

PhD Thesis

**CENTRAL NERVOUS EFFECTS OF MANGANESE IN RATS
INVESTIGATED BY REPEATED SIMULTANEOUS
ELECTROPHYSIOLOGICAL AND BEHAVIORAL RECORDING**

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Szeged

2012

The Applicant's Relevant Publications

- I. *Takács Sz, Bankó S, Papp A* (2010) Effect of mitochondrial toxins on evoked somatosensory activity in rats. **Central European Journal of Biology** 5, 293–298. IF: 0.685
- II. *Takács Sz, Szabó A, Oszlanczi G, Paulik E, Papp A* (2011) A pilot study with simultaneous recording of changes in motility and cortical electrical activity of rats during four weeks of oral manganese exposure. **International Journal of Environmental Health Research** DOI:10.1080/09603123.2011.643228. IF: 1.090
- III. *Takács Sz, Papp A* (2011) Neurotoxicity of manganese analysed by a novel combined electrophysiological-behavioral system. In Galbács Z ed.: **17th International Symposium on Analytical and Environmental Problems**, Szeged, pp. 210-214.
- IV. *Takács Sz, Szabó A, Oszlanczi G, Pusztai P, Sági A, Kónya Z, Papp A* (2012) Repeated simultaneous cortical electrophysiological and behavioral recording in rats exposed to manganese-containing nanoparticles. **Acta Biologica Hungarica**, accepted. IF: 0.793

Abstracts:

- Takács Sz, Bankó S, Nagymajtényi L, Papp A* (2009) Acute and subacute functional neurotoxicity of manganese in rats. **FEPS 2009**, Ljubljana, Szlovénia, 2009. november 12-15. Book of Abstracts p. 254.
- Takács Sz, Papp A* (2010) Changes of cortical electrical activity and open field motility in WAG rats during several weeks of oral manganese exposure. **IBRO International Workshop 2010**, Pécs, 2010. január 21-23. Frontiers in Neuroscience DOI 10.3389/conf.fnins.2010.10.00067.
- Takács Sz, Papp A* (2010) Antiepileptikumok és anesztetikum hatása a kérgi alapaktivitásra és a spontán motilitásra egy epilepszia hajlamos patkánytörzsben. **Magyar Élettani Társaság LXXIV. Vándorgyűlése és a Magyar Kísérletes és Klinikai Farmakológiai Társaság II. közös konferenciája**, Szeged, 2010. június 16-18. Acta Physiologica Hungarica 97, 480-481.
- Papp A, Takács Sz* (2010) Cortical electrical activity and open field motility of WAG rats during subacute oral manganese exposure. **7th Forum of European Neuroscience**, Amsterdam, 2010. július 3-7. FENS Abstracts vol.5, 164.26.
- Takács Sz, Máté Zs, Horváth E, Papp A* (2010) Behavioral and electrophysiological effects of intratracheal manganese exposure recorded in rats with chronically implanted cortical electrodes. **11th International Symposium Interdisciplinary Regional Research**, Szeged, 2010. október 13-15. Abstracts p.108.
- Takács Sz, Papp A* (2011) Elektrokortikogram és open field motilitás változások patkányban 8 hetes orális mangán expozíció során. **Magyar Farmakológiai, Anatómus, Mikrocirkulációs, Élettani Társaságok Közös Tudományos Konferenciája (FAMÉ)**, Pécs 2011. június 8-11. Acta Physiologica 202 Suppl 684, 116-117.

SUMMARY

Human environment (including workplaces, homes, outdoor areas, etc.) can contain various neurotoxicants, being of both natural and synthetic origin, and exposing humans by inhalation, by consumption of foods and drinking water, or via the skin. The major groups of relevant toxicants include both organics and inorganics, mainly metals and metal compounds.

Manganese (Mn) has, in form of alloys and compounds, numerous practical applications from steelmaking to nano-electronics. It is an essential trace element for all living organisms – present, among others, in glial glutamine synthetase, and in mitochondrial complex II and III – but has toxic effects when overdosed. This intestinal absorption is a self-regulated process whereas parenteral absorption after inhalation etc. may be much more efficient, which has toxicological relevance.

Occupational Mn exposure typically means chronic inhalation of dusts and fumes. The resulting neurological disorder is called manganism; with nonspecific symptoms (apathy, anorexia, asthenia, headache, hypersomnia, spasms, etc.) in the first stage but progressing to a Parkinson-like syndrome with its associated symptoms. The similarities with Parkinson's disease are the presence of generalized bradykinesia and widespread rigidity; and the dissimilarities, less-frequent resting tremor, more frequent dystonia, a particular propensity to fall backward, and failure to achieve a prolonged therapeutic response to levodopa. Such Parkinson-like disorder due to Mn was also observed in patients undergoing maintenance hemodialysis, or after high oral intake (drinking water, health supplement, etc). Epileptic symptoms were primarily observed in Mn-overexposed children or young adults. So, Mn is among the environmental factors occasionally causing epilepsy (defined usually as transient occurrences of excessive or hypersynchronous activity of brain neurons).

The functional neurotoxicity of Mn has been investigated at the Department of Public Health for ca. 15 years. Electrophysiological and behavioral methods both brought a substantial amount of new information. However, comparison of effects on electrical activity and open field motility was encumbered by the fact that electrophysiological recording was done in anaesthesia and hence its data could not be directly put in parallel with the behavioral effects. In the meantime, it became possible to perform repeated simultaneous recording of cortical electrical activity (electrocorticogram, ECoG) and of open field (OF) motility in awake rats. In the research work described in this thesis, rats prepared for such chronic recording were

used, and Mn was administered to them for 4 to 8-10 weeks orally by the drinking water or via the airways by intratracheal instillation. The particular aims of the work were:

- To observe the development of the CNS effects of Mn in time;
- To examine to what extent changes in motor behavior and in cortical electrical activity develop in parallel, and what relationship can be detected between the two; and
- To examine whether the epileptogenic effect of Mn, mentioned in the literature, can be observed under the experimental conditions applied.

To implement these aims, four experiments (summarized below) were performed on adult male rats. The WAG/Rij animals in Experiment 4 were from a strain specially developed to model human temporal lobe epilepsy, showing short bursts of spike-and-wave discharges (SWD) in their cortical electrical activity.

Experiment No.	Rat strain	Manganese treatment			Number of rats evaluated			
		Substance	Dose	Time	Open Field and general tox.		ECoG	
					<i>Treated</i>	<i>Control</i>	<i>Treated</i>	<i>Control</i>
1	Wistar	MnCl ₂ , oral, by drinking water	2.5 mg/mL water	4 weeks	4	4	3	3
2	Wistar	MnCl ₂ , oral by drinking water	7.5 mg/mL water	8 weeks	4	4	4	3
3	Wistar	MnO ₂ nano intratracheal	2.63 mg/kg. b.w.	8 weeks	7	4 (UnT) 3 (VT)	5	3 (UnT) 2 (VT)
4	WAG/Rij	MnCl ₂ , oral by drinking water	7.5 mg/mL water	8 weeks	4	3	4	3

UnT, untreated control; VT, vehicle-treated control in Experiment 3.

For repeated recording of ECoG, the rats were implanted with chronic electrodes and a connector “crown”. The operation was carried out under aseptic conditions. After ca. 10 days recovery, one recording session per week was made. The first two were control sessions with one week interval and without any treatment. Then, Mn administration for the treated rats (and vehicle administration for the controls in Experiment 3) was started, and lasted as given

in Table 1 with further recording sessions in one week intervals. The sessions lasted 30 (Experiment 1) or 60 (Experiment 2, 3 and 4) minutes. The rat was in an OF box, detecting and analysing its movements based on interruptions in an array of infrared sensor gates; while ECoG was recorded via a cable attached to the crown. Beam interruptions were processed to time and count of ambulation, local activity and immobility (but no rearing because of inevitable false signals of the ECoG cable). From the ECoG records, the software calculated the continuous power spectrum with 0.5 Hz resolution between 1 and 49 Hz, as well as the total power in this range, in pre-set bands (delta, between 1 and 3.5 Hz; theta, between 4 and 7.5 Hz; alpha, 8-12 Hz; low beta, 12.5-15; mid beta, 15.5-18; high beta, 18.5-31, and gamma, 31.5-44 Hz). Evaluation was based on the shape of the power spectrum curve, and on difference spectra obtained as the ratio of a spectrum from a given phase of treatment and a control spectrum. Correlation between OF, ECoG and tissue Mn level data was tested by the “linear fit” function of Excel after plotting corresponding data pairs in an X-Y plot. Epileptic bursts were detected on the basis of ECoG power peaks around 13 Hz.

Mn treatment caused significantly elevated levels of the metal in the blood and brain samples, but had no noteworthy effect on body and organ weights as indicators of general toxicity. The numerical parameters of OF motility and ECoG were, however, significantly affected.

In Experiment 1, low dose Mn for a shorter time span (4 weeks) caused a relative increase of OF ambulation time and decrease of immobility time, and both showed less habituation during the 30 min recording session. ECoG total power decreased in the treated rats, at all frequencies but especially around the 7 Hz peak. The OF and ECoG parameters changed in parallel in the treated rats and were correlated.

In Experiment 2, a higher dose of Mn was given longer (8 weeks) via the drinking water. There was no effect on body weight, but the Mn content and absolute weight of the brain as well as blood Mn and liver weight were in correlation ($R^2 > 0.5$). The motility of the treated rats was moderately decreased; mainly the time of immobility became longer but the increase of immobility during the 60 min recording session was less in the treated than in the control rats. ECoG power decreased only in the last two weeks of Mn exposure (which indicated a two-phase development of Mn effect, observed in clearer form in Experiment 3). The decrease was seen mainly around 7 and above 30 Hz, the latter correlated well with the changes of motility in the treated, but not in the control, rats. Two Mn-treated rats in Experiment 2 showed short bursts of spikes in the ECoG towards the end of treatment and a coincident peak on the ECoG spectrum around 13 Hz.

In Experiment 3, Mn was administered into the rats' trachea in form of MnO₂ nanoparticles, resulting in higher Mn levels in blood and brain samples and more expressed functional changes. An obvious hypomotility appeared; most of the decrease developed in the first 4-5 weeks of treatment with little change afterwards. The increase of immobility during one recording session (habituation) was also stronger in the treated rats. ECoG total power increased in the treated rats from the 4th week, with similar time trend as the OF parameters, mainly in the range above the 7 Hz peak but below 30 Hz. Several OF parameters and ECoG total power had good correlation ($R^2 \geq 0.5$) with brain Mn levels. Blood Mn levels during the treatment period and the corresponding ambulation distance and ECoG power data were also correlated, suggesting that the time course of functional changes reflected that of the inner Mn dose.

Experiment 2 and 3 did not provide enough data on the epileptogenic effect of Mn, described in the literature. In Experiment 4, the genetically epilepsy-prone WAG/Rij rats strain was used, treated with Mn orally as in Experiment 2. In Mn-treated WAG rats, decrease of motility was much less than in the controls. Total ECoG power increased massively in the treated rats in the first weeks of Mn exposure. Their OF motility was strongly correlated to ECoG total power and to the power of the low beta band (showing mostly the bursting-related activity). OF parameters were also strongly correlated to brain Mn level, whereas the correlation of ECoG low beta power to brain Mn was weaker. Mn treatment caused increase in the bursting activity of WAG rats. ECoG low beta power during the bursts, and especially the power for one bursting second, increased more than the number of bursts itself.

In all four experiments, administration of Mn in different chemical forms and doses resulted in significant tissue Mn deposition (inner dose) and in alterations of CNS functional parameters. Inner Mn dose and neuro-functional alterations developed in parallel and the final strength of the latter depended on the dose and length of exposure; not only in terms of strength but also in direction. The mostly fair correlations among changes in the OF or ECoG parameters and the measured brain Mn levels raise the question of common mechanisms. These can include the action of Mn on astrocytic glutamate metabolism, leading to imbalance between excitation and inhibition and/or excitotoxicity. Oxidative stress due to mitochondrial damage, inhibition of Mn-SOD, etc., also must be considered, together with the especial sensitivity of the dopaminergic system, responsible for motivation and OF motility. The alterations observed in the Mn-treated rats were to some extent analogous to the effects in exposed humans, regarding both cortical electrical activity and motor behavior. Functional biomarkers of Mn effect, to be developed in the future on the basis of experimental results

similar to those presented in this Thesis, may be more suitable for detection and follow-up of such effects than standard tests like blood Mn level.

The particular points of aims can finally be answered as follows:

- The CNS effects of Mn developed in a non-monotonous way. Depending on the dose and length of exposure, the direction of changes varied (Experiment 1 vs. Experiment 2 and 3); and in case of longer (8-10 weeks) exposure, the effects developed mostly in the first 4-5 weeks and showed little change thereafter.
- The changes in motor behavior and in cortical electrical activity developed in parallel in time. Usually there was a fair correlation between numerical parameters of OF motility and of ECoG (total or band power), and inner Mn dose (brain Mn level) at the end of Mn treatment. In a few cases, the parallel changes of OF and ECoG parameters with blood Mn level during the treatment period could be showed.
- The epileptogenic effect of Mn was occasionally observed in Wistar rats in Experiment 2 and 3. In the epilepsy-prone WAG/Rij rats in Experiment 4, Mn treatment intensified bursting activity.
- Beside the above points, a further noteworthy conclusion could be drawn, namely that intratracheal administration of Mn in form of MnO₂ nanoparticles was far more efficient in causing high inner Mn doses than dissolved Mn applied via the drinking water.

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ABBREVIATIONS

Mn-SOD	Mn-containing superoxide dismutase
CNS	central nervous system
ECoG	electrocorticogram
EEG	electroencephalogram
OF	open field
UnT	untreated control
VT	vehicle-treated control

1. INTRODUCTION

1.1. Neurotoxicity

Neurotoxic studies represent an especially important part within the whole complexity of investigating the biological interactions of living organisms and their environment.

In the life of all creatures possessing some form of a nervous system, this system plays an essential role in perception of relevant external stimuli and in shaping the organism's adequate response to these in order to secure its survival. In case of humans, an improperly functioning nervous system may greatly affect the quality of life at individual level, and cause a loss of mental power, being perhaps the most valuable human resource at population level. The ability of not only humans but various animals to distinguish hundreds of hues by vision or to execute delicate movements shows, among others, that the nervous system is a highly fine-tuned one: a system where apparently minor external influences can cause significant functional alterations.

So, for example, in rat cerebellar Purkinje cells, modification of less than 1% of the Na-channels by tetramethrin (a pyrethroid-type insecticide: Lah, 2011) is enough to induce abnormal, repetitive firing (Song and Narahashi, 1996). Such repetitive firing is also the elementary mechanism of epilepsy, one of the well-known abnormalities of central nervous functions which still represents unanswered questions in spite of decades of intensive research (Fisher et al., 2005), and in the causation of which the role of external, environmental factors is becoming more and more acknowledged.

1.2. Environmental neurotoxicants

The list of known or suspected environmental neurotoxicants is long and keeps growing. It includes substances of both natural and synthetic origin, the presence of which in various media can likewise be natural or man-made. Environment is meant here in a broad sense that includes workplaces, homes, outdoor areas, etc. Exposure of humans to environmental neurotoxicants can happen by inhalation, or by consumption of foods and drinking water (which, originating from the environment, transmit the toxic effect to humans). In occupational environment, dermal exposure may also be of interest. The major groups of

relevant toxicants include both organics (pesticide agents, volatile solvents, monomers, etc.) and inorganics, mainly metals and metal compounds.

Under the original, prehistoric natural conditions, many of the metals known now to be neurotoxic had minimal bioavailability for humans. With the very first steps towards what we call now technology, this was profoundly changed. The appearance of metallurgy was on one hand a major technical achievement, but on the other hand it was a source of human exposure and environmental pollution. Lead was, e.g., discovered around 3500 BC and became perhaps the most important metal of antique technology, with a yearly production of 80,000 tons in the prosperous period of the Roman Empire. Symptoms of lead poisoning – loss of appetite, fatigue, lead colic, irritability, nervous spasm – in lead-mine workers were observed e.g. by Hippocrates, and have been described the same way since then. The environmental effects of lead industry were not recognized in those times but the emissions from ancient mines and smelters can be identified even today (Breitenlechner et al., 2010; Thevenon et al., 2011).

The advances of analytical chemistry in the 18th and 19th century led to the discovery of numerous chemical elements, including metals. Several of them found industrial application sooner or later so that their toxicity (having the typical workplace conditions of that era in mind) became an important issue. Manganese was discovered in 1774 by the Swedish chemist Johan Gottlieb Gahn, and a report on “manganese madness” was published as early as in 1837 (Couper, 1837).

1.3. Manganese: applications, toxic effects, neurotoxicity

In pure state, manganese (Mn) is a silvery-grey, hard and brittle metal. In such form it is not used but its alloys and compounds are found in numerous practical applications from steelmaking to nano-electronics (e.g. semiconductor nanocrystals: Yang et al., 2005; or ZnS:Mn²⁺ nanoflowers, three-dimensional synthetic nanostructures used in supercapacitors: Chen et al., 2005). Mn shows various oxidation states between 2 and 7; a property with, among others, toxicological importance. It is an essential trace element for all living organisms but has toxic effects when overdosed. For adult humans, the daily demand is 2-5 mg (Greger, 1998), and the amount stored in the body (first of all in the liver and kidneys) is ca. 10 mg. The Mn content of ingested food and drinking water is absorbed to 5-15% only (Greger, 1998). This intestinal absorption, the physiological way of covering Mn demand, is a well-regulated process where Mn overload leads to decreased absorption rate. Parenteral

absorption, e.g. after inhalation or intravenous administration, can be much more efficient which has toxicological relevance.

Numerous enzymes have Mn in their active centre. Glutamine synthetase, e.g., is a glia-specific Mn-protein that catalyses the conversion (that is, inactivation) of the excitatory transmitter glutamate to glutamine. Although this enzyme requires Mn, it is inhibited by its excess which is one of the mechanisms of Mn-induced neurotoxicity (Normandin and Hazell, 2001).

Also, two enzymes of the mitochondrial electron transport chain, succinate dehydrogenase (complex II) and ubiquinol–cytochrome-c reductase (complex III) can be inhibited by Mn excess (Malecki, 2001; Zhang et al., 2003), leading to energy shortage and oxidative stress. The latter is counteracted (among others) by another Mn-enzyme, Mn-containing superoxide dismutase (Mn-SOD). Further Mn-enzymes include oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, lectins and integrins. In the nervous system, disturbed energy metabolism (in itself a systemic effect) can alter excitatory transmission by the abnormal release of glutamate, by blocking glutamate reuptake, and by increasing postsynaptic responses to glutamate receptor activation (Calabresi et al., 2001; Normandin and Hazell, 2001). Inhibited mitochondrial function probably blocks tyrosine hydroxylation, a crucial step of dopamine synthesis, contributing to dopaminergic dysfunction (Parenti et al., 1988). Dopamine depletion can also happen by Mn-induced autooxidation of dopamine. In addition, Mn^{2+} was found to interfere with Ca-channels of neurons and presynaptic endings (Takeda et al., 2002), resulting in generally abnormal conduction and transmission of excitation.

