in the rats treated with nano-Mn. Other reasons of body weight gain reduction include organ damage – liver is attacked by both metals – and oxidative stress (ROS interfere with normal metabolism: Merry, 2002).

In the investigated functional alterations of the treated rats' nervous system, there were some general trends, such as decreased OF motility of the treated rats or slowed electrophysiological responses, indicating possible common mechanisms in the background. That oxidative stress may be one such mechanism was suggested, besides a number of papers ("final common pathway": LeBel and Bondy, 1991), also by earlier results of the Department (Oszlánczi, 2011). Both As and Mn, as inorganic chemical agents, are known to induce the generation of ROS in living tissue (Mn: Hamai and Bondy, 2004; As: Jomova et al., 2011), and this was seen also in the present study (Tables 9 and 11).

Induction of oxidative stress by Mn-containing welding fumes (in which NPs are always found: Antonini, 2003) has been proven in animal experiment in vivo (inflammation markers in the bronchoalveolar lavage fluid) and in vitro (depletion of glutathione) by McNeilly et al. (2004). Concerning As, biochemical signs of oxidative stress were found in occupationally exposed humans (Halatek et al., 2009) and in animal experiments (Yadav et al., 2009).

The negative effect of oxidative stress on CNS functionality has been repeatedly described for both metals. In Mn-exposed rats' brains, ROS generation and membrane lipid peroxidation was seen (Avila et al., 2008). For As, similar effects were described by Flora (2011), and García-Chávez et al. (2006). Damage to membrane lipids results, in turn, in changes of fluidity and probably in altered neuronal membrane functions. Lipid peroxidation is in fact present in those human CNS diseases (Farooqui and Horrocks, 1998) where the role of oxidative stress in the pathomechanism has been supposed (Valko et al., 2007; Chaturvedi and Beal, 2013).

Cortical evoked potentials are those electrophysiological phenomena, recorded and analyzed in this thesis, which reflect alterations in synaptic transmission (a likely consequence of membrane damage, see above) the most directly. Increased latency observed in our rats treated with As or nano-Mn might be, at least partly, due to decreased synaptic efficiency. The parallel dependence of cortical TBARS level and SS EP latency on the local dose of both As and Mn (Figs. 18 and 19) in Experiment 3 suggested such a relationship. In other rat-based

disease models involving oxidative stress (induced hypertension: Göcmen et al., 2014; sulfite exposure: Derin et al., 2009) TBARS level and visual EP latency increased in correlation, and in the latter report protection by lipoic acid, an antioxidant, was demonstrated. Synaptic damage appears to be present also in chronic human CNS diseases, like Parkinson's or Alzheimer's disease. Products of lipid peroxidation, such as 4-hydroxy-nonenal (Valko et al., 2007) may be responsible for damages of synaptic structure and function (LoPachin et al., 2008). Besides synaptic transmission, regenerative nerve pulse conduction also depends on normal membrane functioning which explains why the pattern of changes of EP latency and tail nerve conduction velocity were similar.

The above mechanistic explanation is, of course, by far not exclusive. There are further ways for both As and Mn to influence neuronal activity. Mn^{2+} (released from phagocyted NPs: Lundborg et al., 1985) can block Ca-channels in presynaptic endings but can also activate maze. In As-treated guinea pigs, neuronal apoptosis resulted from ROS-induced Ca influx via voltage-gated L-type Ca-channels and concomitant mitochondrial damage (Pachauri et al., 2013). So, abnormal intracellular Ca level probably causes not only synaptic dysfunction but also oxidative stress.

Significant changes in open field motor behavior were seen only in Experiment 1 and 3, but the general effect of As and nano-Mn was similar: decreased ambulation and rearing, that is, motor hypoactivity. Motivation, determining spontaneous locomotion in the OF, 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). Rodríguez et al. (2001) described hypomotility in rats after subacute oral exposure to inorganic As in doses comparable to ours. A human analogue of that might be impaired motor control and body coordination in Bangladeshi schoolchildren with elevated internal As load (Parvez et al., 2011). In case of Mn, reduced motility observed in Experiment 1 and 3 might be analogous to that in welders with manganism (Bowler et al., 2007).