Occupational Mn exposure typically means chronic inhalation of dusts and fumes, and the first documented cases of Mn-induced central nervous disease (see above) were indeed miners who inhaled Mn ore dust. The resulting neurological disorder is today called manganism, and it usually progresses in three stages (Saric et al., 1977; Calne et al., 1994). The first symptoms are nonspecific like apathy, anorexia, asthenia, headache, hypersomnia, spasms, arthralgia, weakness of the legs, and irritability. In the second stage, psychomotor and psychic disturbances dominate, such as dysarthria, excess salivation, and difficulty in walking. The third stage represents a Parkinson-like syndrome with its associated symptoms. One of these symptoms is bradykinesia but in the early phase behavioral disinhibition with hypermotility also occurs (Calabresi et al., 2001). And, in spite of the similar symptoms, the site of damage in manganism and in Parkinson's disease is different, since Mn affects the striatal, and not the mesencephalic, dopaminergic neurons (Erikson and Aschner, 2003). The similarities with Parkinson's disease are the presence of generalized bradykinesia and widespread rigidity; and

the dissimilarities, less-frequent resting tremor, more frequent dystonia, a particular propensity to fall backward, and failure to achieve a prolonged therapeutic response to levodopa (Takeda et al., 2002).

Besides metallurgy, Mn inhalation is also typical in welding jobs because the coating of welding rods often contains Mn in order to protect the glowing hot welded metal parts from oxidation. Spray of Mn containing agricultural fungicides (Maneb, Mancozeb; Ferraz et al., 1988) can also be inhaled accidentally. Non-occupational airborne Mn exposure might result from car fuel (petrol) containing methylcyclopentadienyl manganese tricarbonyl (MMT) as anti-knock agent (by now, it has been mostly phased out).

Inhalation is, however, not the only way of Mn exposure leading to central nervous system (CNS) damage. Parkinson-like disorder caused by Mn was also observed in patients undergoing maintenance hemodialysis (Ohtake et al., 2005), or after inadvertent overdosing due to long-term consumption of a health supplement which contained high levels of Mn. The latter case indicates that oral exposure to Mn can also be relevant in terms of health of the CNS, and hence deserves being included in neurotoxicological studies. Unusually high Mn levels in drinking water were observed due to geological reasons (e.g. in Greece: Kondakis et al., 1989) or to anthropogenic pollution (such as improper disposal of used dry cells in Japan: Kawamura et al., 1941); and in the affected population, CNS symptoms were detected. In regions of the USA with high-Mn drinking water, loss of visual and verbal memory, typical consequences of Mn-induced brain damage, was described (Woolf et al., 2002). Foodborne overexposure by Mn in babies fed on cow milk- or soybean-based formulas was reported (Marlowe and Bliss, 1993) as was hypermanganesaemia following long term parenteral nutrition (Crook, 2001). Another, although infrequent, way of non-ingestional Mn exposure is the application of trisodium mangafodipir (Mn-DPDP, Teslascan: Rofsky and Earls, 1996) as contrast agent in magnetic resonance imaging. The simple Mn compound MnCl_2 has also been used for magnetic resonance imaging (MRI) contrasting (Rief et al., 2010). MEMRI (manganese enhanced magnetic resonance imaging) is also an animal experimental method.

The neurotoxic spectrum of Mn is variable. It goes beyond classical manganism, and includes, among others, epileptic disorders (see below). In young shipyard workers, electroencephalographic (EEG) and visual evoked potential alterations were observed and blood Mn levels up to 14 $\mu\text{g/L}$ were measured (Halatek et al., 2005), while in reference groups blood Mn is 5–7 $\mu\text{g/L}$ (Bader et al., 1999). Disturbances of EEG and evoked potentials following occupational Mn exposure were also reported by Sinczuk-Walczak et al. (2001) and Sjögren et al. (1996).

1.4. Epilepsy: concept, external causation, role of environmental chemical factors

Epilepsy is, defined by the International League Against Epilepsy, a brain disorder characterized by the prolonged or steady predisposition to produce epileptic seizures. These are in turn defined as transient occurrences of signs or symptoms of abnormal – excessive or hypersynchronous – activity of brain neurons (Fisher et al., 2005). The concept of epilepsy in fact comprises a group of diseases with various causative factors, different manifestations and varied severity. It has always been a major issue during the development of modern neuropsychiatry and neurology, with several questions still unresolved.

External causes, including environmental ones, can be found mainly in cases of human epilepsy appearing in adulthood, while early-onset epilepsy is mostly idiopathic (Fisher et al., 2005). There are exceptions, however: the documented cases of Mn-induced epileptic activity occurred in children or young adults.

Apart from physical phenomena like flickering light, the environmental epileptogenic agents are typically chemicals (metals, solvents, agrochemicals, etc). Most of the modern insecticides, e.g., act on the nervous system (of both target and non-target organisms). Exposed persons were found to show epileptiform symptoms after acute intoxication, with lindane (Hrncic et al., 2011), organophosphates (Brown and Brix, 1998), or cypermethrin (Condés-Lara et al., 1999). The common basis of epileptogenicity seems to be here a dysbalance between excitation and inhibition. Lindane is a GABA_A antagonist (Maurissen and Fonnum, 2006), similarly to the well-known laboratory convulsant picrotoxin. Organophosphates cause primarily cholinergic hyperfunction, and the resulting status epilepticus in experimental animals (known also from human victims of severe poisoning) can be reversed not only by cholinergic antagonists (such as atropine: Shih and McDonough, 1999) but also by GABAergic agonists like diazepam or midazolam (Gilat et al., 2005), and both drugs are included in the standard treatment protocol of organophosphate poisoning (Bencze and Göbl, 1998). Pyrethroids cause abnormally long open state of the neuronal Na⁺ channels (Narahashi, 1987), leading to repetitive discharges. A comprehensive paper by Colosio et al. (2003) however, does not mention seizures or convulsions among the significant consequences of insecticide poisoning.

Exposure to neurotoxic industrial solvents also caused such problems. There were reports about trichloroethylene-induced myoclonic encephalopathy (Sanz et al., 2008), as well as general seizures following ingestion of 1,4-butanediol (Mégarbane et al., 2002) and styrene (Welp et al., 1996).

The metals brought into connection with epileptogenesis include one with pharmaceutical and a few with industrial application. Lithium (Li) has been used to treat bipolar psychotic disorders for over 50 years. In a few cases of overdose, epileptic activity was reported (Yip and Yeung, 2007). It is noteworthy here that Li is also applied in the pilocarpine-based animal epilepsy model (Curia et al., 2008).

Iron (Fe) contributes to epileptogenesis in cases of brain haemorrhage or trauma, with release of free Fe into the cortical tissue. In animal experiments, application of a soluble Fe compound, like FeCl₃, in or on the brain induces recurrent seizure activity, in the development of which oxidative stress resulting from the redox activity of Fe²⁺/Fe³⁺, and glutamatergic hyperactivity, play a role (Willmore and Ueda, 2009). Inhalation of dust and/or fumes of Fe, although a frequent occupational exposure, has not been brought into connection with epileptic disorders.

Concerning manganese, however, a number of cases have been reported where Mn exposure of environmental or occupational origin resulted in an epileptiform disease (Gonzalez-Reyes et al., 2007). Accidental inhalation of welding fumes caused hypermanganesemia and led to progressive generalized seizures in a three years old child (Hernandez et al., 2003). An even younger child developed seizures after a long period of total parenteral nutrition delivering too much Mn (Komaki et al., 1999). A 17 years old welder developed myoclonus and his Mn-overload was verified (Ono et al., 2002). As mentioned above, children or young adults were the victims in these cases.

Abnormally high activity of the glutamatergic excitatory transmission seems to be a common point between epileptogenesis and Mn neurotoxicity. On the one hand, Eid et al. (2008, 2012) described downregulation of glutamine synthetase in patients with mesial temporal lobe epilepsy, thought to originate from the hippocampus, and modelled the human disorder by inhibiting the same enzyme in the hippocampus of rats. On the other hand, glutamine synthetase is a known point of attack in the neurotoxicity of Mn (Normandin and Hazell, 2001).

1.5. Structures and functions of the rat nervous system involved in the study

1.5.1. Cortical electrical activity

The central nervous system processes sensory input from the outside and inside environment of an individual and initiates adequate motor and other responses. The cerebral cortex, serving the highest level of information processing, is uninterruptedly active throughout the whole life

of the organism. This is reflected in continuous electrical signals which can be recorded from the human head skin (electroencephalogram, EEG) or, typically in animal experiments, from the exposed surface of the cortex or the dura mater (electrocorticogram, ECoG). ECoG (and EEG) can be defined as a stream of partly wave-shaped, partly irregular electrical deflections, and be characterized by its spectral composition, that is, by the presence of waves of various frequencies as components. It is the result of spatiotemporal summation (at large scale, as several ten millions of cells – or tens of cortical columns – are “seen” by a conventional human scalp electrode) of postsynaptic potentials of the cortical pyramidal cells. According to the idealized, simplistic model, the apical dendrite of a pyramidal cell rises at right angle to the cortical surface, and when a synapse somewhere on the dendrite induces a local postsynaptic potential, a transient dipole is formed, with the polarity depending on the location and character of the synapse (Fonyó, 2004). The mentioned spatiotemporal summation results in waves because in the nonspecific thalamic nuclei, from which the axons synapsing at the apical dendrites in the cortical layers I and II originate, a cyclic rhythm of inhibition is generated by inhibitory interneurons driven by the collaterals of the thalamocortical axons. Likewise, the spread of specific thalamocortical activation within the cortex (or an activation of local origin) is limited by surround inhibition from the activated column to its neighbours. If there is excess activation or if inhibition is weakened, more and more local and distant neurons can be recruited to this hyperactivity, and this is how the development of the primary epileptic focus and its generalization is explained (Lothman and Collins, 1990).

At the elementary level, all electrical phenomena of the nervous system arises from the activity of neurons, which results from transmembrane ionic currents. In the membrane, the ions flow through specific channels the opening and closing of which is controlled either by the local transmembrane potential or by ligands (transmitters, regulatory substances) binding to the receptor the given channel belongs to. All the channels and receptors are possible sites of action for neurotoxicants, and because the ions carrying the membrane currents are mostly metal ions, the role of toxic metals (including Mn) in disturbing nervous functions is obvious (and has been verified many times). Ca-channels provide a good example for that (Büsselberg, 1995) especially with the role of Ca in intracellular signalling.

1.5.2. Behavioral phenomena and the underlying structures

Behavioral tests have long been applied in assessing the effects of substances acting on the CNS. In contrast to cellular and molecular mechanisms, behavior is an integrated output of a

vast array of chemical and electrophysiological changes in the nervous system (Paul et al., 1997).

Open field (OF) tests are widely used to study locomotor and other activities that comprise exploratory behavior of the rat (Clark et al., 2005; Nemati and Whishaw, 2007). There are several theories about what motivates locomotor behavior in animals that are not deprived of food or water. According to one, exploration is motivated by fear and the information acquired during exploration serves to reduce fear. Others stated that exploration is only a response to unpredicted stimulation, and functions to gather information about that stimulation; and still others that exploration is not a manifestation of a single motivation but a product of the combined influences of curiosity and fear (Whishaw et al., 2006).

The motor systems involved in OF activity comprise the cortex, especially the precentral gyrus, subcortical structures like the basal ganglia, the cerebellum, the spinal cord and the peripheral nerves down to the neuromuscular junction. The basal ganglia (substantia nigra, caudate, putamen and globus pallidus) have been found to contribute to the initiation and execution of movements, sequencing of movements, automatic execution of routine movements, inhibition of competing motor programs, motor learning and reward mechanisms (Hauber, 1998; Herrero et al., 2002). Basal ganglia damages (generated among others by Mn exposure) can appear in changes of muscle tone, of speed and quantity of movement, and in incoordination. Akinesia and bradykinesia (observed among the symptoms of both Parkinson's disease and manganism), related to slowed motor execution, are the major impairments associated with basal ganglia dysfunctions (Hauber, 1998).

1.6. Aims

The functional neurotoxicity of Mn and other heavy metals has been investigated at the Department of Public Health for ca. 15 years. Both the electrophysiological and the behavioral methods brought a substantial amount of new information. However, even in those works where both methods were applied (e.g. Vezér et al., 2005; Horváth et al., 2012) the comparison of effects on cortical (or peripheral) electrical activity and OF motility was encumbered by the fact that electrophysiological recording was done in anaesthesia and hence its data could not be directly put in parallel with the behavioral effects. Moreover it was hard to make any statement on how the Mn-induced functional alterations evolved in time.

In the meantime, a methodological development – realized in cooperation with Experimetria Ltd, Hungary – enabled us to perform repeated simultaneous recording of ECoG and of OF motility in awake rats (Papp, 2009; Takács and Papp, 2010b).

So, in the research work described in this thesis, rats prepared for this chronic recording were used, and Mn was administered to them for 4 to 8-10 weeks orally by the drinking water or via the airways by intratracheal instillation; that is, in forms assumed to be more or less realistic models of human exposure. The particular aims of the work were:

- To observe the development of the CNS effects of Mn in time;
- To examine to what extent changes in motor behavior and in cortical electrical activity develop in parallel, and what relationship can be detected between the two; and
- To examine whether the epileptogenic effect of Mn, mentioned in the literature, can be observed under the experimental conditions applied.

To implement these aims, four experiments (summarized in Table 1 on page 13) were performed.

2. MATERIALS AND METHODS

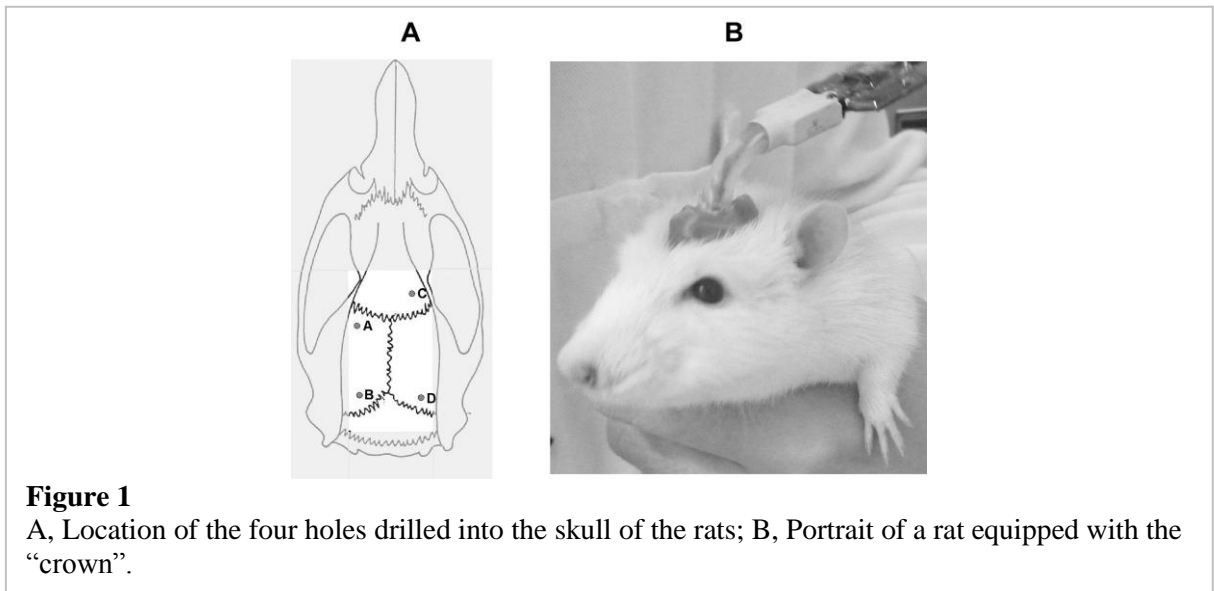
2.1. Animals and preparation

The experiments were done on adult male rats. In Experiment 1, 2 and 3 (Table 1) these were Wistars of 10-11 weeks of age, with ca. 350 g body weight, and were obtained from the breeding centre of the University of Szeged. Rats of this age were needed because their skull bones are strong and no more growing.

In Experiment 4, WAG/Rij rats were used (their age and body weight was about the same as of the Wistars). A stock of parent males and females was obtained from Charles River, Germany (so, these precisely were WAG/RijCrl). They were bred several times with the maximal exclusion of in-breeding, and the male offspring were used in the experiments.

WAG/Rij (Wistar Albino Glaxo from Rijswijk) is a strain specially developed to model human temporal lobe epilepsy (Coenen and van Luijtelaar, 2003). These rats show characteristic absence states and short (5-15 s) bursts of spike-and-wave discharges (SWD) in the cortical electrical activity. This strain has been extensively used for screening and testing antiepileptic agents (Marescaux et al., 1984).

For repeated recording of ECoG, the rats were implanted with chronic electrodes and a connector “crown” (Fig. 1). The operation was carried out under aseptic conditions.



For anesthesia, isoflurane was used in 100% O₂. For induction, 4.5% isoflurane was given by saturating the interior of a glass jar. The animal was put in the jar, and on reaching total analgesia (tested by hind foot pinching) it was quickly clamped into a head fixing frame, and

the anesthesia was maintained with 2-3% isoflurane, administered from a custom-made orofacial mask in open system (at 400-500 ml/min oxygen flow, the amount escaping was negligible). The mask was shaped so that it did not interfere with the incisor bar of the frame. The fur was shaved from the skull cap, the skin was disinfected with Betadine, and a mid-sagittal skin cut was made, starting between the eyes and ending behind the occiput, down to the bone surface. The skin was pulled aside and fixed, and the dorsal surface of the skull was scratched free of soft tissue, over the complete frontal, parietal and interparietal bones to the occipital edge, and laterally between the two temporal ridges (Fig. 2A). Care was taken not to damage the muscles lateral to the temporal ridge. The surfaces were repeatedly rinsed with 3% H_2O_2 , and bleeding was stopped by cautery. Finally the bone surface area to be used later for gluing was etched with concentrated (30%) H_2O_2 (Fig. 2B) and was primed with cyanoacrylate adhesive.

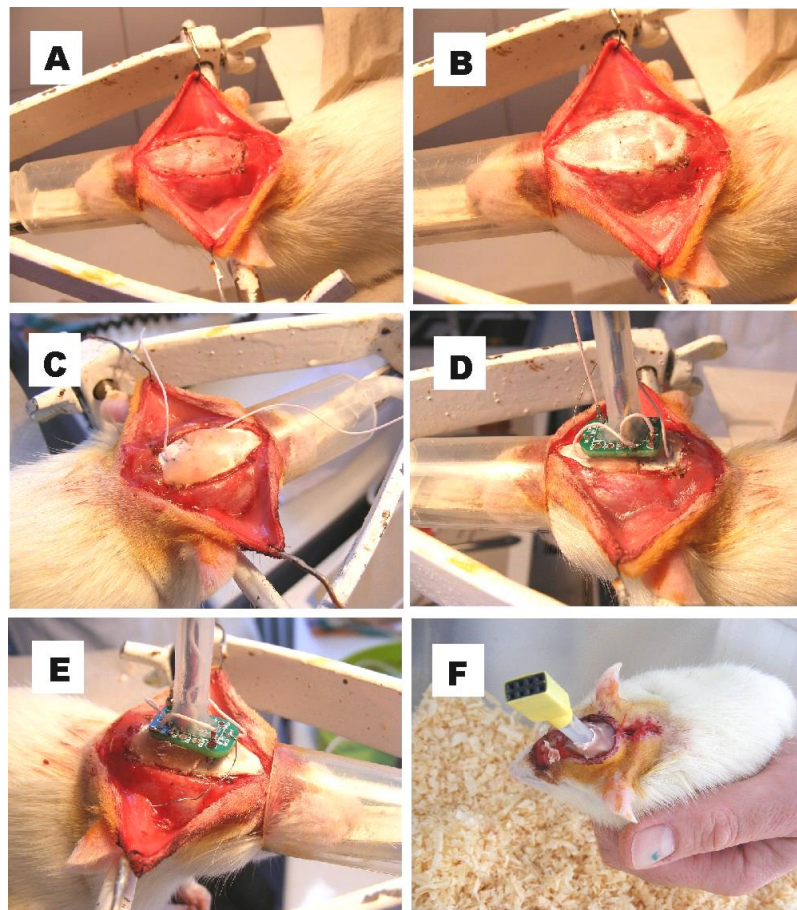


Figure 2

Steps of implanting the cortical electrodes. A, skull bone cleaned; B, bone treated with H_2O_2 ; C, silver wire electrodes glued in the drilled holes; D, crown base fixed with screws driven into the remaining holes; E, crown base secured with dental acrylic; F, operation ready.

Four holes of ca. 0.6 mm diameter were drilled in the skull over the right and left frontal and parietal lobe, down to the epidural space, at the locations shown in Fig. 1. In two holes (B and C in Fig. 1A), the stainless steel screws fixing the crown and serving as electrodes were driven, and in the other two (A and D in Fig. 1A), silver wire electrodes were placed. The position of hole B and C was dictated by the size and shape of the crown base; the other two holes were placed so that the activity of the whole hemispheres could be recorded.

It was tested previously on separated skull bones, how long the screws had to be driven to touch the dura without puncturing it, and the silver wires were bent back in 180° to be non-traumatic. The silver wires were placed first (Fig. 2C) and fixed with dental acrylic (Duracryl) then the crown base was fastened with the two screws (Fig. 2D). The screws and the silver wires were connected to the crown base and the base was secured to the skull with dental acrylic (Fig. 2E) and covered from above with the glue. Dilute H₂O₂ was applied again, the skin was sutured (Fig. 2F), and the rat was allowed a recovery period of 10-14 days. Before and after surgery, sufficient analgesic and antibiotic treatment was given (30 mg/kg b.w. amoxicillin plus 0.2 mg/kg. b.w. meloxicam, injected subcutaneously 2 hours before the intervention and repeatedly after it as necessary).

The “crowned” animals had to be kept one-by-one in separate high-walled cages but needed no extra care. They were kept under standard conditions (22±1 °C, 30-60 % relative humidity, 12-h light/dark cycle with light on at 06:00), and free access to standard pellet and water (tap water or MnCl₂ solution, see below). Every rat was marked with a letter or number written on the base of tail with permanent marker. The procedures applied were approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged. During the whole experimental work, the principles of the Committee were strictly followed.

2.2. General description of the experiments: time scheme and manganese exposure

Altogether four experiments are included in this thesis. Their general description is given below and in Table 1.

The experiments for each rat started after the mentioned recovery period. First, two control recording sessions (see 2.3.) were held with one week interval (that is, 7 days apart) without any treatment (that is, free of any Mn effect). Then, Mn administration for the treated rats (and vehicle administration for the controls in Experiment 3) was started, and lasted as given in Table 1 with further recording sessions in one week intervals. Body weight of the orally exposed rats (and their parallel controls; Experiment 1, 2 and 4) was measured at every

change of the Mn solution, and of the intratracheally treated rats (Experiment 3) before every administration. When the treatment period was over (or when an animal fell out of the experiment because of crown loss or damage) the rats were overdosed with urethane, dissected, and organ weights (brain, lungs, liver, heart, spleen, kidneys, adrenals, thymus) were measured. Whole brains and lungs, a ca. 1 g slice from the liver, and 2-5 ml blood samples were taken and stored at -20 °C for Mn level determination.

Table 1 General data of the experiments

Experiment No.	Rat strain	Manganese treatment			Number of rats evaluated			
		Substance	Dose	Time [§]	Open Field and general tox.		ECoG	
					<i>Treated</i>	<i>Control</i>	<i>Treated</i>	<i>Control</i>
1	Wistar	MnCl ₂ , oral	2.5 mg/mL water	4 weeks	4	4	3	3
2	Wistar	MnCl ₂ , oral	7.5 mg/mL water	8 weeks	4	4	4	3
3	Wistar	MnO ₂ nano intratracheal	2.63 mg/kg. b.w.	8 weeks	7	4 (UnT) 3 (VT)	5	3 (UnT) 2 (VT)
4	WAG/Rij	MnCl ₂ , oral	7.5 mg/mL water	8 weeks	4	3	4	3

UnT, untreated control; VT, vehicle-treated control in Experiment 3.

[§]The length of time axis in Figs. 4, 9, 16 and 23 corresponds to these time spans.

2.2.1. Oral Mn exposure

Mn was given to the rats in two forms. In Experiment 1, 2, and 4, a water-soluble form, MnCl₂·4H₂O (analytical grade, Reanal, Hungary) was used, administered via the rats' drinking water. For that, MnCl₂ was diluted in normal tapwater. In Experiment 1, 2.5 mg/mL was used (based on Máté et al., 2009), and in Experiment 2 and 4, 7.5 mg/mL. This higher dose was chosen because the effects in Experiment 1 were mild and because literature data (Ávila et al., 2008) showed that higher doses are technically possible and are tolerated by the rats. To prevent pH-dependent precipitation, 0.125 mg/mL (Experiment 1) or 0.375 mg/mL (Exp. 2 and 4) citric acid was also added to the Mn solution. Parallel control rats had plain tapwater (the Mn concentration of which was very low, 0.03 µg/mL; Szeged Vízmű, 2010).

The Mn solution was refreshed twice a week; the bottles were weighed at every change to see the fluid consumption of the rats. The solution had apparently no aversive taste because the rats' measured consumption was not significantly different between the exposed and control. Individual Mn exposure of each rat was calculated from the daily consumed volume and the Mn concentration.

2.2.2. Intratracheal Mn exposure

The other mode of Mn exposure, used in Experiment 3, was instillation of a suspension of MnO₂ nanoparticles (NPs) into the rats' trachea. The NPs were synthesized at the Department of Applied and Environmental Chemistry, University of Szeged; their diameter was 30.9±9.9 nm. The NPs were suspended in a lightly viscous vehicle (1% hydroxyethyl cellulose dissolved in phosphate-buffered saline; pH 7.4) which was physiologically neutral and slowed the aggregation of the NPs. The NP suspension was intensively sonicated as it was prepared, and was sonicated again before administration. Instillation was done once daily, 5 times per week. For that, the animals had a brief anesthesia in a glass jar (with isoflurane, the same way used for introduction in the operation described above). The completely anesthetized rat was suspended on a board tilted to 60° 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 brought into direct contact with 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 NP suspension (or the vehicle in the parallel controls) 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. The dose, 2.63 mg/kg b.w., was based on other experiments of the laboratory (Horváth et al., 2012).

2.3. Recording

The recording sessions, one per week for each rat, lasted 30 (Experiment 1, a "pilot experiment") or 60 (Experiment 2, 3 and 4) minutes. Such long sessions were supposed to show the own effects of Mn, after all novelty effects ceased, and to be able to detect any rare event. The data were collected in a combined system (provided by Experimetria Ltd,

Hungary) capable of recording motility and cortical electrical activity in parallel. The rat was in an OF box, detecting and analysing its movements; while ECoG was recorded via a cable attached to the crown.

2.3.1. Open field

The OF component of the system was a black plastic box with 48x48x40 cm inner space, equipped with an array of infrared light gates at floor level. The instrument recorded and analyzed the rat's horizontal motor activity based on the interruptions of the infrared beams, using the software Conducta 1.3 (Experimetria). Detection of vertical activity (rearing) was deliberately omitted because of the false signals the crown and cable would have produced. The room used for recording was lit dimly.

2.3.2. Electrocorticogram

The rat's cortical electrical activity was taken up by a preamplifier mounted on the end of the flexible lead-off cable, which also served as counterpart of the crown connector (Fig. 1B). The two lead-off points on the left and right hemisphere, respectively, provided one bipolar channel each. The amplified signals were fed via swivel contact in the main amplifier. Overall amplification was $10^4 \times$ with high- and low-pass filters set to 1.6 and 75 Hz. The ECoG signals were visualized on the monitor of a PC in real time and stored on the hard disk, using the software Neurosys EEG v1.1.0.72 of Experimetria.

No major difference between the electrical activity on the two channels was seen. So, signals of channel 1 (left hemisphere) were used for analysis of ECoG.

2.4. Analysis

2.4.1. Open field

The software automatically processed the beam interruption data to numerical description of motility. More than 40 mm shift in the location of interrupted beams during a time unit of 1 s was interpreted as horizontal activity (ambulation); less shift, as local activity; and no shift at all, as immobility. These data were obtained and evaluated for the whole 30 or 60 min session, and also in 3 min periods within a session to see short-term changes.

2.4.2. Electrocorticogram

From the ECoG records, the software calculated the continuous power spectrum with 0.5 Hz resolution between pre-set limits which were in the present experiments 1 and 49 Hz. The total power within this frequency range was also calculated. The analysis was based on FFT technique. Evaluation was based on the shape of the power spectrum curve, and on difference spectra obtained as the ratio of a spectrum from a given phase of treatment and a control spectrum.

Band spectra were also obtained, primarily to see any correlation with variables of motility. The band limits were based on monograph references (Kandel and Schwartz, 1985; Szirmai, 2001) and were finally set as follows: delta activity was measured between 1 and 3.5 Hz; theta, between 4 and 7.5 Hz; alpha, 8-12 Hz; low beta, 12.5-15; mid beta, 15.5-18; high beta, 18.5-31, and gamma, 31.5-44 Hz. The lower part of beta was cut in two bands to see better any change around 13-15 Hz where the epileptogenic effect of Mn typically appeared (see. Fig. 15 and 29). Correlation between OF, ECoG and tissue Mn level data was tested by the “linear fit” function of Excel after plotting corresponding data pairs in an X-Y plot.

Spectrum calculations were done for the whole, 30 or 60 minutes long, recording session, or for shorter periods. Three minute periods were used to see the change within one 30 or 60 min session; and 1 s resolution was used in Experiment 4 to obtain numerical data of the visually detected epileptic SWD burst. For that, the maximum and minimum of ECoG power in the 12.5-18 Hz range (low and mid beta) was determined for every 1 s period, and the value of

$$[(max-min)/2 + min]; \text{ called “peak indicator” PI}$$

was calculated to indicate how peaked the maximum was. These values were averaged for the whole 60 min session (PI_{60}). A second in which

$$PI \geq 8PI_{60}$$

was considered a bursting second, and a series of consecutive seconds where

$$PI \geq 2PI_{60} \text{ for every second, and } PI \geq 8PI_{60} \text{ for at least one second}$$

was considered a bursting period. The numerical criteria were set to achieve maximal agreement between the bursts observed on the ECoG and the calculations (illustrated by Fig. 3). These calculations were used to quantify the occurrence and intensity of bursts. (The first idea was to use low beta ECoG power only but mid beta was finally included in order not to omit bursts with a slightly higher frequency.)

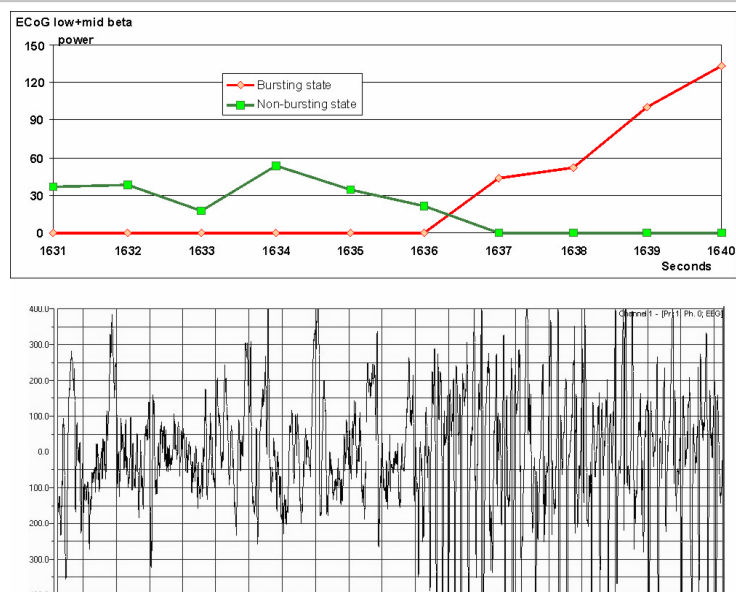


Figure 3

Top: ECoG power of the low + mid beta band in one-second bins, selected to bursting/non-bursting by the criterion described in the text. Bottom: ECoG record of the same period.

2.4.3. General toxicity

Body weight data were plotted against the days of the experiment to obtain weight gain curves. The organ weight data in Experiment 2 and 3 were transformed to relative weights with 1/100 body weight or brain weight as calculation basis.

For Mn level determination, the stored tissue samples were dried at 80°C to constant weight, and were digested in 5 ml 65% HNO₃ at 90°C for 90 min. Mn determination was finally done by inductively coupled plasma mass spectrometry (at the laboratory of the MOL Hungarian Oil and Gas Company). For financial reasons, not all samples underwent Mn level determination.

2.4.4. Statistical evaluation

Due to the small data pool, a simple statistical evaluation was done. Corresponding data of different groups (treated or control) were taken as one data set each and were compared by means of two-sample t-test (Experiment 1, 2 and 4) or one-way ANOVA (Experiment 3). Significance was accepted at $p < 0.05$.

3. RESULTS

3.1. Experiment 1

In Experiment 1, crowned rats were exposed orally to a lower dose of Mn via the drinking water (see Table 1). It was a “pilot experiment” the experiences of which were utilized in designing the forthcoming experiments.

3.1.1. General toxicity and tissue Mn levels

The general toxicity of oral Mn exposure was assessed on the basis of body weight gain. As shown in Fig. 4, the presence of 2.5 mg/mL MnCl_2 in the treated rats’ drinking water had no effect on body weight gain. The time course of body weight was not identical in all rats but both treated and untreated rats belonged to the groups showing higher and lower weigh gain, as shown by the concordant and discordant curves.

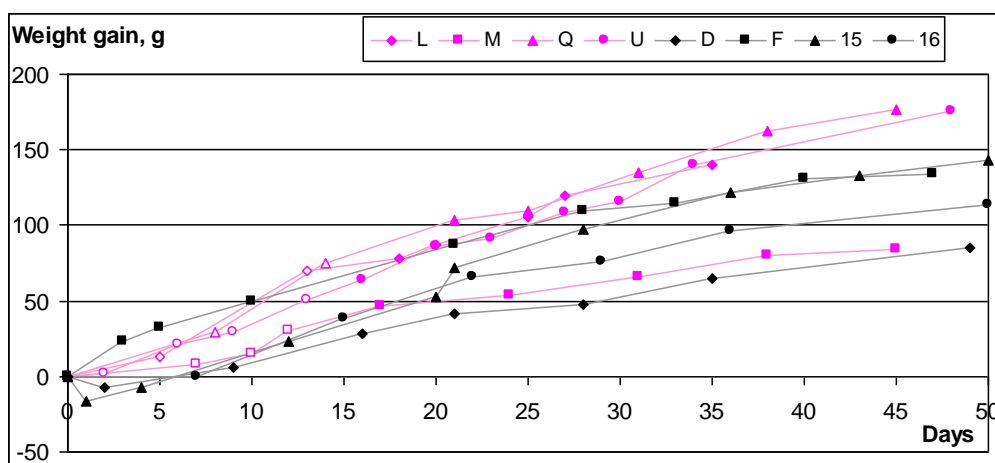


Figure 4

Curves of the rats’ body weight gain in Experiment 1. Mn-treated animals (tail marking: L, M, Q, U) are shown in pink – empty symbols, before Mn application; filled symbols, during Mn application. Control animals (tail marking: D, F, 15, 16) are in black.

Daily water consumption was 56.0 ± 11.6 ml in the Mn-treated and 52.4 ± 8.3 ml in the control group. The values were practically not different from each other and also not much different from that seen in previous experiments with oral Mn exposure (Máté et al., 2009). This indicated that MnCl_2 and citric acid present in the treated rats’ drinking water caused no major off-taste or other sensory effect acting on drinking habits. The Mn uptake, resulting from the calculated summed dose of ca. 1100 mg Mn in 28 days, did not influence body weight gain

(see above) but deposition of Mn in the rats' tissues was detected in the samples taken at the end of the experiment. The data in Table 2 show that the increase of brain and blood Mn levels, vs. control rats consuming normal tapwater, was significant.