Each antioxidant used in this study had some counter effect on the electrophysiological and/or behavioral alterations induced by As and nano-Mn, but to a dissimilar extent. The effect of ascorbic acid was in most cases less than that of rutin or green tea brew, although the measured antioxidant capacity of ascorbic acid solution was higher (Tables 9 and 11). Protective actions, other than reduction of oxidized biomolecules, of rutin and green tea flavonoids include first of all chelation of metal ions. Metal chelating ability of tea flavonoids (Weinreb et al., 2004) may act directly on the exogenous toxicants – here: As, or Mn set free

form NPs – or on endogenous reaction partners like Fe released from ferritin by methylated As (Jomova et al., 2011). Rutin, and its aglycone quercetin, also bind transition metal ions and reduce so oxidative load (Omololu et al., 2011). This may explain that, in Experiment 3, As levels in cortex and liver in groups *AsR* and *AsT* were significantly lower than in group *As* (Table 10). In the nano-Mn treated rats, no such effect was seen, however. Flavonoids, including rutin and EGCG, were shown also to enhance endogenous antioxidant defense including activity of superoxide dismutase and catalase, and anti-inflammatory pathways (Mandel et al., 2005).

Local bioavailability of the agents can influence the observed protective effect. Uptake of flavonoids to human plasma (Nakagawa et al., 1997) and to the brain of animals is promoted by the somewhat lipophilic character of the molecules (Mandel et al., 2005) providing easier (and, theoretically, unlimited) access to the CNS. Vitamin C, on the contrary, is brought to the brain by regulated active transport which depends on local demand but not on supply via blood (Harrison and May, 2009). But if these "protective" agents are to be given orally, intestinal absorption comes into play. For rutin and quercetin, poor intestinal absorption, and degradation by the gut microflora, was reported (Formica and Regelson, 1995) – all the same, rutin was found neuroprotective in animal experiments (Javed et al., 2012; Gomes-Rodrigues et al., 2013). Absorption of green tea polyphenols was also low (0.2 to 2%) in humans consuming dosed amounts of tea extract, but was sufficient to provide antioxidant defence (Nakagawa et al., 1997).

That curcumin had virtually no effect in Experiment 1 (except on nerve conduction, see Fig. 5) was a bit of odd, in view of the literature data (Kunchandy and Rao, 1990; Daniel et al., 2004; El-Demerdash et al., 2009; Yadav et al., 2009, 2011). Our dose was in the same range as in the mentioned papers, so an explanation might be that sunflower oil, used as vehicle in Experiment 1, dissolved curcumin well but also impeded its intestinal absorption.

Regarding the body of literature on the protective effect of antioxidants both in vitro and in vivo, the easy availability of many such agents as food ingredients or additives, and the number of humans subject to oxidative challenge due to heavy metal (As, Mn and others) exposure, it is surprising that there have been apparently no studies testing the putative protective effect of antioxidants in exposed populations, and only a few studies on the neuroprotective effect of antioxidants altogether (Albarracin et al., 2012). Ideas like "functional drinks in neurodegenerative diseases" (Zafrilla et al., 2009) still await implementation, although millions are exposed to As via drinking water and Mn via workplace air pollution – not to mention other exposures leading to CNS damage by a

mechanism involving oxidative stress – and chronic degenerative brain diseases represent a huge and growing problem. However, it must not be forgotten either that outcome of studies in which protective factors from "healthy diets" were given in pure form was often disappointing.

Based on the results, and keeping the above mentioned problems in mind, the particular questions set in 1.5. can be answered as follows:

- In Experiment 3, the used physicochemical form of As and Mn were investigated in terms of neurotoxicity and general toxicity together in the same experiment. Identical but separate experiments with the two metals were not made, but the answer to the first question is essentially positive.

- The antioxidants included in the experiments had some clearly detectable effects on the alterations induced by the two metals, regarding both nervous system effects (electrophysiological and behavioral) and general toxicity, but:

- There were marked differences between the effects of the antioxidants: 1/ Curcumin had practically no effect. 2/ The protective effect of vitamin C was weaker than that of the applied flavonoids – rutin and green tea constituents. The cause of the difference was apparently not the antioxidant capacity but probably the metal chelating ability of the flavonoids and/or their better local availability in the CNS.

- The examined antioxidants are all easily available natural compounds. As a chemically defined compound, rutin showed better effect than ascorbic acid. Green tea infusion (or an extract or concentrate) is chemically complex but the main constituents have been chemically identified. An "optimal" choice of antioxidant has to consider, beyond verified in vivo protective effect, also technical (sources, processing, formulation) and social (acceptance in the population to be protected) aspects, and requires further studies both within and outside the scope of environmental neurotoxicology.

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