Table 2 Mn levels in the control and treated rats' tissue samples, Experiment 1

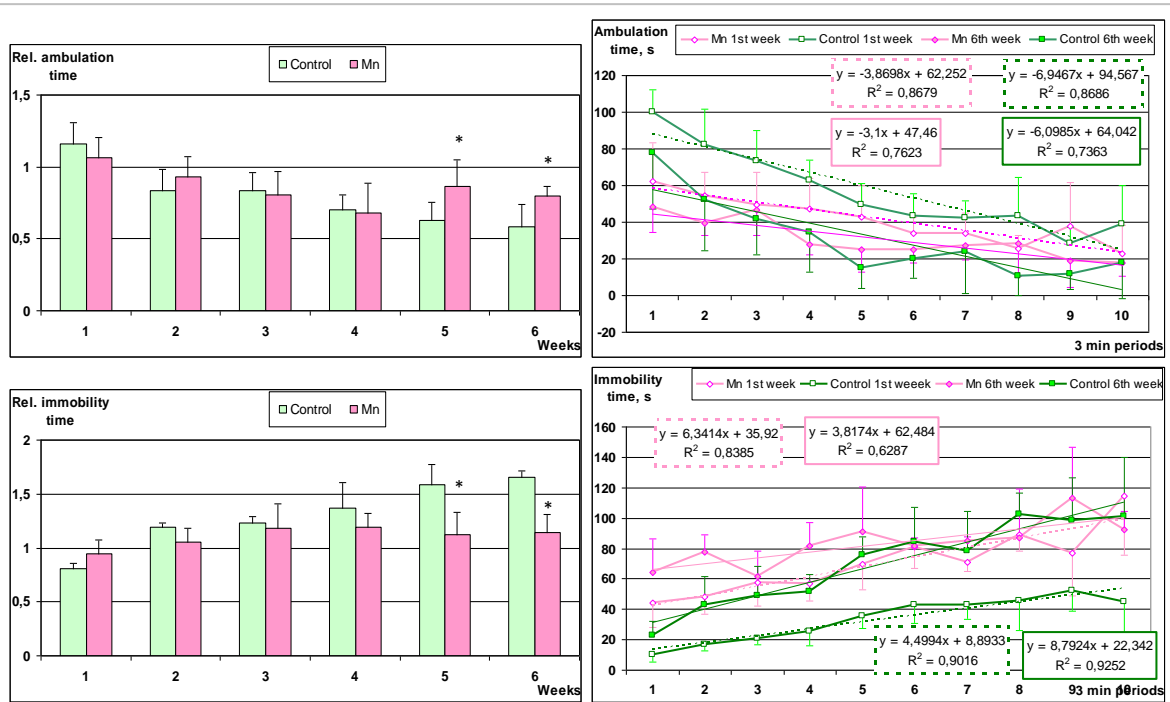
	Mn level ($\mu\text{g/kg}$)	
	Treated	Control
Blood	281.35 \pm 63.62*	181.46 \pm 60.17
Brain	1931.88 \pm 156.93***	984.71 \pm 132.56
Liver	8022.97 \pm 609.25*	7132.24 \pm 471.74

Mean \pm SD, n= 4 (see Table 1). *, ***: p<0.05, 0.001 vs. Control.

3.1.2. Open field motility

A general trend of decreasing motility over the 6 weeks of the experiment was present in the OF data. From the 4th week on, however (i.e. after 2 weeks Mn exposure in the treated rats) a difference developed and reached significance by the end of the experiment: the treated rats' time spent in ambulation remained stable with even a minimal increase while that of the controls decreased substantially, as seen in Fig. 5, upper left graph. The upper right graph shows that the trend of motility decrease within the 30 min session, characterizing the level of habituation to the unchanged environment, was somewhat less expressed in the treated rats. The slope of the fitted lines (see equations in Fig. 5, right graphs) changed less between week 1 and 6 in the Mn-treated than in the control rats, that is, the treated rats remained more alert in the low-stimulus environment of the OF box.

In the trends of immobility, the very opposite of ambulation in the OF tests, this difference was more pronounced. The lower left graph in Fig. 5 shows a continuous increase of the time spent in immobility by the control, but not by the treated, rats during the weeks of oral Mn exposure. The lower right graph shows that immobility increased during the 30 min OF sessions both in control and treated rats, but in the treated the slope of increase was lower after 4 weeks Mn exposure than in the 1st week (decreased habituation), whereas in the controls the slope became higher (increased habituation).

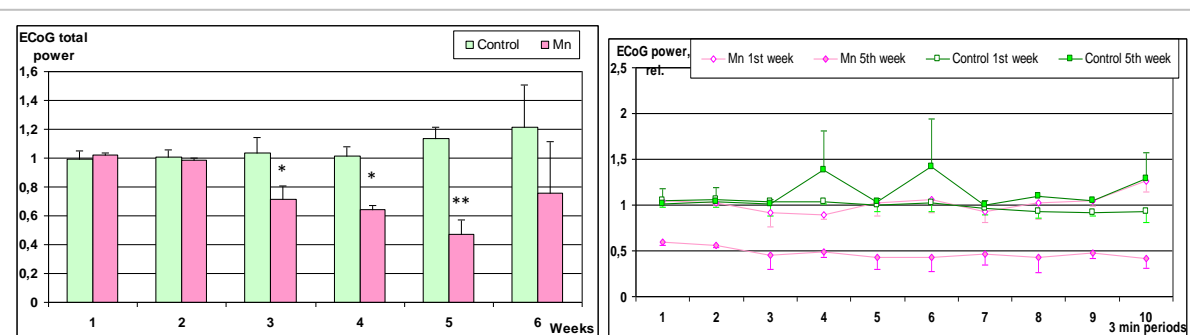
**Figure 5**

Group means of the time spent in ambulation (upper graphs) and in immobility (lower graphs) by the control and treated rats in the 6 weeks of Experiment 1. Left, values normalized to the average of the two control weeks; right, time course of time spent in ambulation or immobility within the 30 min recording session in the 1st and 6th weeks. Trend lines and the corresponding equations have the same line and color and style.

Mean and SD (plus or minus), n=4 (see Table 1). *: p<0.05 vs. Control.

3.1.3. Electrocorticogram

Overall cortical electrical activity, quantified as the total power in the 1-49 Hz range, decreased substantially in the treated rats by the end of the 4th treatment week but did not change much in the controls (Fig. 6, left graph).

**Figure 6**

Group means of ECoG total power (in the 1-49 Hz range) in the control and treated rats in the 6 weeks of Experiment 1 (left graph, values normalized to the average of the two control weeks), and time course of ECoG total power within the 30 min recording session in the 1st and 5th weeks (right graph).

Mean and SD (plus or minus), n=4 (see Table 1). *, **: p<0.05, 0.01 vs. Control.

During one 30 min recording session, there was no trend of change in the ECoG total power either in the control or in the treated rats, in contrast to what was seen in the OF activity (Fig. 6, right graph; cf. Fig. 5).

The ECoG power decrease was present over the whole frequency range in the Mn-treated rats, as shown by the continuous spectra in the 1-49 Hz range (Fig. 7). The basic ECoG spectra had the same general shape irrespective of the Mn exposure, but difference spectra – generated as the ratio of the spectrum of a treatment week to the mean spectrum of the two controls (1st and 2nd week) – showed in the Mn-treated rats a decrease around the typical 7 Hz maximum to develop first, followed by general decrease towards the end of treatment. In controls, this difference spectrum remained closely around 1.0, indicating no change in the shape of the curve.

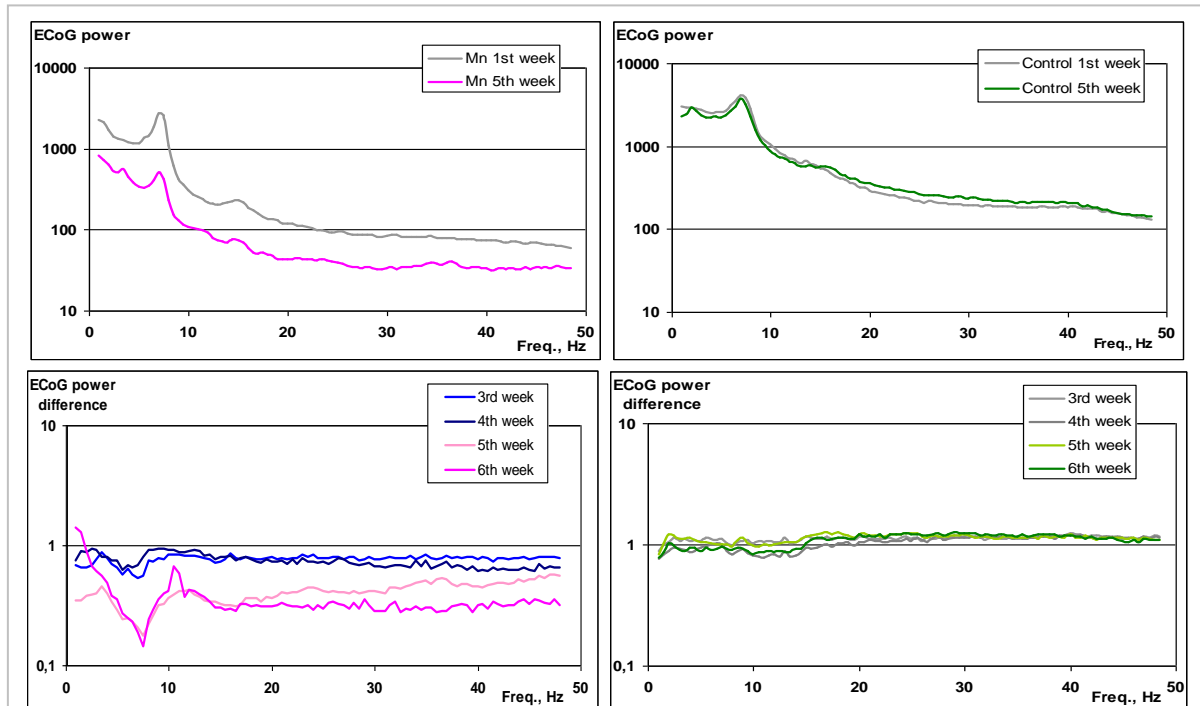


Figure 7

Top: ECoG spectra (group means) in the 1-49 Hz range from the Mn-treated (left) and control (right) rats in the control period (1st week) and towards the end of Mn treatment (5th week).

Bottom: Difference spectra: the ratio of the spectrum in the four treatment weeks to the average spectrum of the control period (left: Mn-treated, right: controls).

3.1.4. Correlations

The time course of the OF parameters and the ECoG power during the 6 weeks suggested a correlation between the two (which, if present, would be biologically plausible). As seen in Fig. 8, this correlation was present indeed, with fair R^2 values, and clearly dissimilar trends in the treated vs. control rats. Taking power of single ECoG bands for this correlation analysis, $R^2 > 0.5$ was found for theta, high beta and gamma in the Mn-treated and for theta, alpha, mid beta and high beta in the control rats, for the correlation with the time of ambulation and immobility (not shown).

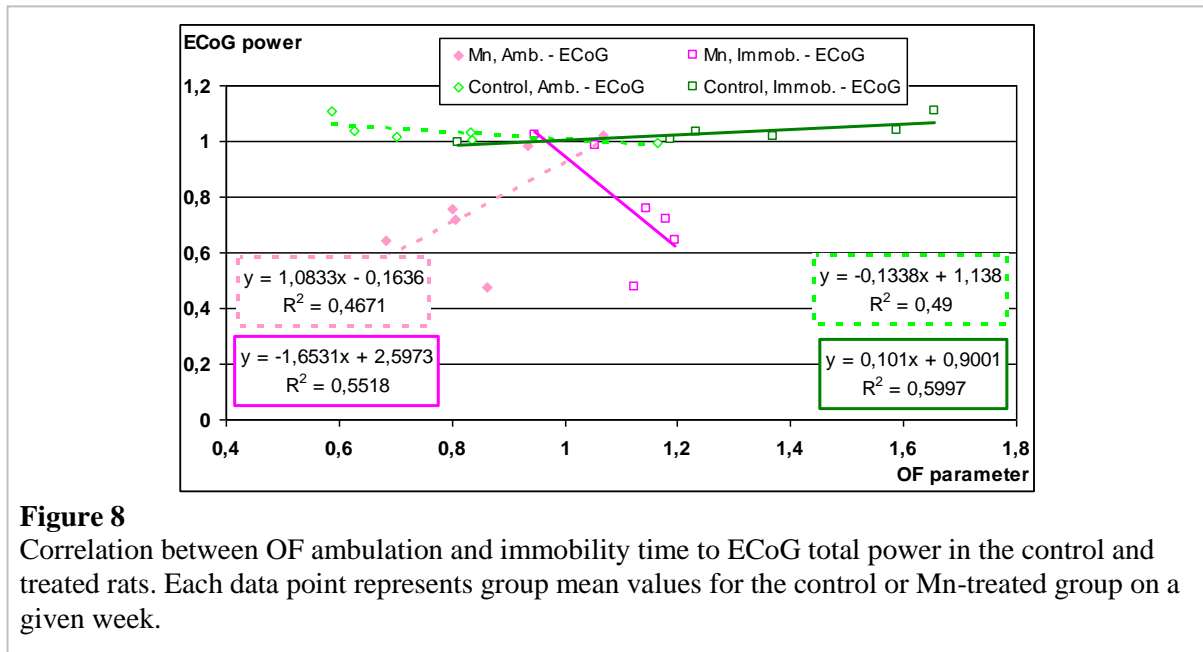


Figure 8

Correlation between OF ambulation and immobility time to ECoG total power in the control and treated rats. Each data point represents group mean values for the control or Mn-treated group on a given week.

3.2. Experiment 2

In this experiment the way of exposing the rats to Mn was identical to that in Experiment 1 but the dose, 7.5 mg MnCl_2/mL water, was higher, and the treatment period was ca. 10 weeks long, giving a calculated summed dose of ca. 5700 mg Mn. The daily drinking volume of the rats was unaffected by Mn also in this experiment. Due to uncertainties towards the end of the treatment, ECoG and OF data of the first 8 weeks of treatment were used for evaluation.

3.2.1. Indicators of general toxicity

Due to the mentioned higher summed external dose (higher Mn concentration in the water, applied for longer time) the mean summed brain and blood Mn levels at the end of the treatment period were higher than in Experiment 1 (Table 3). All the same, the rats' body weight gain was not significantly influenced by the Mn exposure. The control and treated weight curves were in the same range (Fig. 9) and, again, rats with different rates of weight gain were more distinct than treated vs. untreated ones. Of the relative organ weights, only that of the lungs showed some significant alteration both with body weight and with brain weight as calculation basis. There was fair correlation ($R^2 > 0.5$) between the Mn content and absolute weight of the brain, and between blood Mn level and the body weight-relative liver weight.

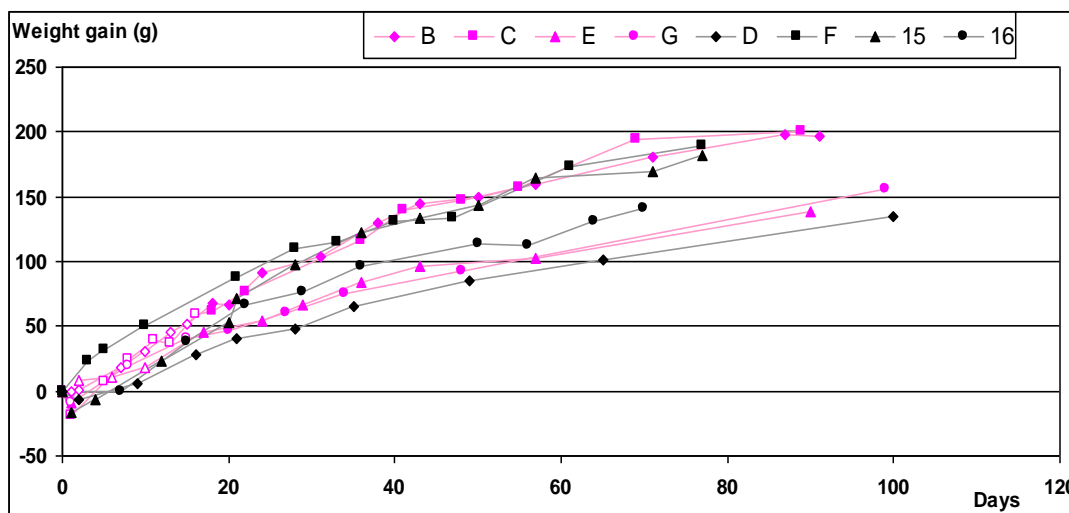


Figure 9

Body weight gain curves of the rats in Experiment 2. Mn-treated animals (B, C, E, G) are shown in pink – empty symbols, before Mn application; filled symbols, during Mn application. Control animals: black.

Table 3 Mn levels in the control and treated rats' tissue samples, Experiment 2

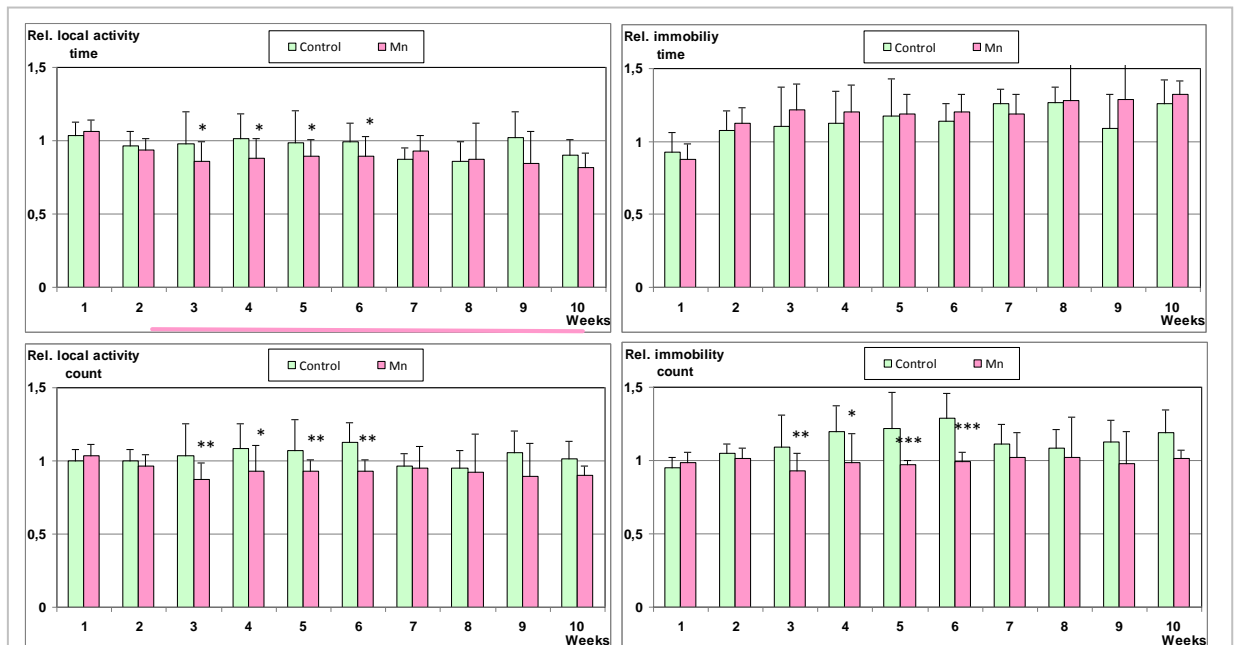
	Mn level ($\mu\text{g/kg}$)	
	Treated	[§] Control
Blood	502.42 \pm 114.98**	181.46 \pm 60.17
Brain	3394.55 \pm 358.72***	984.71 \pm 132.56
Liver	11304.12 \pm 3042.25*	7132.24 \pm 471.74

Mean \pm SD, n: see Table 1. *, ***, $p < 0.05$, 0.001 vs. Control.

[§]The identical way of exposure allowed us to use the control Mn level data of Exp. 1 also to Exp. 2.

3.2.2. Open field motility

The Mn-treated rats in Experiment 2 showed, in contrast to Experiment 1, decreased motility but this change was not very explicit (and might be interpreted as transition to the later phase of Mn-induced Parkinson-like disorder, in correspondence with the higher brain Mn level). Decrease of local activity and increase of immobility was detectable (Fig. 10). Event counts were also informative in this experiment, and showed that the (non-significantly) longer overall immobility time of the treated rats resulted from longer (but not more numerous) immobile periods. Ambulation time data of individual rats on the 10th (last) week and Mn levels in the brain samples were correlated ($R^2 > 0.5$).

**Figure 10**

Group means of time and event count of local activity (left) and immobility (right) for the control and treated rats in the 10 weeks of Experiment 2. The pink strip in the top left graph shows the period of Mn exposure.

Mean \pm SD, n: see Table 1. *, **, ***, $p < 0.05$, 0.01, 0.001 vs. Control.

The trend of change in the OF activity within the 60 min recording sessions was partly similar to that seen in Experiment 1: at the end of Mn exposure (10th week), immobility in the treated rats decreased less steeply than in the controls (Fig. 11). Some indication of the non-linear development of Mn effect, seen first of all in Experiment 3, can be seen here also.

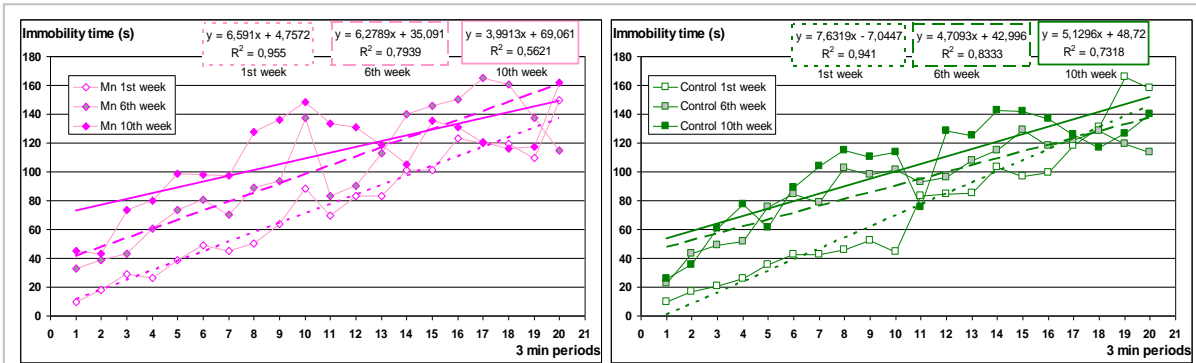


Figure 11

Time course of time spent in immobility within the 60 min recording session in the 1st, 6th and 10th weeks. Left: Mn-treated; right: Control. Trend lines and the corresponding equations have the same line color and style.

Mean values (error bars omitted for clarity), n: see Table 1.

3.2.3. Electrocorticogram

A decrease in the ECoG total power, similar to that seen in Experiment 1, developed only towards the end of the treatment period (Fig. 12). This was, in itself, in line with the less clear-cut changes of motility. During one 60 min recording period, total ECoG power had, similarly to what was found in Experiment 1, no noteworthy time trend either in the treated or in the control rats (hence, not shown).

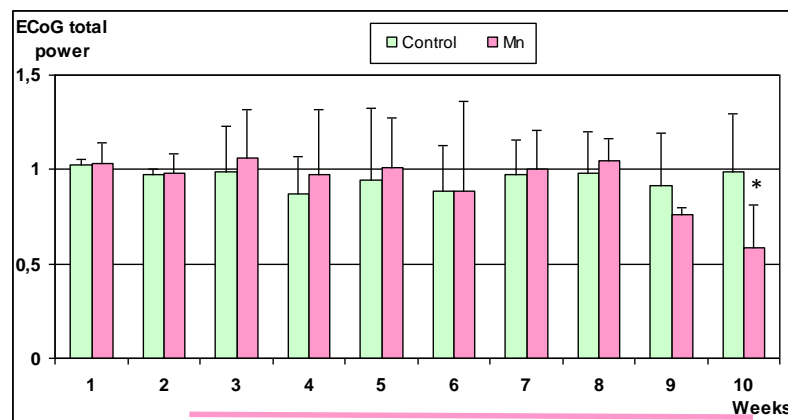


Figure 12

Group means of ECoG total power (in the 1-49 Hz range) in the control and treated rats in the 10 weeks of Experiment 2. Mn exposure marked as in Fig. 10.

Mean+SD, n: see Table 1. *: $p < 0.05$ vs. Control.

The difference spectra in Fig. 13 show a gradually developing decrease of ECoG power in the Mn-treated rats, first of all around the 7 Hz peak and above 30 Hz, while in the controls no such change can be observed.

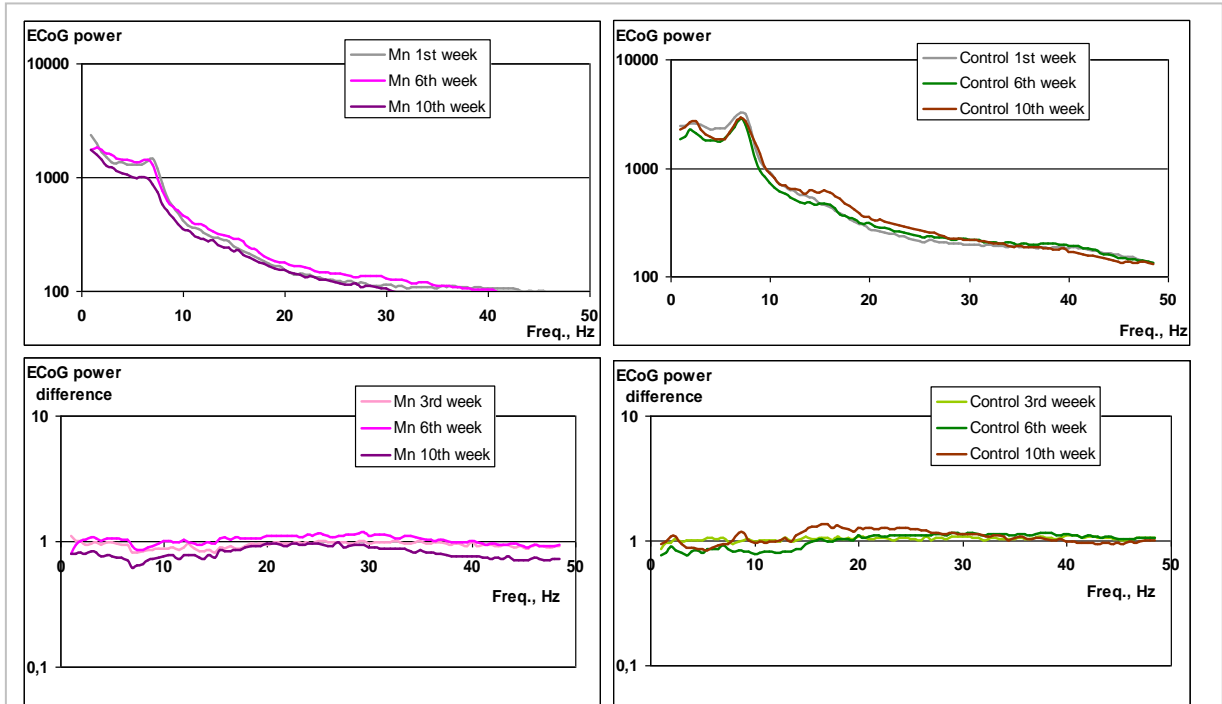


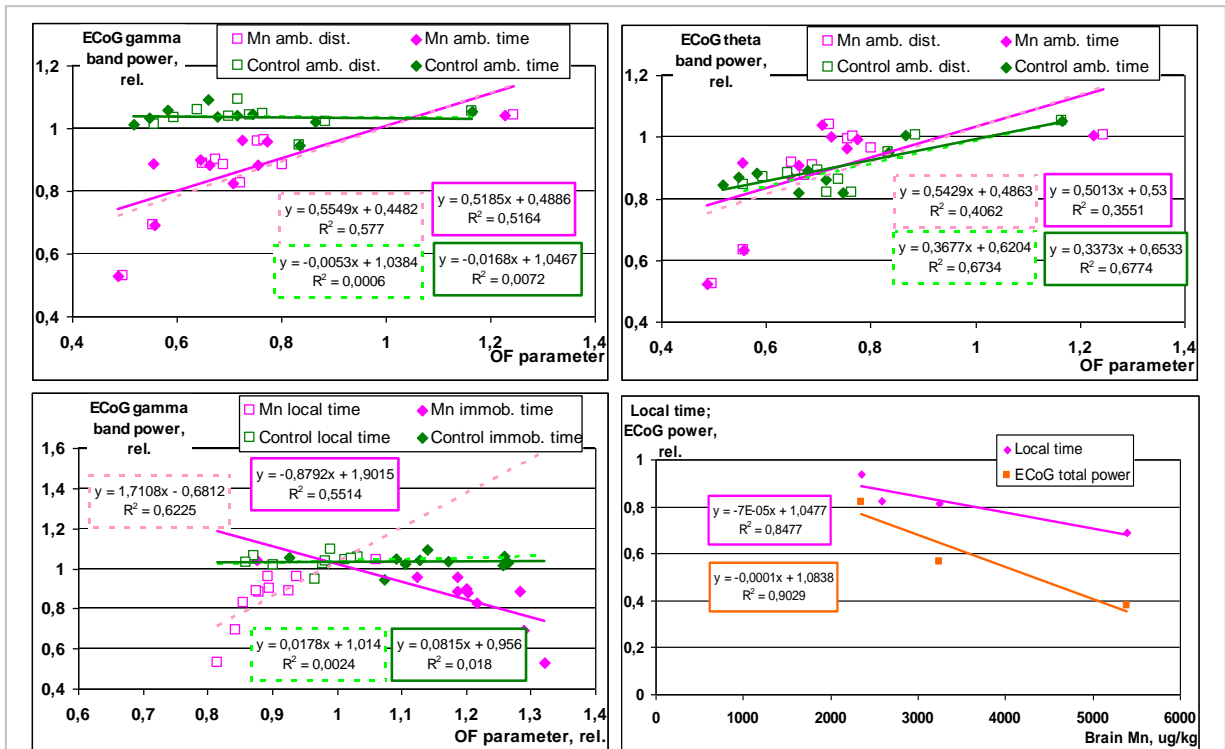
Figure 13

Top: ECoG spectra (group means) from the Mn-treated (left) and Control (right) rats in the control period (1st week) and after 4 and 8 weeks of Mn exposure (6th and 10th week).

Bottom: Difference spectra: the ratio of the spectrum in the 3rd, 6th and 10th week to the average spectrum of the control period (left: Mn-treated, right: Control).

3.2.4. Correlations

In correspondence with the decreased gamma activity, correlation with good R^2 values was found between the time trend of ECoG gamma band power and several OF parameters in the treated rats (Fig. 14). This held true for some of those OF parameters which themselves did not change significantly during the weeks of Mn exposure, and this indicated that the correlation in values of two functional parameters, supposedly influenced by Mn, might be more sensitive than the parameters alone. With ECoG theta band power, the correlation of treated and control rats' ambulation distance and time was nearly identical, although this band was also affected by Mn. The bottom right graph in Fig. 14 shows the relationship of local activity time and ECoG total power to brain Mn levels.

**Figure 14**

Top row and bottom left: Correlation between certain OF parameters and the ECoG power in the theta and gamma band. Data points represent group means in a given week.

Bottom right: Correlation between local activity time and ECoG total power and brain Mn levels. Data points represent individual Mn-treated rats.

Trend lines and the corresponding equations have the same line color and style, dashed lines belong to empty symbols.

3.2.5. Signs of epileptogenic action of Mn

In Experiment 2, Mn dose and treatment time were apparently sufficient to provoke epileptiform activity. Towards the end of the treatment period, short series of spikes (Fig. 15) were observed on the ECoG in two of the four exposed rats (tail mark C and E). These bursts were similar to those seen in the WAG/Rij rats (used in Experiment 4); and were coincident with decreased motility, and a typical peak on the ECoG spectrum around 13 Hz.

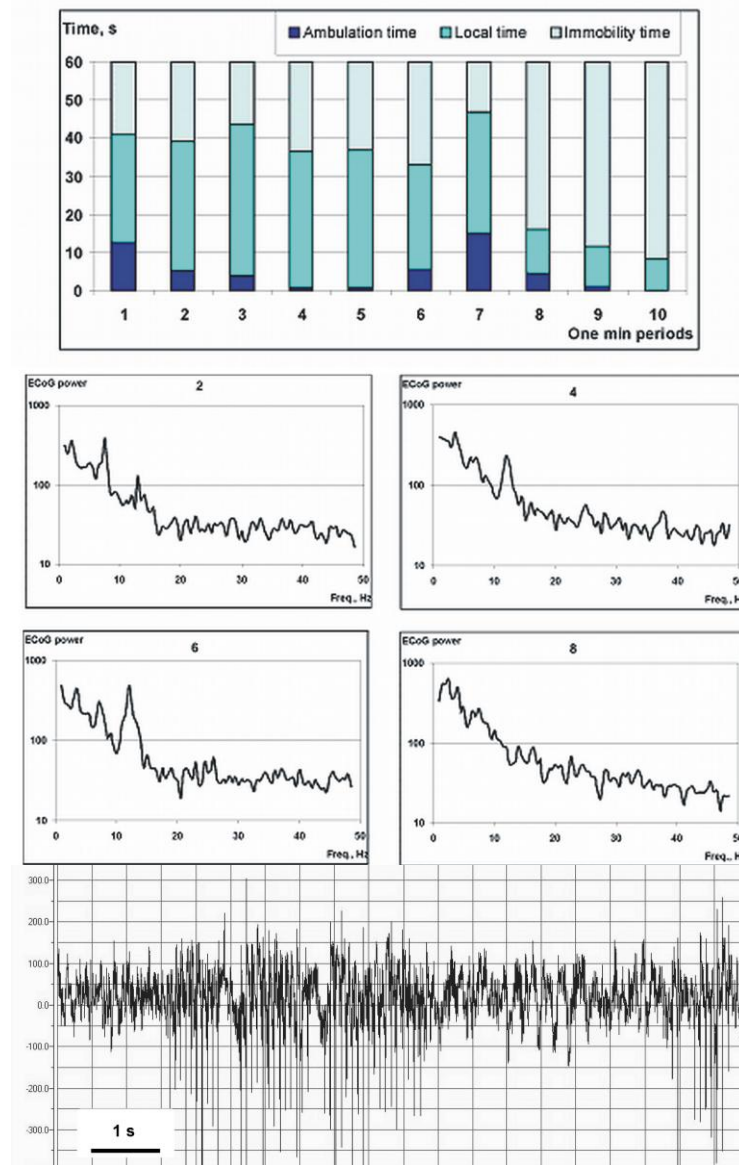


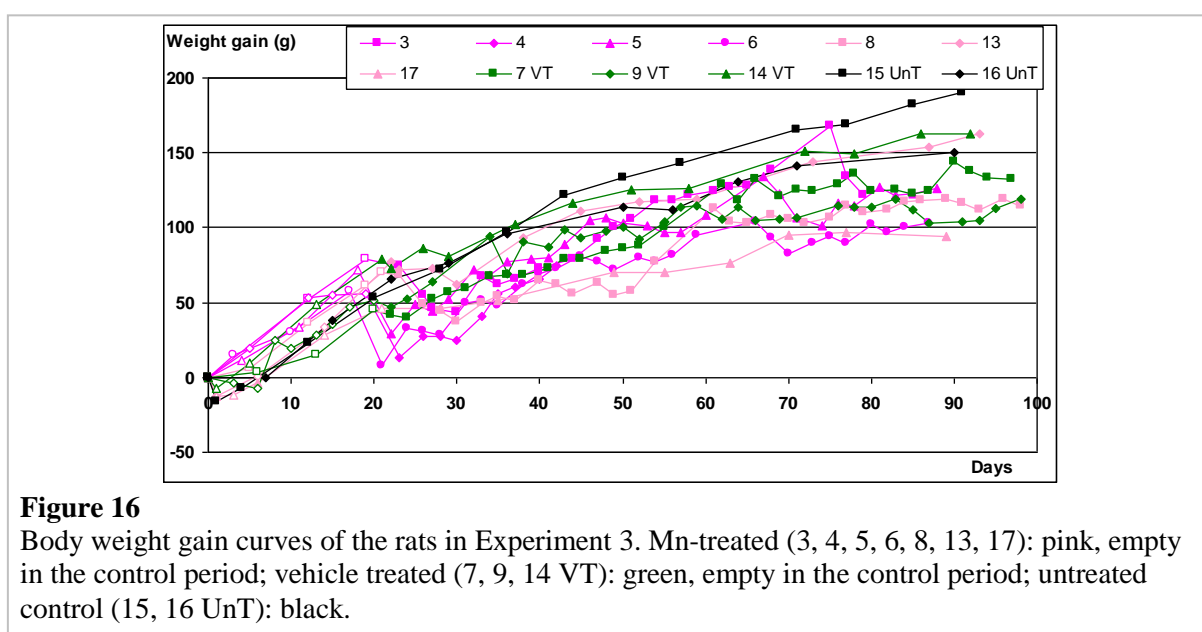
Figure 15

Distribution of the 3 forms of OF activity in a 10 min period where epileptic ECoG activity was observed in rat „E”. Top: OF activity; below: the corresponding ECoG spectra in the minutes indicated above each curve. Bottom: ECoG record with spike bursts, 6th minute.

3.3. Experiment 3

3.3.1. Indicators of general toxicity

As shown in Fig. 16, starting the intratracheal administration of MnO₂ NPs, around the 20th day, caused a sudden drop in the treated rats' body weight. This disappeared later and the trend of weight gain was similar in the Mn-treated and vehicle treated (VT) rats (in the untreated control, UnT, weight gain was a bit more intense). In the VT rats, the drop on starting the treatment was much less expressed (so it must have been largely due to nanoparticulate Mn, not to the procedure itself).



The only significant change in relative organ weights was increase of the lung weight (related to body weight or brain weight) and of the thymus (to brain weight). The calculated summed Mn dose in this mode of exposure was ca. 40 mg only, but it caused higher inner doses than orally applied Mn did in Experiment 2 (Table 4).

Table 4 Mn levels in the Mn-treated and vehicle-treated rats' tissue samples, Experiment 3

	Mn level (µg/kg)	
	Mn-treated	Control (VT)
Blood	979.08±389.31**	285.43±133.21
Brain	11210.89±1393.34***	2072.53±358.16
Liver	12309.07±1553.89*	7369.43±1837.86

Mean±SD, n: see Table 1. *, **, ***: p<0.05, 0.01, 0.001 vs. Control.

3.3.2. Open field motility

In this experiment, the inner Mn dose (higher than in Experiment 2, see Table 3 and 5) was apparently sufficient to induce significant hypomotility. As seen in Fig. 17, the decrease of ambulation time could be detected already in the 3rd week of the experiment, after only 5 Mn administrations. The decrease developed further in the first 4-5 weeks of treatment but showed minimal change afterwards. A similarly manifest but opposite trend (increase from the 4th week on) was seen in the immobility time. The change/no change of event counts was like in Experiment 2: less ambulation meant less events while the longer total immobility time resulted from not more numerous (but, necessarily, longer) motionless events. In the treatment period, longer overall motionless time was paralleled by more intense increase of immobility during a 60 min recording session (Fig. 18). This was a difference vs. Experiment 1 and 2, but fit well within the general picture of hypomotility.

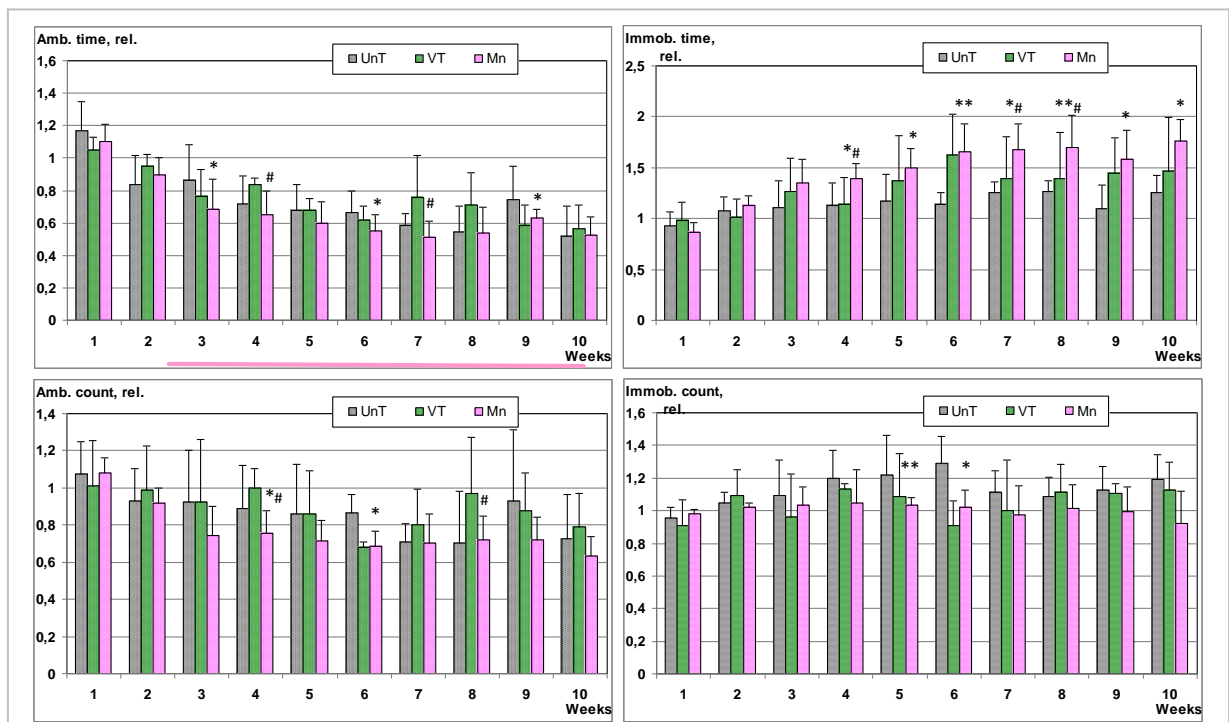


Figure 17

Group means of time and event count of ambulation (left) and immobility (right) for the untreated control (UnT), vehicle control (VT) and Mn-treated (Mn) rats in the 10 weeks of Experiment 3.

Period of Mn exposure marked as in Fig. 10.

Mean+SD, n: see Table 1. *, **: $p < 0.05, 0.01$ vs. UnT control; #: $p < 0.05$ vs. VT control.

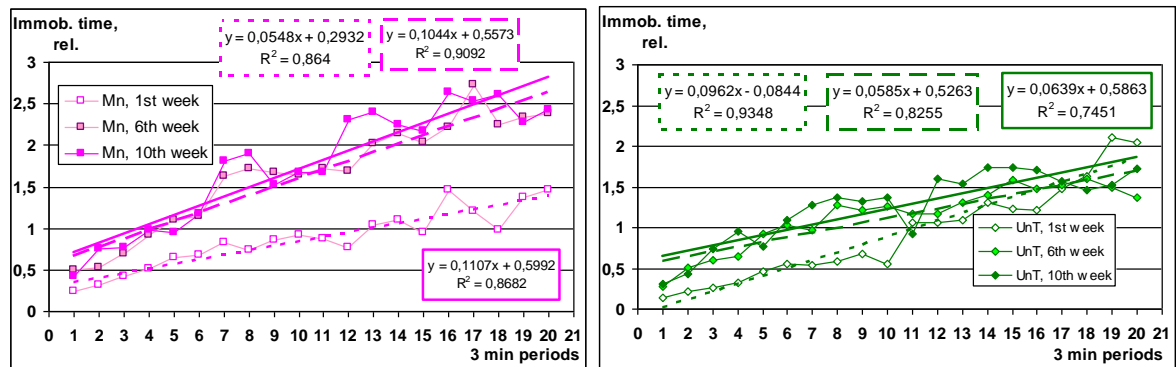


Figure 18

Time course of time spent in immobility within the 60 min recording session in the 1st, 6th and 10th weeks in Experiment 3. Left: Mn-treated; right: control. Trend lines and the corresponding equations have the same line color and style.

Mean values (error bars omitted for clarity), n: see Table 1.

3.3.3. Electrocorticogram

ECoG total power increased on the effect of Mn but this change was noteworthy only from the 4th week on, i.e. it developed later than the change in OF motility (Fig. 19). Maximal increase was seen in the 7th week; after that, some decrease followed. The direction of ECoG total power change was opposite to that seen in the previous experiments and supported what was suggested by the OF data viz. that with various inner Mn doses not only the strength but also the direction of the Mn-induced functional changes can vary.

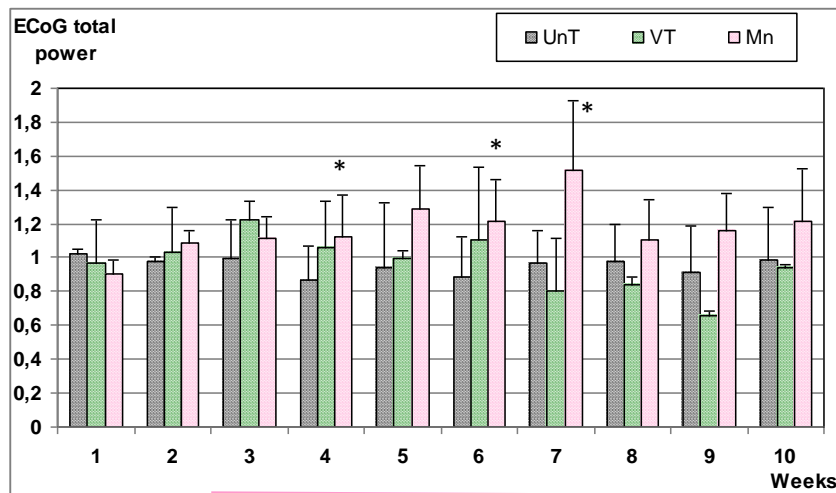


Figure 19

Group means of ECoG total power (in the 1-49 Hz range) in the 10 weeks of Experiment 3.

Mean±SD, n: see Table 1. *: $p < 0.05$ vs. UnT control.

The difference spectra in Fig. 20 showed stability in UnT. In the VT rats, there were deviations indicating alteration vs. the pre-treatment period but without a clear trend, in accordance with Fig. 19. In Mn-treated rats, however, the difference curves showed a gradually developing power increase which resulted in the relative reduction of the 7 Hz peak.

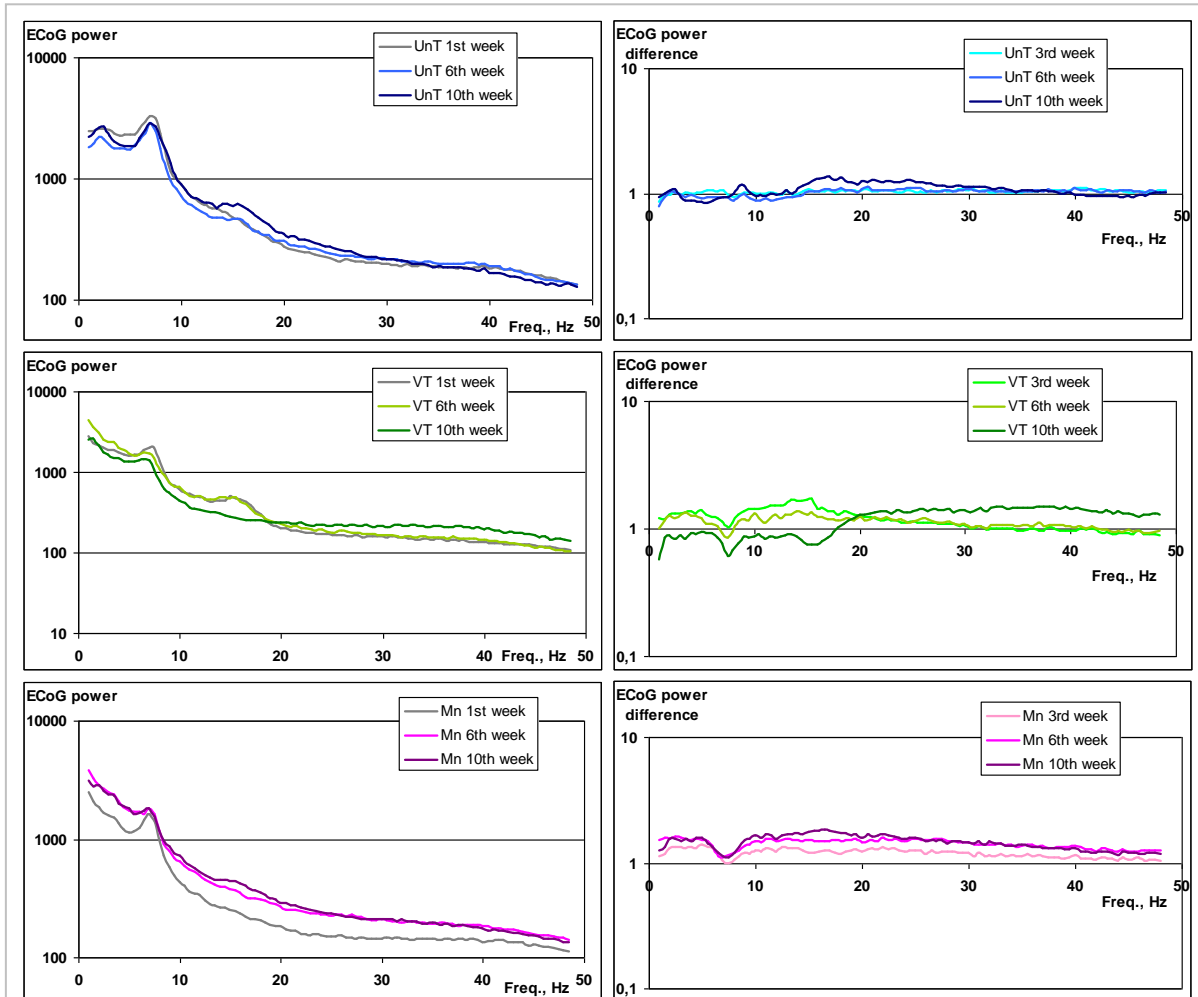


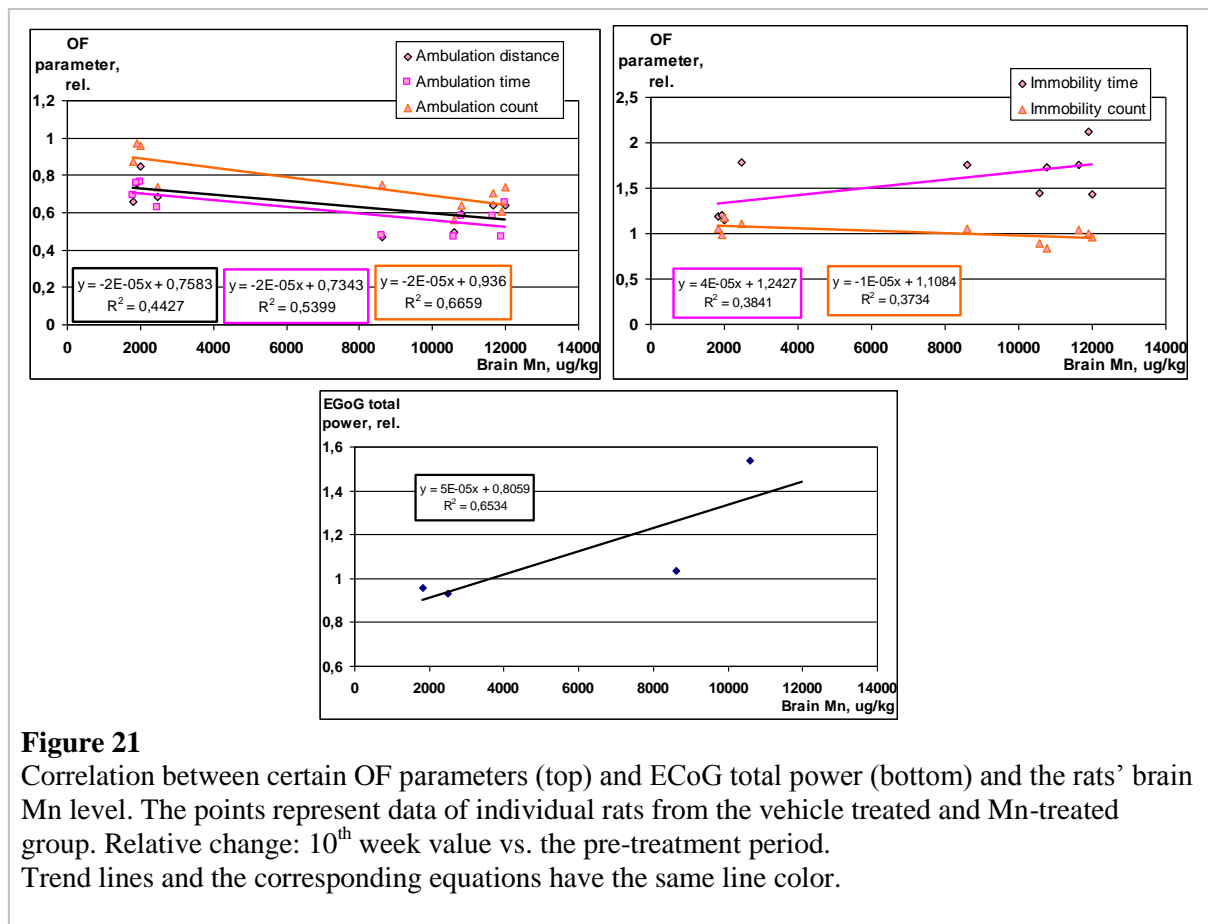
Figure 20

Left: ECoG spectra (group means) from the UnT (top), VT (middle) and Mn-treated (bottom) rats in the control period (1st week) and after 4 and 8 weeks of Mn exposure (6th and 10th experimental week).

Right: The corresponding difference spectra: the ratio of the spectrum in the 3rd, 6th and 10th week to the average spectrum of the control period.

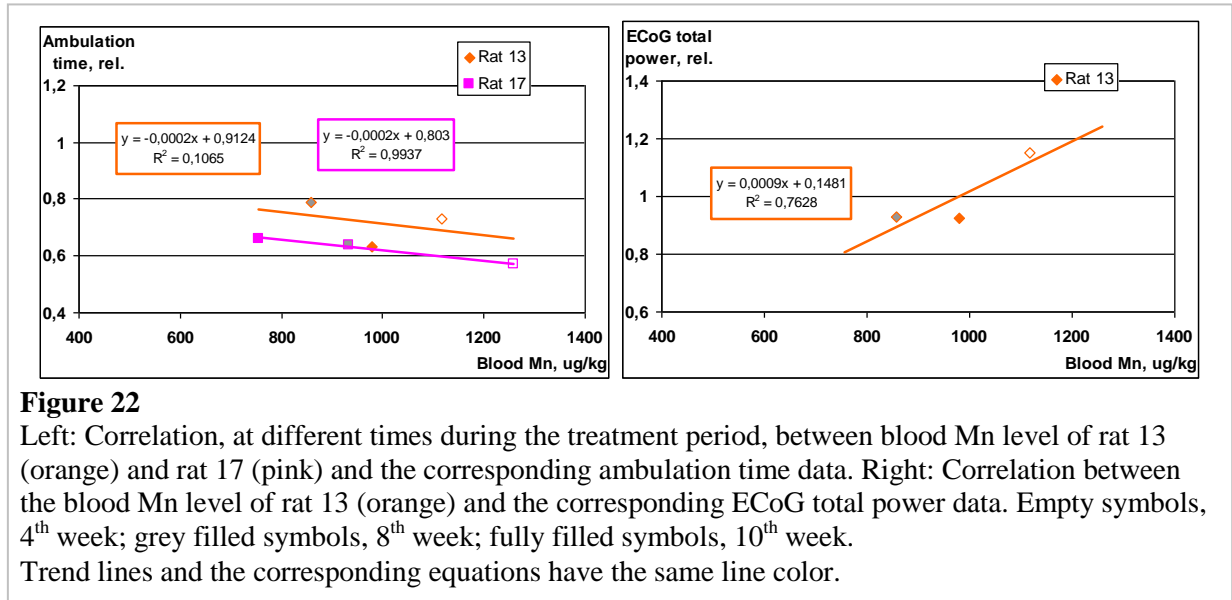
3.3.4. Correlations

Based on the clear changes of OF and ECoG parameters in the Mn-treated rats in Experiment 3, the correlation of these neuro-functional parameters and inner Mn dose was tested. As shown in Fig. 21, ambulation distance, time and count had fairly good correlation to brain Mn levels. Immobility time was less well correlated and the no-change of immobility count is also seen. The correlation of ECoG bands and OF parameters was strong ($R^2 > 0.5$) between ambulation distance and time, and immobility time, and low beta, mid beta and high beta power in the treated rats. In both controls (UnT and VT) only high beta and ambulation time were strongly correlated.



In Experiment 3, both the OF and ECoG data showed a two-phase change on Mn application: a stronger effect in weeks 3 or 4 to 6 and not much change afterwards. This was similar to certain earlier observations (Vezér et al., 2005). The method of repeated recording lent itself to test if the non-linear time trend of OF and ECoG was due to a non-linear trend of Mn deposition in the rats' body. From two rats in the treated group of Experiment 3, rat "13" and rat "17", tail vein blood was taken on the 4th and 8th week. The measured Mn level in these

samples and in the final blood sample (10th week) were correlated to ambulation distance (Fig. 22, left; note that the weeks are indicated by the symbol filling colors) and to ECoG total power (Fig. 22, right, rat 13 only).



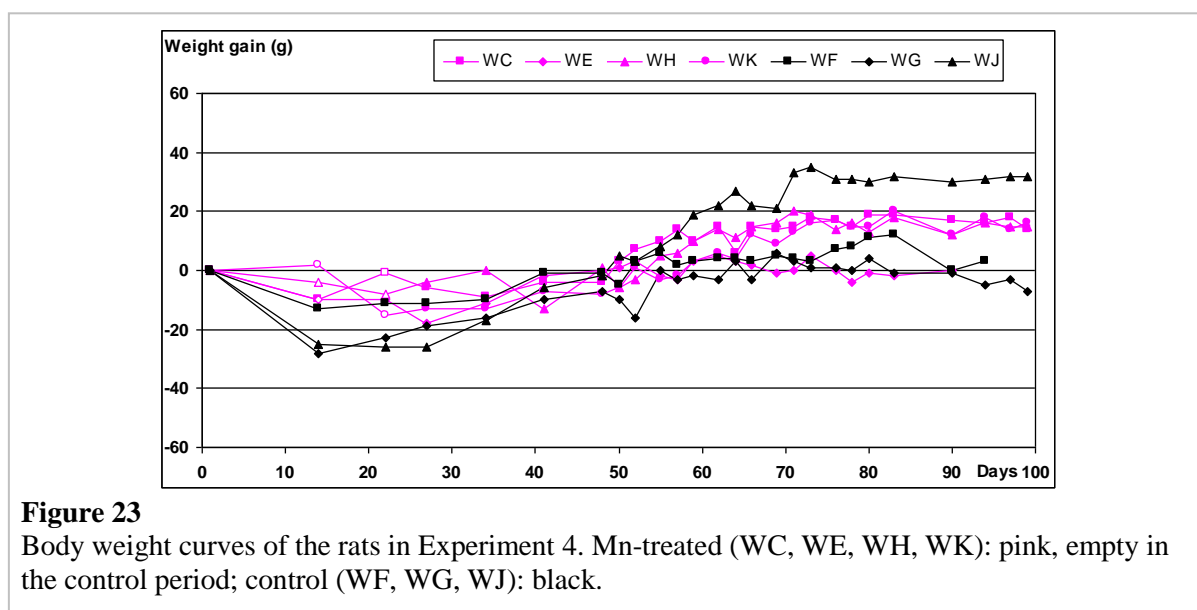
3.3.5. Signs of epileptogenic action of Mn

In Experiment 3, the kind of epileptiform activity observed in Experiment 2 (short series of spikes on the ECoG) were occasionally seen. Such events indicated the epileptogenic action of Mn but were not consequent enough to allow any deeper analysis. Hence it was investigated in the next experiment whether and how Mn exposure alters the spontaneous epileptic activity of WAG/Rij rats.

3.4. Experiment 4

3.4.1. Indicators of general toxicity

The time course of the WAG/Rij (short, WAG) rats' body weight was markedly different from that of the Wistars. Fig. 23 shows that these rats showed only a minimal weight gain during the whole experiment, but also that the weight of control and treated rats were in the same range. There was also no noteworthy difference in the organ weights.



The tissue Mn levels (Table 5) showed the effect of oral Mn exposure and were comparable to those in Experiment 2 (in which Wistar rats had an identical exposure via the drinking water).

Table 5 Mn levels in the control and treated rats' tissue samples, Experiment 4

	Mn level ($\mu\text{g/kg}$)	
	Treated	Control
Blood	440.95 \pm 88.18	385.86 \pm 131.78
Brain	4983.24 \pm 2051.48*	2269.57 \pm 680.70
Liver	11671.06 \pm 3192.37*	6865.94 \pm 920.33

Mean \pm SD, n: see Table 1. *: $p < 0.05$ vs. Control.

3.4.2. Open field motility

In the Mn-treated WAG rats, decrease of ambulation and increase of immobility during the weeks of Mn exposure – due partly to habituation and partly to aging – was much less than in

the controls (Fig. 24); in spite of the motility minimum observed in both groups in the 5th and 6th week.

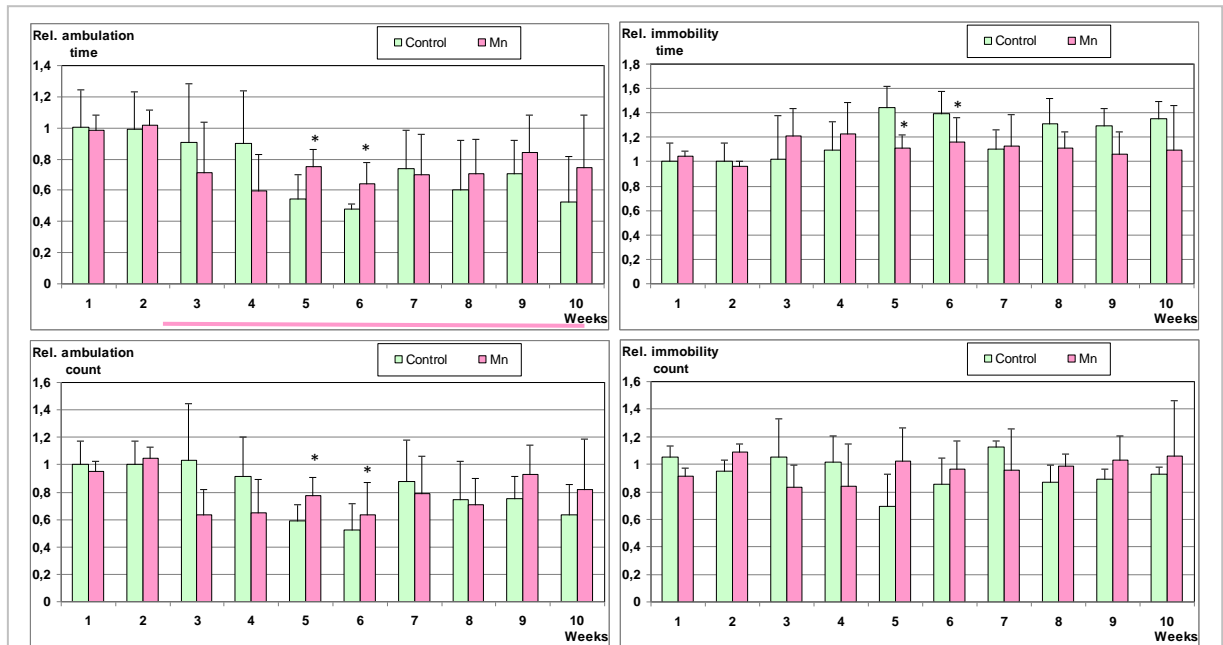


Figure 24

Group means of time and event count of ambulation (left) and immobility (right) for the control and treated rats in the 10 weeks of Experiment 4. Mn exposure period marked as in Fig. 10. Mean+SD, n: see Table 1. *: $p < 0.05$ vs. Control.

Also, the increase of immobility within one 60 min recording session became more pronounced in the controls as the weeks elapsed but in the treated rats the trend was opposite (Fig. 25, note that the slope factors in the trend line equations decrease in the treated and increase in the control group).

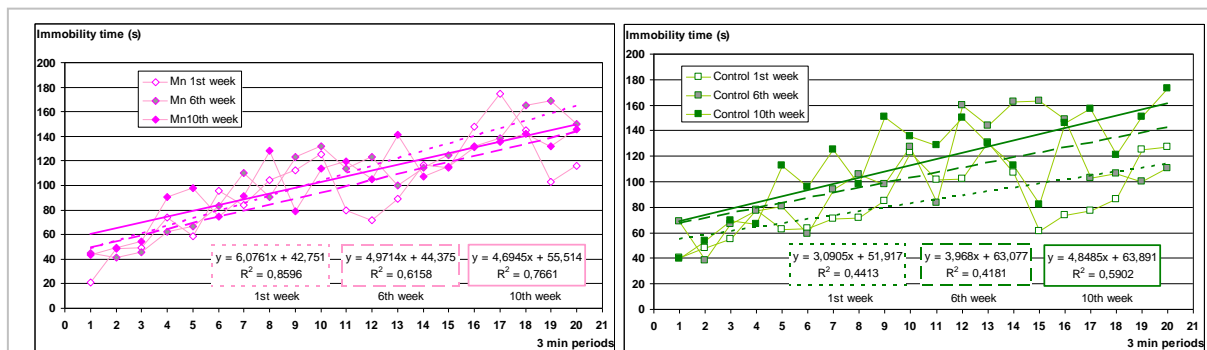


Figure 25

Time course of time spent in immobility within the 60 min recording session in the 1st, 6th and 10th week of Experiment 4. Left: Mn-treated ; right: control. Trend lines and the corresponding equations have the same line color and style.

Mean values (error bars omitted for clarity), n: see Table 1.

3.4.3. Electrocorticogram

Total ECoG power increased massively in the treated rats on commencing the Mn exposure (3rd week, Fig. 26). This was not reflected in the general shape of power spectra but the difference spectra and their time trend were markedly dissimilar (Fig. 27). The difference spectra of the Mn-treated rats had also some similarity to those in Experiment 2 and 3 which is another indication of the latent epileptogenic effect of Mn in those experiments.

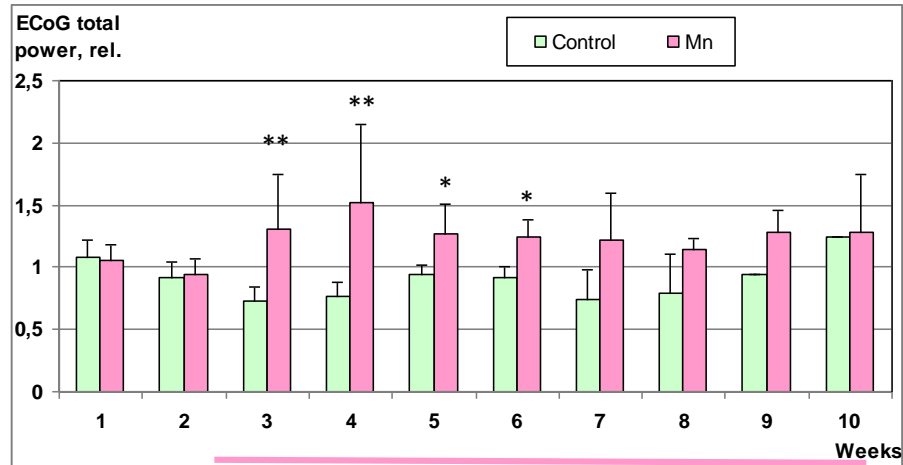


Figure 26

Group means of ECoG total power (1-49 Hz) of control and Mn-treated WAG rats in the 10 weeks of Experiment 4.

Mean+SD, n: see Table 1. *, **: $p < 0.05, 0.01$ vs. Control.

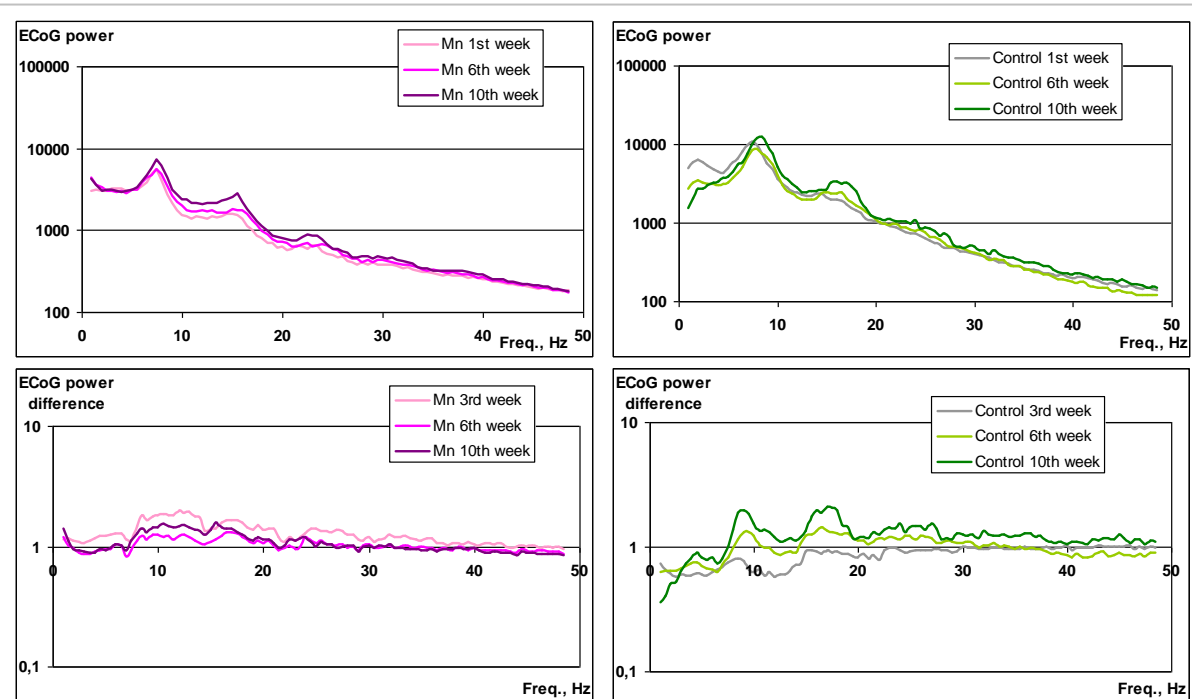


Figure 27

Top: ECoG spectra (group means) in the 1-49 Hz range from the Mn-treated (left) and control (right) rats in the weeks indicated in the insert.

Bottom: Difference spectra; left: Mn-treated, right: control.

3.4.4. Correlations

In the treated rats, the time spent in ambulation and in immobility was strongly correlated to ECoG total power and to the power of the low beta band (Fig. 28). These OF parameters were also strongly correlated to brain Mn level, whereas the correlation of ECoG low beta power to brain Mn was weaker.

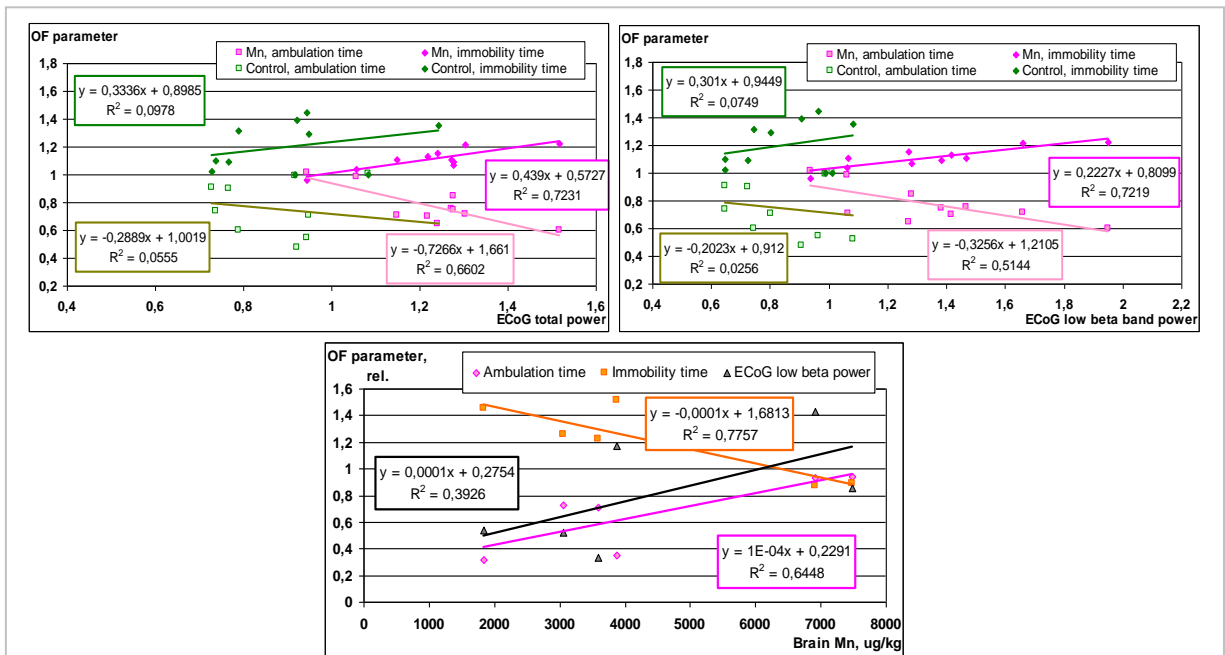


Figure 28

Top: Correlation between OF ambulation and immobility and ECoG total (top left) and low beta (top right) power. Bottom: Correlation between OF and ECoG parameters and the rats' brain Mn level. The points represent data of individual rats from the Mn-treated and control group. Relative change: 10th week value vs. the pre-treatment period.

Trend lines and the corresponding equations have the same line color and style, dashed lines belong to empty symbols.

3.4.5. Effect of Mn on the spontaneous epileptic activity

The ECoG of WAG rats is characterized by short (5-15 s) bursts of SWDs, during which a peak appears on the ECoG power spectrum around 13-15 Hz. This gave the importance of the low beta band (12.5-15 Hz), mentioned also above (2.4.2.; 3.2.5.; Fig. 15). The electrographic picture and ECoG power spectrum of various phases of activity in WAG rats (Fig. 29) underlined the importance of low beta. In silent phase, the peak around 7 Hz was visible, while in high-amplitude irregular activity the elevation of the spectrum curve right of the peak made it disappear. Typically this activity appeared when the initial exploratory reaction of the animal put into the OF box was over. During a burst, the 13-15 Hz peak was present. Hence, the power in the low and mid beta band was used to measure the intensity of epileptic activity (see 2.4.2.).

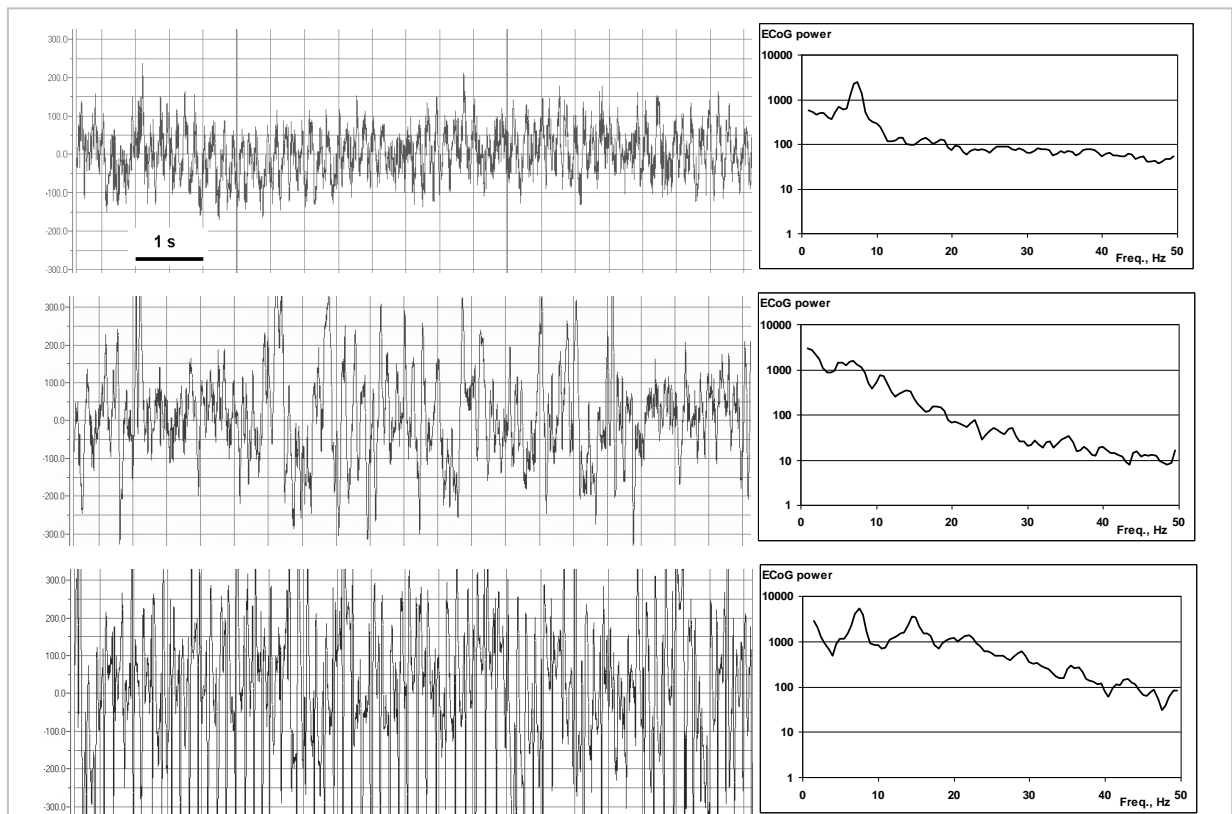


Figure 29

Sample ECoG records (left) and ECoG spectra (right) in the three typical states of cortical activity of WAG rats. Top: silent phase; middle, high-amplitude irregular activity; bottom, burst. Note the spectrum peaks around 7 and 13 Hz.

Mn treatment caused increase in the bursting activity. As seen in Fig. 30, ECoG low beta power during the bursts increased more than the number of bursts itself. Especially the power for one bursting second increased in the treated rats, indicating that bursting – manifested in frequent and high-amplitude deflections from the isoelectric line – was more intense under Mn influence. The ratio of low beta power in bursting/silent seconds was, on the contrary, higher in the controls, probably because in the Mn-treated rats the high-amplitude irregular cortical activity was more abundant in non-bursting periods.

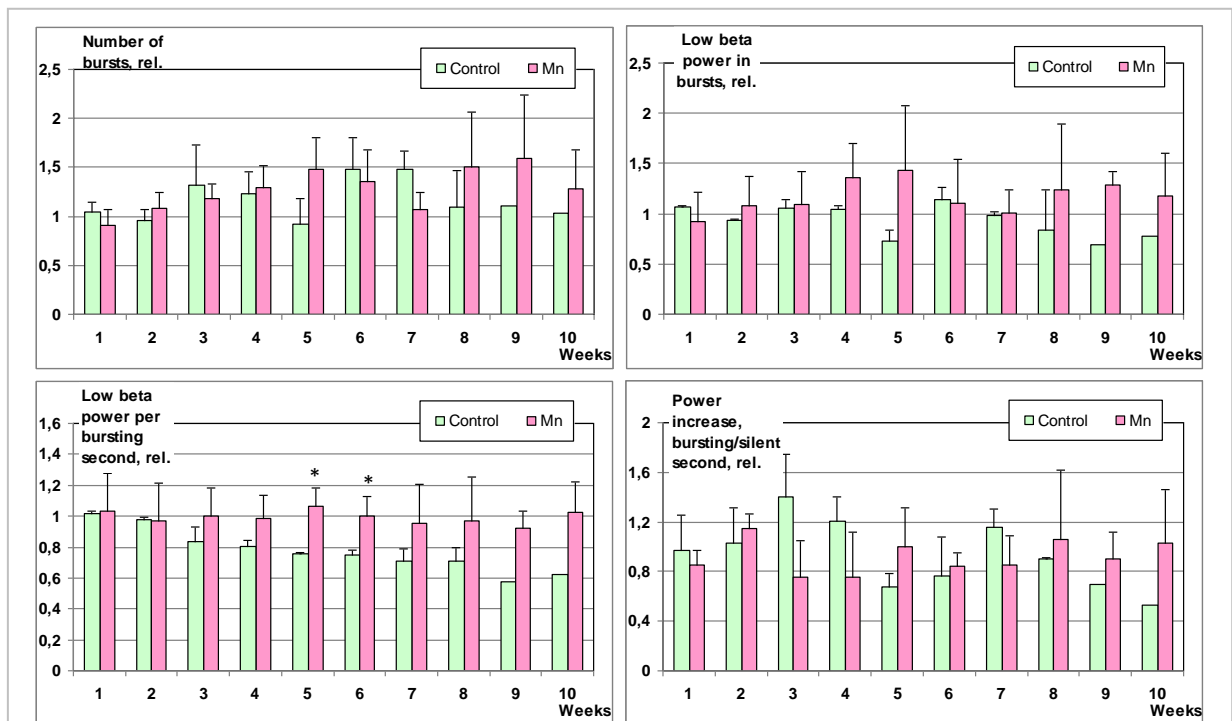


Figure 30

Numerical data on bursting activity in control and Mn-treated WAG rats.

Mean+SD, n: see Table 1.

*: $p < 0.05$ vs. Control.

4. DISCUSSION

In all four experiments, administration of Mn in different chemical forms and doses resulted in significant tissue Mn deposition (inner dose) and in alterations of CNS functional parameters. Body weight gain and organ weights indicated no noteworthy general toxicity even at the highest inner doses in Experiment 3 (except for the lungs) which suggested there was no major general toxic effect of Mn present that could be responsible for the observed neuro-functional effects.

Based on the correlations observed in each experiment, one can broadly state that the changes in OF motility and in cortical electrical activity developed in parallel. In comparable previous experiments of the Department this could not be ascertained so this was one of the achievements of the study presented in this thesis.

The neuro-functional effects were apparently highly dependent on the inner Mn doses and (not independently of that) on the length of exposure; not only in terms of strength but also in direction. The general expectation was, based on earlier results (Vezér et al., 2005; Oszlanczi et al., 2010; Horváth et al., 2012) and on literature data (Normandin et al., 2004; Calne et al., 1994) decreased motility in the Mn-exposed rats. In contrast to that, in Experiment 1 (where the dose and time of Mn exposure, and the resulting inner dose, was the lowest) an opposite alteration was seen: treated rats spent more time in ambulation and less in immobility than the controls towards the end of the 4-week exposure; and the decrease of motility during the 30 min OF sessions, signaling habituation, was also less than in the controls. All the same this could be interpreted within the framework of Mn neurotoxicity because the treated rats' increased (more exactly, less strongly decreased vs. control) motility could be likened to the early phase of adult human manganism, characterized by behavioral disinhibition (Calabresi et al., 2001; diminished habituation was also reported in this paper). In children exposed to high Mn level via drinking water, attention deficit and hyperactivity (Bouchard et al., 2007) or low performance in intelligence tests (Wassermann et al., 2006) was described, and the disturbance of higher order functions was correlated to the bodily Mn load.

In Experiment 2, the dose and length of Mn exposure was longer and the final tissue Mn levels were higher than in Experiment 1. OF activity was mildly decreased, so the inner doses might be marginally sufficient to induce the late “established” stage of Mn-induced Parkinson-like disorder. There were also indications of the two-phase development of Mn effect, with more expressed alterations in the first ca. 4 weeks of exposure than afterwards,

which was observed in full-blown form in Experiment 3. The change of motility within a 60 min session showed the same effect as the week-to-week changes also in Experiment 2 and 3 (Fig. 11 and 18), similarly to what was seen in Experiment 1 (Fig. 5).

Taking Experiments 1 to 3 together, an up-and-down shape of the dependence of OF motility on the (presumable) total inner Mn dose could be conceived. In certain published experiments where rats were treated with 3-nitropropionic acid, a standard mitochondrial toxin used to model Huntington's disease, initially hyper- then hypomotility was found (Borlongan et al., 1995) which can be likened to the above mentioned variations of effects of Mn on motility. It is noteworthy here that Mn and 3-nitropropionic acid have at least one effect in common, mitochondrial toxicity – Mn acts on complex II (Malecki, 2001) and complex III (Zhang et al., 2003) – and were found to cause similar changes in cortical evoked responses (Takács et al., 2010a).

In Experiment 3, the inner Mn doses were the highest of all, and the two-phase development of both OF and ECoG changes was clearly seen. By taking blood samples during the treatment period (and not only at the end) in that experiment, we could show parallelism (with fair correlation) between the inner Mn doses and the extent of change in ambulation time and in ECoG total power; demonstrating directly what was suggested by earlier result of the Department (first of all in Vezér et al., 2005) namely that the deposition of Mn during a longer treatment period was not linear in time and that the functional alterations depended directly on brain Mn level. One possible cause of that could be that elimination of Mn from the brain substance, normally a slow diffusion process (half life around 60 days in rats; Takeda et al., 1995) might have been increased in case of high Mn loads (Mena et al., 1967). Alternatively, the Mn transport to and storage in the brain might have been saturated, similarly to the findings by Oszlanczi et al. (2010) where MnO₂ NPs were applied to rats for 6 and 9 weeks with the dose used in this work and a twice higher one. In that work, the brain Mn level in the higher dose rats was only slightly higher than in the lower dose group, and the levels after 9 weeks were even slightly lower than after 6 weeks. The mechanisms limiting the inner Mn dose may have been active also in Experiment 2 (with oral Mn exposure) as suggested by the slight indication of two-phase time course, and even more by the self-regulated character of intestinal Mn uptake (Davis et al., 1993; Greger, 1998).

Numerical parameters of OF activity and ECoG were fairly well correlated to brain Mn levels at the end of the experiments (that is, at the time when tissue samples were taken: Fig. 14, 21 and 28); and the above mentioned blood samples taken during the treatment period showed parallel development of bodily Mn load and neuro-functional changes (Fig. 22). It is

reasonable that CNS functions be influenced by the local deposition of Mn; conversely this means that certain functional parameters may be useful indicators of CNS Mn burden and may be developed to practical biomarkers. An interesting collateral result of the present study was that intratracheal administration of Mn in form of MnO₂ nanoparticles was far more efficient in causing high Mn level in the brain (and other organs) than dissolved Mn applied via the drinking water. This may be explained by the bodily need dependent, regulated character of intestinal absorption, mentioned above, opposed to the non-functional absorption from the alveoli.

The typically fair correlations among changes in the OF or ECoG parameters and the measured brain Mn levels raise the question what beyond the presence of Mn in the brain could be the common background.

One such mechanism is the effect of Mn on astrocytic glutamate metabolism. Astrocytes not only take up glutamate and convert it to glutamine but supply that as transmitter precursor for both glutamatergic and GABAergic neurons (Coulter and Eid, 2012) closing thereby the glutamine cycle. On action of Mn²⁺, both glutamate uptake (Erikson and Aschner, 2003) and the transformation to glutamine (Normandin and Hazell, 2001) is reduced. The result may be the imbalance between excitation and inhibition, not only because of excess free glutamate but because of loss of negative feedback between glutamate and GABA if glutamate-uptake induced GABA release from the astrocytes (Héja et al., 2012) is lost. This feedback is supposed to act mainly when excitation is elevated, such as in (true or modelled) epilepsy, and might provide explanation to the increased intensity of bursting in the Mn-treated WAG rats in Experiment 4 – but the case is more complex because in WAG rats systemically given GABA agonists do strengthen, and not suppress, bursting (Coenen et al., 1995). The source of the bursts (SWDs) in WAG rats is apparently the perioral area within the somatosensory cortex of the rats where the motor impulses to drive the exploratory sweeping movements of the whiskers, with 7-12 Hz frequency, are generated (van Luijtelaar and Sitnikova, 2006). Under natural (no stimuli, no motion) or artificial (the GABA agonist thiopental, in 40 mg/kg b.w. dose, failed to suppress SWDs but turned them to continuous hyperactivity: Takács et al., 2010b) diminution of sensory input, this oscillatory tendency can come to effect and spread the synchronic activity to the whole cortex.

In Experiment 2 and 3, the occasional SWD-like bursts in a few of the Mn-treated rats always appeared when the animal was beyond the initial exploratory phase and was more or less motionless. Also with the WAG rats, the bursts were rare in the first 10-15 min of the 60 min session and became abundant later. Thus, Mn could promote bursting also by reducing the

treated rats' motility, besides the effect on transmitters described above. The fact that the intensity of bursts (quantified by ECoG low+mid beta power) increased more significantly than their overall time (Fig. 30) suggests mainly increase of glutamatergic transmission (involving more neurons in the rhythmic hyperactivity) and less effect on GABA (which would more likely result in altered number of bursts in a given period). Inhibited function of astrocytic glutamine synthetase is probably involved also in human CNS disorders, including various forms of epilepsy (such as temporal lobe epilepsy: Eid et al., 2012). There are a few reports on the epileptogenic effect of abnormally high Mn levels in humans, all in children (Hernandez et al., 2003; Komaki et al., 1999) or young adults (Ono et al., 2002). At this point it is of interest that absence seizures, to which WAG rats serve as a model and in which the intensity of seizures was elevated by Mn exposure in Experiment 4 of the present study, also occur typically during childhood (Coenen and van Luijtelaar, 2003). Mn deficiency also seems to induce epileptic activity (Gonzalez-Reyes et al., 2007) and the common point is apparently astrocytic glutamine synthetase which requires Mn but is blocked by an overdose of this metal (Normandin and Hazell, 2001).

High extracellular glutamate level is universally deteriorative for the CNS – due to imbalance between excitation and inhibition (resulting e.g. in epileptic activity: Eid et al., 2012) and to excitotoxicity (manifested also in electrophysiological abnormalities: Nagy et al., 2010). These mechanisms are present, according to the above, also in the neurotoxicity of Mn. Another general harm concomitant with Mn exposure is oxidative stress, resulting from mitochondrial dysfunction due to Mn effects on complex II and III (mentioned above) and Mn-SOD (Morello et al., 2007). Further, Fenton reaction, a redox cycle characteristic for transition metals with several oxidation states including Mn, can generate dangerous hydroxyl radicals (Valko et al., 2005). Reactive oxygen species in the brain lead to membrane lipid peroxidation (Avila et al., 2008) and to downregulation of glial glutamate transporter (Willmore and Ueda, 2009) the latter contributing to epileptogenesis. Damaged lipids lead to changes of fluidity and other membrane properties which, in turn, disturb all membrane- and receptor-bound phenomena (Coyle and Puttfarcken, 1993).

The dopaminergic system is especially sensitive to oxidative stress, due to the autooxidizing tendency of dopamine and to the presence of monoamine oxidase producing hydrogen peroxide (Alexi et al., 2000). Motivation, determining spontaneous locomotor activity in the OF test, is regulated by mesolimbic/mesocortical dopaminergic structures (Alexander et al., 1990). Increased time and/or count of immobility, seen in Experiment 2 and even more in

Experiment 3, was an indication of general hypomotility, an effect in line with the symptoms of heavily Mn-exposed welders suffering from Parkinson-like syndrome (Bowler et al., 2006). The neuro-functional alterations observed in the Mn-treated rats were to some extent analogous to the effects in exposed humans, regarding both cortical electrical activity (Hernandez et al., 2003; Sinczuk-Walczak et al., 2001; Sjögren et al., 1996) and motor behavior (Bowler et al., 2006; Bouchard et al., 2007; Saric et al., 1977). As blood or urine Mn levels as routine measurements do not adequately characterize CNS damage (Manzo et al., 1996; Myers et al., 2003), functional biomarkers of Mn effect, to be developed in the future on the basis of experimental results similar to those presented in this Thesis, may be more suitable for this purpose.

The particular points of aims (1.6.) could finally be answered as follows:

- The CNS effects of Mn developed in a non-monotonous way. Depending on the dose and length of exposure, the direction of changes varied (Experiment 1 vs. Experiment 2 and 3); and in case of longer (8-10 weeks) exposure, the effects developed mostly in the first 4-5 weeks and showed little change thereafter.
- The changes in motor behavior and in cortical electrical activity developed in parallel in time. Usually there was a fair correlation between numerical parameters of OF motility and of ECoG (total or band power), and inner Mn dose (brain Mn level) at the end of Mn treatment. In a few cases, the parallel changes of OF and ECoG parameters with blood Mn level during the treatment period could be showed. This raises the possibility to develop neuro-functional biomarkers of effect for human Mn poisoning.
- The epileptogenic effect of Mn was occasionally observed in Wistar rats in Experiment 2 and 3. In the epilepsy-prone WAG/Rij rats in Experiment 4, Mn treatment intensified bursting activity.
- Beside the above points, a further noteworthy conclusion could be drawn, namely that intratracheal administration of Mn in form of MnO₂ nanoparticles was far more efficient in causing high inner Mn doses than dissolved Mn applied via the drinking water.

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ACKNOWLEDGEMENT

First, I would like to thank to Dr. László Nagymajtényi for providing the opportunity to perform my experiments at the Department of Public Health, and for always improving my work with his wise foresight and useful advices.

I am especially grateful to my supervisor, Dr. András Papp, who was always willing to help, continually smoothed the way for the completion of my thesis, and always guided my progress to the right direction. His support and guidance was vital in the thesis coming into existence.

I am thankful to my colleagues, Dr. Andrea Szabó, Dr. Edina Horváth, Zsuzsanna Máté and Dr. Gábor Oszlanczi, for their support and help in the experimental work. Many thanks to Dr. Zoltán Kónya, Dr. András Sápi and Péter Pusztai at the Department of Applied Chemistry, University of Szeged, for providing the applied nanoparticles, and to Dr. Attila Szőke, József Koszta and Edit Pálincás at the laboratory of the MOL Hungarian Oil and Gas Company for the metal level determinations.

I also would like to thank to Mihályné Németh, Anita Balázs, Gyuláné Kiss and János Kis for their assistance in handling the animals, and to Imre Gera and Lászlóné Szalai for their help in technical questions and documentation.

APPENDIX

- I. Takács Sz, Bankó S, Papp A (2010) Effect of mitochondrial toxins on evoked somatosensory activity in rats. **Central European Journal of Biology** 5, 293–298.*
- II. Takács Sz, Szabó A, Oszlanczi G, Paulik E, Papp A (2011) A pilot study with simultaneous recording of changes in motility and cortical electrical activity of rats during four weeks of oral manganese exposure. **International Journal of Environmental Health Research** DOI:10.1080/09603123.2011.643228.*
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